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Invited Review

Otoconia biogenesis, phylogeny, composition and functional attributes

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Summary. This work consolidates data about these interesting organic crystals of vertebrate inner ears. It addresses 5 aspects of inner ear otoliths not completely understood to date: 1) embryological data that explains the formation of the crystals, 2) the significance of the organic and the inorganic phase of the otolith and the changing patterns of otoconia formation along the evolutionary tree, 3) otoliths contribution for detecting linear acceleration, 4) the effect that altered gravity and aminoglycosides have on the development and adult shape of the crystals, and the evolutionary significance of a changing shape of the crystals from primitive forms (lamprey) to high vertebrate birds and mammals is discussed, 5) functional attributes of the otolithic organs and morphological modifications of the otoliths by physical and chemical insults are presented with an extensive discussion of the most relevant literature published and available to us.

Key words: Otoliths, Otoconial membrane, Biogenesis, Maturation, Composition, Evolution, Gravity, Hypergravity, Aminoglycosides, Evoked potentials

Introduction

The working hypothesis accepted today for vestibular function, is that mechanical displacement of hair cells stereocilia initiates transduction of physical into neural energy (Pickles, 1982; Hudspeth, 1983). Preferential sensitivity of hair cells (Ashmore, 1994) according to directional displacement of the hair bundles has been known for decades, and the diversity of hair cells and afferent neurons morphology (Engstrom et al., 1966; Hamilton, 1968; Ballantyne and Engstrom, 1969; Bergstrom, 1973a-c) suggests that vestibular receptors acquired distinct phenotypes that imparted to them specific antigenic identity (Fermin and Martin, 1995; Fermin et al., 1997). Acceleration of the head provides the inertia necessary to displace the stereocilia against the otolithic membrane (Israel and Berthoz, 1989) and thus, it is reasonable to assume that the loading mass of the otolithic membrane was shaped by the progressive varying size of the vestibular end organs during phylogeny. The elegant work of Lewis et al. (1985) showed that newer species progressively acquired refinement features (Vinnikov, 1965) not only at the level of the sensory epithelia, but also at the level of the otoliths. In the portion of this review that addresses the evolutionary significance of the otoliths it is clear that the transition from single otoliths to compound and multiple single crystals conglomerate invariably goes hand in hand with the refinement of the epithelia and design of the vestibule.

The transition from "more to a less" bulky vestibular structures probably occurred with the transition from water to terrestrial evolution, but the relationship remains unproven. In higher vertebrates utricular and saccular volumes varies among species, with a gradual increase proportional to body weight (Igarashi et al., 1984). An in depth analysis of the auditory end organs refinement and function during phylogeny was done in birds and reptiles by Manley (1990c), who indicated that one of the first changes occurred when transformation from pressure to motion began at the surface of the swimbladder (Manley, 1990b). The Weberian ossicles that connect the bladder to the hearing apparatus are found in living fishes attesting to the transition of swimbladder from a barometer-sensing organ into a modified auxilary sensory structures. Further modifications continued with the emergence of newer

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species of fishes in which the saccules and macular lagena (Jorgensen, 1970; Lavigne-Rebillard et al., 1985) probably share a combined auditory and vestibular function (Fay and Popper, 1980). Vestibular functions of inner ear receptors probably evolved from the lateral line system (Budelmann et al., 1991) in which the organization of sensory receptors resembles present inner ear sensory epithelia and hair cells, stereocilliary bundles, nerve endings and actuating masses, which are all present in living vertebrates (Manley, 1990a).

Not only did vestibular sensory epithelia have to adapt to the changing environments during evolution of the organism, but the accessory structures such as the otoliths had to adapt as well (Lychakov, 1988c). In newer species the fine and delicate arrangements (Lim, 1979) of the vestibular epithelia and the overlying actuating masses (gelatinous cupula or semicalcifed otolithic membrane) had to change its final mature shape and density, while retaining certain key features necessary for directional sensitivity. For instance, sensory receptor for most sensory functions (vision, smell, etc) have stereocilia and/or kinocilia (Vinnikov, 1965), which in the inner ear of vertebrates are strategically positioned as to allow proper detection of stimulus in many directions (Lewis et al., 1985) and invertebrates (Budelmann et al., 1973; Budelmann, 1979, 1987). The transition from large otoliths to single polycrystaline otoliths required the modification of the organic matrix with deposition and mineral sequestration to the organic matrix (Fermin and Igarashi, 1985b, 1986; Fermin et al., 1990, 1995b; Fermin, 1993). Mutant mice and treatment of normal animals with drugs that affect mineralization of the otoconia exhibit crystal heterogeneity such as that found along the evolutionary ladder. For instance, mutant pallid mice develop multi facet otoconia; and vaterite otoconia (Lim, 1984) otoconial forms that are found in children (Wright and Hubbard, 1977, 1981; Wright et al., 1979a,b, 1982) with diseases such as Potter' syndrome. On the other hand, acetazolamide (a carbonic anhydrase inhibitor) greatly affect otoconia formation in chicken, but it does not affect formation and maturation of the sensory and secretory epithelia (deVincentiis and Marmo, 1968; Fermin and Igarashi, 1986) at least ultrastructurally. In lower species such as the red-bellied newt (Wiederhold et al., 1986; Steyger et al., 1995) different crystaline forms may be found in normal animals, a situation that is only seen in the otolith of birds and mammals after chemical insults (Fermin and Igarashi, 1986). The existence of more than one otoconia crystaline polymorph in the same species was elegantly demonstrated by (Pote and Ross, 1993), who showed that utricular otoconia of amphibians are primarily calcitic just as in birds and mammals, whereas saccular otoconia of reptile are aragonitic. While unproven, it is possible that these forms reflect the evolutionary age of the utricle and saccule.

Crystal lattice arrangement leads to different forms of calcium carbonate and related compounds, which in turn contributes to varying shape and composition of otoconia (Carlstrom et al., 1953; Carlstrom, 1963; Carlstrom and Engstrom, 1955). A polymorph of calcium carbonate is the primary source of birds and mammalian otoconia (Ross and Pote, 1984). The organic and inorganic components of otoconia were characterized in many animals including chickens (Fermin, 1993), but the disparity between the perfect crystallographic projections of calcitic crystals and chemically fixed otoconia has not been completely resolved (Fermin and Martin, 1994; Fermin et al., 1995b). There is evidence to suggest that seeding of a nucleus may occur in each otoconion before mineralization takes place, but mineralization of biological crystals (Mann, 1986, 1988; Addadi and Weiner, 1992), is a very complicate process that requires more accessability to fresh tissues in situ, than is allowed by the hidden and well protected inner ear structures. In these works the authors cautioned about the many structural artifacts that are invariably introduced by preparatory techniques and imaging processing of crystal lattices (Pradere and Thomas, 1990). In addition, crystal lattice organization is affected by impurities incorporated into the crystals, and possibly by mechanical stress (Tozeren, 1988) during formation. Moreover, crystal formation may follow rules dictated by pre-existing patterns as those offered by pre-existing organic matrix fibrils arrangements (Fermin et al., 1987), or self organized according to laws governing the assembly of matter in general (Bak and Chen, 1991). In normal developmental studies and adults the endolymphatic sac seems to play a role in otoconia fate and turnover (Imoto et al., 1983; Yamane et al., 1984; Yoshihara et al., 1992), but the exact role the sac plays in the emergence of new otoconia, removal of defective otoconia and/or replacement of lost ones is not clear.

Since the inital analyses of otoconia formation (Belanger, 1953, 1956, 1960), it is clear that otoconia sequester calcium which is concentrated away from the endolymphatic fluid (Anniko, 1980), but turnover rate of otoconia has not been clarified despite several studies on the subject (Ballarino and Howland, 1982, 1984; Kido, 1985, 1997; Kawamata, 1987, 1990c; Kawamata and Igarashi, 1995; Kido and Cohen, 1995). Most of these studies however point to an exquisite organization of the hexagonal crystals found in mammals and birds, such that a central region remains less dense than the peripheral region of each crystal. The otoconia central core (Igarashi and Kanda, 1969; Marco et al., 1971; Nakahara and Bevalander, 1979; Campos et al., 1984; Fermin, 1993; Kawamata and Igarashi, 1993) may be related to the density of each crystal. If such is the case, buoyancy of single and groups of otoconia (or layers) may be affected accordingly. For instance, regional variability of otoconial membrane thickness of birds and mammals in the striolar and extra striolar regions (Wersall, 1956; Wersall et al., 1965; Wersall and Lundquist, 1966; Ross, 1983) may be influenced by crystal size and buoyancy, which in turn may be related to the types of hair cells and nerve fibers contacting

them (Fernández et al., 1972; Goldberg, 1991).

Some of the results presented here and in previous works by one of us suggests the face angles of each hexagonal crystal is influenced by the turning directions of organic fibrils seen in chemically fixed otoconia (Fermin, 1993). To reconcile the previously observed disparity between the crystallographic diffraction pattern of calcite (polymorph of hexagonal otoconia in birds and mammals) and the ultrastructural organization of fixed fibrils (Fermin and Martin, 1994) either: 1) single nucleation sites lead to individual planes of calcification of the organic matrix which intercept in such a way as to produced the perfect diffraction pattern of calcite; or Second, every otoconion is an aggregation of individual single units of calcite. Unfortunately, the possibility for the second postulate is not supported by either: 1) the biogenesis of otoconia in birds (Fermin and Igarashi, 1985b, 1986; Fermin et al., 1987) and mammals (Lim, 1973; Salamat et al., 1980); or 2) the ultrastructural arrangement of organic fibrils left behind after chelation.

As far as the first postulate (Fermin, 1993), one can envision bricks, layers upon layers forming individual planes that cross at the angle faces of hexagons in the case of birds and mammals otoconia. If one assumes that each brick corresponds to a molecular subunit of micron size otoconia, it is possible to imagine the assembly of the otoconia by filling in of the submolecular spacing between the organic matrix that is first laid down during biogeneis. Ordered and staggered piling of the bricks could lead to assembly of crystals with crystallographic properties similar to the native polymorph that characterizes them. In this manner fracture of crystals could produce surfaces that follow (Mann, 1986), or completely miss the alignment (Mann, 1988) one would expect from perfect crystals such as calcite (Addadi and Weiner, 1992). Filling of the organic fibrils spaces by calcium carbonate in such manner could conceivably explain how the angles needed to construct each hexagon appears and the difference between the peripheral and the central portion of each crystal originates.

Several macromolecules were involucrated with the organic phase of otoconia including glycoproteins, glycosaminoglycans (Fermin et al., 1990) and proteins (Pote and Ross, 1986; Drescher et al., 1989; Pote et al., 1993a,b). Increased mineralization of otoconia was postulated to relate to a differential distribution between positively charged and negatively charged macromolecules (Fermin et al., 1995b) because the proximity of the uncalcified horizontal cupula to the calcified utricular otoconia demands a very exquisite homeostatic control difficult to imagine through free diffusion of ions in the endolymph. A more sensible hypothesis would involve attraction (sequestration) of calcium to one (otoconial membrane) and not the other (cupula) after all gellatinous masses of the inner ear are secreted. Quoting from (Mann, 1988): "The key features of the interface are that it is chemically, structurally and topographically organized for site-directed nucleation, and that the molecular interactions at each nucleation site involve a high degree of complementarity between the electrostatic, structural and stereochemical requirements of functional groups at the matrix surface and ions in the crystal faces of the developing nuclei".

The molecular organization of the organic matrix that is first secreted at the site of otoconia formation and mineralization may play a crucial role in the formation, maturation and turnover, if indeed turnover of otoconia does exist in normal adult animals. In any event, the ultrastructural arrangement of crystal subunits resembles granular structures in fishes (Gauldie and Xhie, 1995) similar to those described earlier in birds (Fermin and Igarashi, 1985a) with and without histochemical precipitation of the macromolecules involved. Those studies and more recent analyses of birds otoconia (Fermin et al., 1995b) suggest that the organic matrix influences mineralization of crytals.

Otoconia formation

The vertebrate inner ear transforms from a simple bulb (otocyst) to a complex structure. Initially, the ectodermic placode invaginates near the developing hindbrain or rhomboencephalon (Fig. 1). From this simple bulb or otocyst a series of evagination appears and the endolymphatic duct forms. Subsequently, thickening of the epithelia in its most interior and posterior divisions occur at the point of contact with a nerve and the presumptive epithelia begin to form (Fig. 2). From this seemingly simple structure all the components of the inner ear appear, including the vestibular structure and the cochlear structures. The inner ear is housed in the temporal bone, and contains a series of twisted conduits where the membranous components of the inner ear are located (Fig. 3). The properties of the mature inner ear components were discussed previously by many authors and will not be dealt with in this work.

The membranous structure that contain the gravity receptors (maculae) gradually appear over the hyaline cartilage of the developing structure. By five and a half days of incubation (stage 28) the presumptive hair cells of the maculae are distinguishable from the supporting cells and mitosing figures are clearly seen at the apex of the epithelium (Fig. 4). As development proceeds and the distinction between hair cells and supporting cells advances in the areas of the macula closest to the nerve, the epithelial cells still retain permanent nucleoli and mitotic figures. The overlying otoconial membrane displays a more mature appearance, and the fibers traveling toward the epithelia become more prominent (Fig. 5). At seven and one-half days of incubation (stage 31), abundant afferent temporary synapses are observed at the base of the hair cells with the electron microscope. By ten days of incubation (stage 36), otoconia are well-formed and distinction between support and hair cells is very obvious (Fig. 6). By twelve days of incubation (stage 38), calcification of the crystals is prominent as indicated by lack of staining properties of

the otoconia. Even though numerous afferent expanded growth cones are clearly seen inside the epithelia around the bases of the hair cells, mitotic figures are still present at the apex of the epithelia. Characteristics of this stage are the presence of myelin around the afferent fibers and thickening of the hayline cartilage that supports the epithelia (Fig. 7).

The appearance of a secretory material over the presumptive macula epithelia is gradual. When Figs. 4 and 6 are compared one can clearly see the distinction between immature and more advanced forming otoconia. The gradual maturation of otoconia is best illustrated by thin sections at the microscopic level of early biogenesis of the otoconial membrane. The organic matrix is laid down and is supposedly secreted by the supporting cells of the macula and other cells of the membranous duct. The mass closest to the hair cells stereocilia (Fig. 8) begins to segment (or fragment) and perfect hexagonal crystals appear. Otoconia farthest away from the stereocilia are more developed, whereas those closest to the stereocilia at this early stage of development are only beginning to form (Fig. 9). When decalcification with the EDTA chelator is used it becomes apparent that granular calcific components abundant in the endolymphatic space intermingle within the organic matrix (Fig. 10). Five distinct regions are seen as one moves away from the stereociliary bundle toward the outer edge of the otoconial membrane. The highest extraction of calcium occurs in the crystals that are farthest away from the stereociliary bundle, and the newly laid organic matrix has virtually no extraction by the chelation procedure.

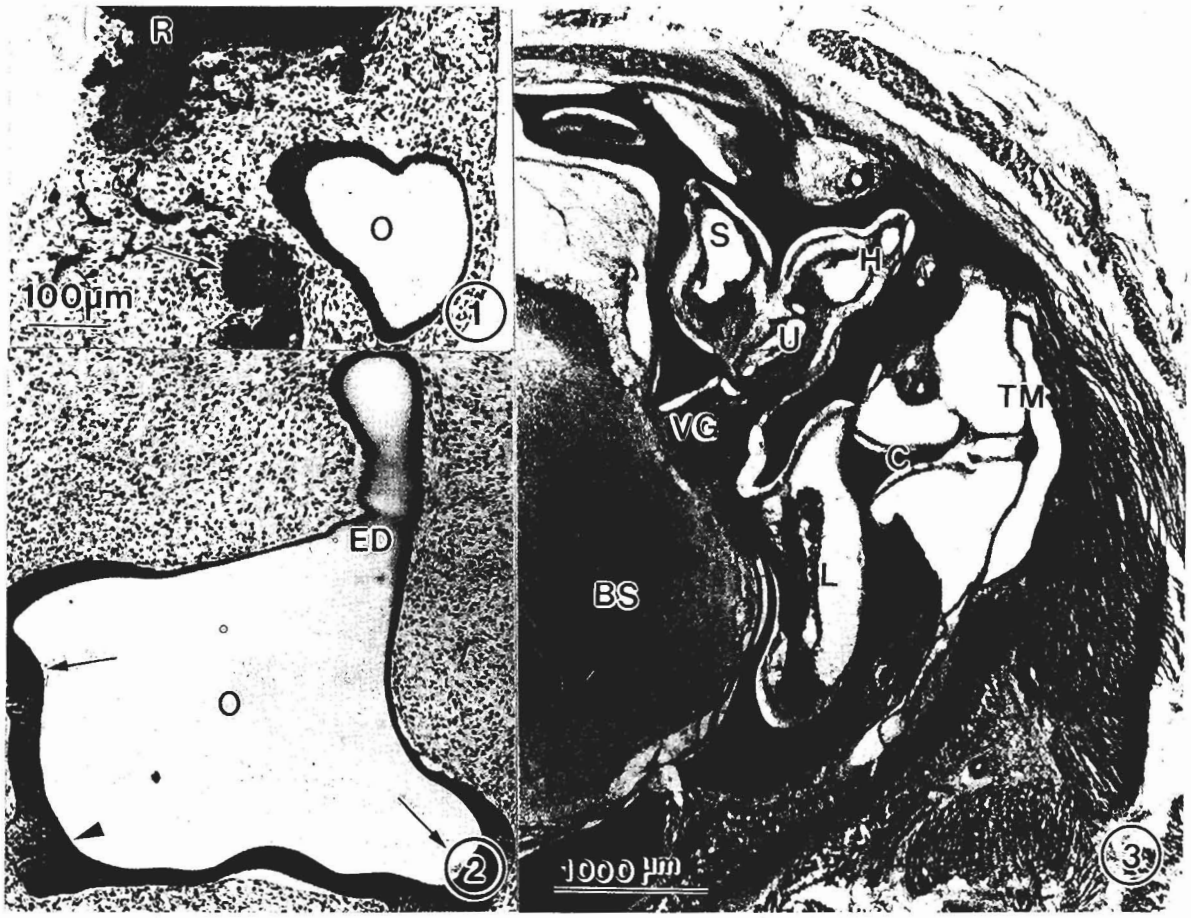


Fig.1. Three day old otocyst (O) near the rhomboencephalon (R). The presumptive VIIIth nerve is indicated by an arrow.

Fig. 2. Four day old otocyst with endolymphatic duct (ED) in the upper portion of the figure. The arrows indicate accumulation of organic secretory material over the presumptive crista (left) and saccule (right). The arrowhead points to the presumptive utricle.

Fig. 3. Near hatching the chick, which is a precocious animal containing all of the structures of the adult inner ear. Cross section of the head through the brain stem of the inner ear illustrates the main components. The brain stem (BS) is connected to the vestibular ganglion (VG) which sends bundles of fibers toward the utricle (U), the horizontal canal crista (H), and the superior canal crista (S). The inferior portion of the VIIIth nerve sends fibers toward the lagena (L) where the auditory organ is found. The modified stapes or columnella (C) connects the tympanic membrane (TM) to the oval window of the lagena.

Calcification of the otoconia

The gradual formation of the otoconia by the aggregation of organic components with subsequent calcification of the fibrils can be modified and sometimes halted by treatment of the embryos with substances known to interfere with calcium metabolism in the inner ear. Of those substances, the most commonly

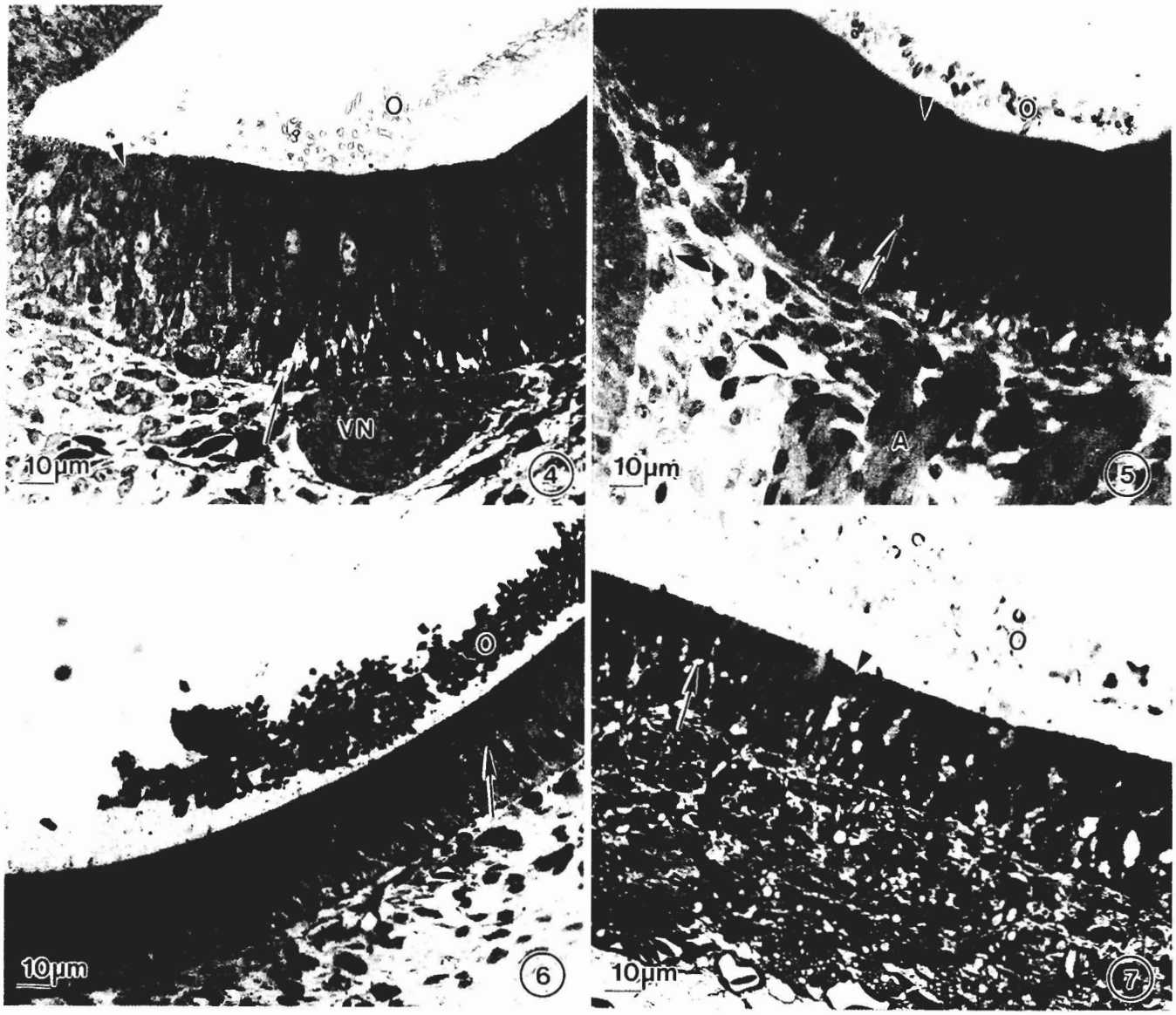


Fig. 4. Stage 29 (E5-6) the epithelia of the saccular macula with developing otoconia (O). The afferent dendrites are recognized as large spaces between the supporting cells, arrows inside of the basement membranes. The vestibular nerve (VN) is seen. Mitotic figures are identified at the apex of the epithelia when hair cells will appear.

Fig. 5. Short after stage 29, when afferent fibers (A) penetrate the basement membrane through the habenula perforata in travel toward the hair cells (arrow), mitotic figures (arrowhead) are found at the apex of the macula near the otoconia (O). Distinction between hair cells and supporting cells is already possible.

Fig. 6. By stage 36 (E10) when myelination has begun, distinction between supporting and hair cells (arrow) is very clear and otoconia are clearly distinguishable from the surrounding and non-calcified organic matrix.

Fig. 7. By stage 38 (E12), when afferent fibers are myelinated, and afferent fibers are now seen as swollen spaces at the base of the hair cells (arrows), mitotic figures (arrowheads) are still visible at the apex of the epithelia near the otoconial membrane (O). One of us showed that mitotic supporting cells occur even after hatching (Cohen and Fermin, 1978, 1985).

used by us is Diamox. In the presence of Diamox, otoconia organic matrix substances still accumulate (Fig. 11), but crystallization of normal otoconia fails to occur.

Forming otoconia attract granular components that stain positive with potassium pyroantimonate. The granules are of various sizes and usually aggregate at the periphery of crystals (Fig. 12). Chelation by EDTA of previously postassium pyroantimonate treated materials also removed the granules, in addition to calcific components from the peripheral portion of the otoconia. The central core remains relatively innert and unaffected by the procedure (Fig. 13). The calcific granules can be

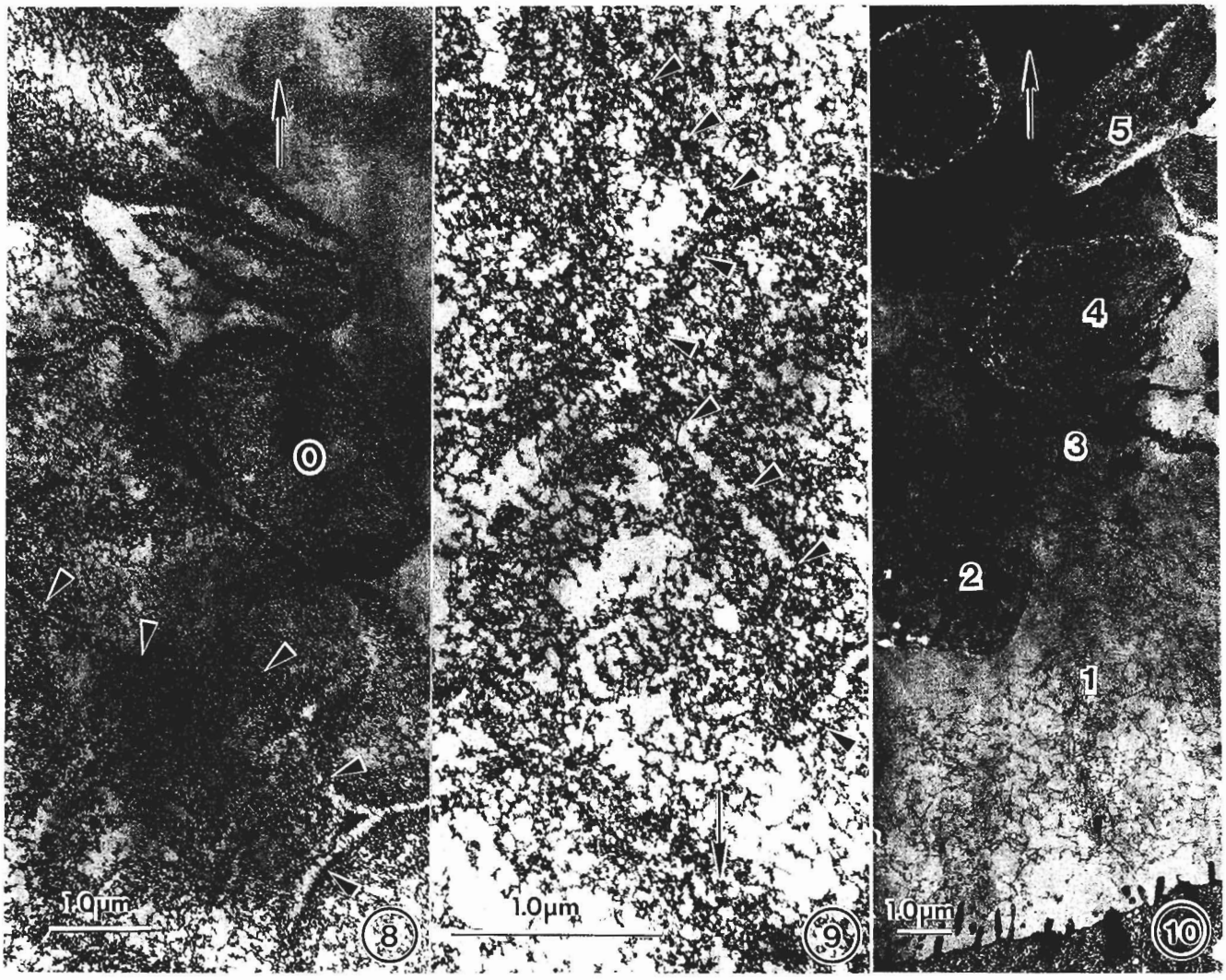


Fig. 8. Segmentation of the initial overlay of the organic matrix above the stereociliary bundle of the early stage 29 (E5-6) otocyst is illustrated in this electronmicrograph. The segmentation line (arrowheads) corresponds to the hexagonal angle face of the individual crystals. As one moves away from the stereociliary bundle toward the endolymphatic space (arrow), newly formed otoconia are seen as detaching from the segmentation lines.

Fig. 9. As one moves closer to the stereociliary bundle (arrow) the segmentation line becomes more disorganized than in the upper area of the otoconial membrane, Stage 29 (E5-6) resembling the fibrillar organization seen in the tectorial membrane (Cohen and Fermin, 1978, 1985).

Fig. 10. Five arbitrary divisions, some also shown by (Lim, 1979) can be made on the otoconial membrane at Stage 38 (E12) developing macular organ. **1.** On the first region near the stereociliary bundle, disorganized newly laid organic matrix that is not calcified is seen among granules of calcium, that were extracted and now appear as empty spaces. **2.** The second layer contains very disorganized fibrils that entrap cellular debris from the epithelium. **3.** The organic matrix cementing the individual otoconia is more organized than the newly laid organic matrix, but it is decalcified as illustrated by the lack of chelation in this area. In layer **(4)** of the otolithic membrane, some of the youngest otoconia have already began acquiring calcium as illustrated by the gradual chelation of the periphery of the otoconia. Note that the simple core remains untouched. Similarly, in layer **(5)** or the farthest area away from the stereociliary bundle (arrows), the central core remains untouched by the chelation. The peripheral area of the otoconia was heavily chelated suggesting that it had more calcium than otoconia on layer **(2)**.

observed in various regions of the otoconial membrane (Figs. 10, 14). Higher magnification of otoconia from the area proximal to the stereociliary bundle demonstrates the granules which are positive for the potassium pryoantimonate reaction (Fig. 15), suggesting that they may be calcific components, because they are readily extracted by the EDTA (Fig. 16). The central core of each crystal contains less calcific components than the peripheral portion. Alcian blue stain (Fig. 17) shows readily both the outside and inside of the otoconia. Since the stain was done prior to sectioning of the material, it is highly unlikely that the precipitate formed inside the central core were contributed by diffusion artifact. Consequently, a transition of the staining pattern should be obvious from the periphery toward the central core if caused by artifact. Therefore, it seems that calcific components that ultimately calcified the organic matrix are secreted in various portions of the membranous labyrinth. Potassium pyroantimonate reaction of the entire utricular area shows high precipitate content on the roof of the utricle, suggesting that the cells in this area may contribute to the production of a calcific component. At least, they may sequester calcium within as yet unidentified new molecular components (Fig. 18). The initial aggregation of organic fibrils is gradually intermixed with calcific components that are heavily stained with potassium pyroantimonate (Fig. 19). The intermixing of the calcific component thoroughly occurred in different directions, because sectioning through different portions of the otoconia do not show a repeating or symmetrical configuration that one would expect from the fractioned pattern of calcite, the prevalent crystal polymorph of birds' otoconia.

Ultrastructural and molecular units of otoconia

The components of the organic matrix and the remaining otoconial membrane substance that cement together the individual crystals is highly negatively charged. It reacts strongly with highly positively stains such as Ruthenium Red (Fig. 20). When the organic matrix that forms a cementing substance between the individual otoconia is removed by chlorox etching, the distinct hexagonal shape of each crystal becomes apparent (Fig. 21) with scanning electron microscopy views. The hexagonal characteristic shape of the large crystals is preserved at the ultrastructural level at very high magnifications. When otoconia are etched in chlorox and subsequently sonicated, pitts are made on the faces of the crystal and the resulting supernatant contains crystals that have the exact hexagonal shape of the large mother crystal (Fig. 22). The pits formed by the sonication procedure also yield hexagonal faces of the smaller subunits, that might represent fracture planes of the large otoconia. Unfortunately, the configuration of the organic material does not resemble that of a perfect crystal. Instead, the organic material is disorganized and resembles the secretion laid down early at Stage 26 (4 days of incubation). Nonetheless, intercollated within the organic material are small hexagonal subunits (Fig. 24), that may represent some of the smaller seeding nuclei for the subsequent formation of the calcific component of otoconia (calcite). It is now indisputible that calcium is the main inorganic component of bird and mammal otoconia. Calcium is detected with elemental anaylsis in dry specimens (Fig. 25) and in wet specimens fixed at -212 °C in liquid propane (Fig. 26). Freshly edged otoconia also appears to contain superimposed layers of combined organic/inorganic substances that may contribute to the orderly appearance of the crystallographic projection of the otoconia (Figs. 27, 28). It is clear, nonetheless, that the perfect crystallographic projection of the otoconia does not match the ultrastructural organization of the organic matrix. When thin sections of the organic matrix are tilted, it becomes clear that the fibrilles bend in different directions. This

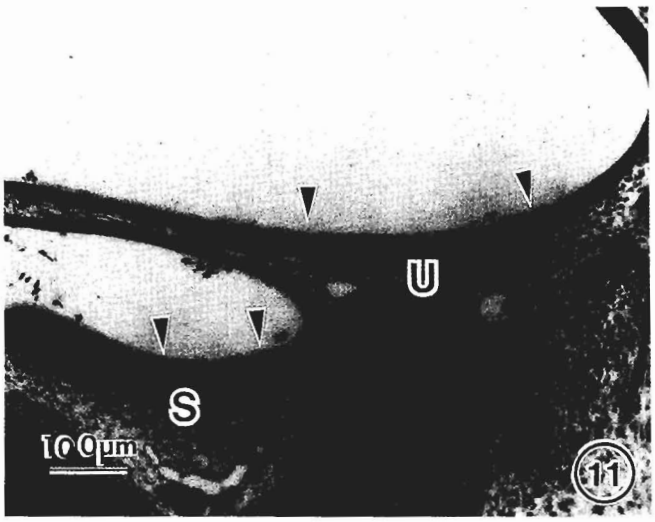
Fig. 11. The macula of the saccule (S) and the utricle (U) are seen in this micrograph devoided of otoconia at stage 39 (E13). A faint secretory layer overlaying the epithelia is seen (arrowheads in both structures) which corresponds to newly laid organic matrix. Sequestration of calcium to the organic matrix was inhibited by the injection of Diamox at Stage 31 (E7). According to previous estimates by others, chick otoconia are calcified at this stage, but we feel that most are not as evidenced by the figure above.

Fig. 12. In block staining of the entire inner ear with potassium pyroantimonate mixed with aldehydes yield calcium precipitate near the otoconia, but not in the endolymphatic space. The granules are of different size (arrowheads) and the largest ones are immediately attached to the periphery of the otoconia at stage 40 (E14).

Fig. 13. These granules are readily chelated from pyroantimonate reacted otoconia. Note (arrowheads) that the large granules on the periphery of this otoconia have been removed. Some still remain on the bottom portion of the otoconia. Nonetheless, the central core (C) remains untouched by the chelation procedure, suggesting that it is devoided of calcium at stage 39 (E13).

Fig. 14. In the standard 1 μm thick section prior to electron microscopy analysis, the central core of each otoconia (O) appear lighter. Some areas of the otoconia cut conventionally through the edge show the characteristic-denser appearance that is often seen on these preparations. The utricular (U) epithelia is well fixed, the fibrous mesh of the otoconial membrane is attached to the microvilli of the supporting cells. Crystals far away from the microvilli appear free and detatched from each other because the intraotoconial organic matrix that hold them together is usually extracted by prolonged aldehyde fixation.

Fig. 15. Enlargement of otoconia shown by arrowheads in Fig. 14. Note the large number of round granules that may correspond to a calcific component being sequestered toward the otoconia. The central core (C) and peripheral (P) of the crystals are preserved as well as the intraotoconial organic matrix that holds them together. The dense granules (arrowheads) may represent calcific components.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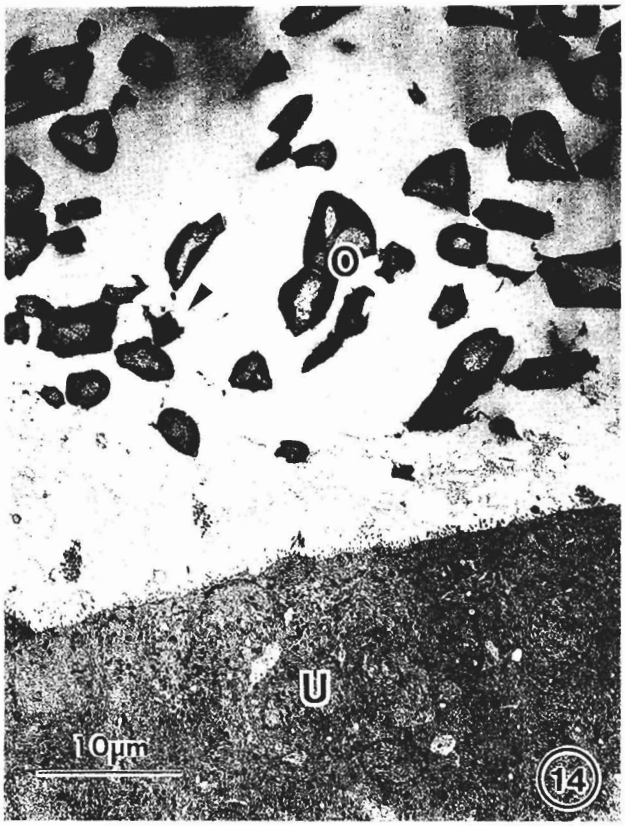
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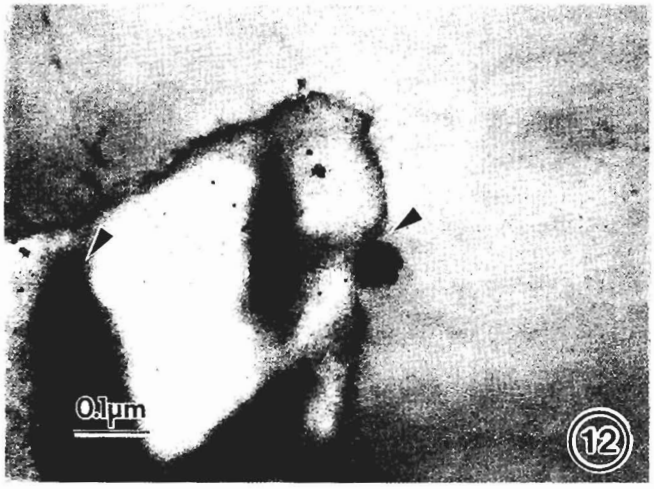
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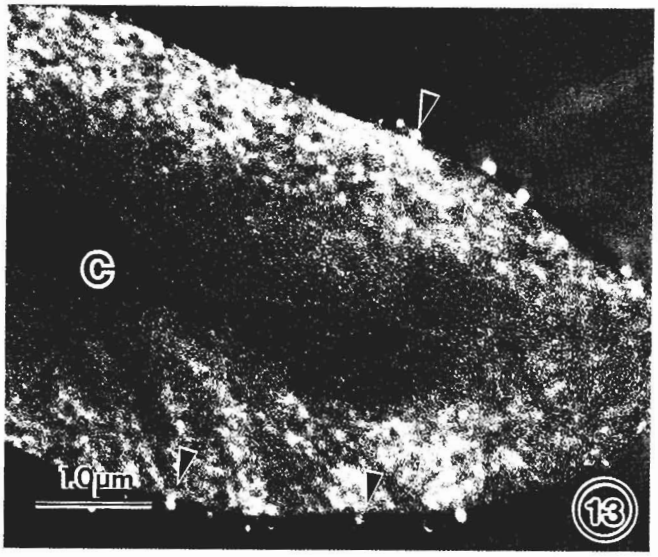
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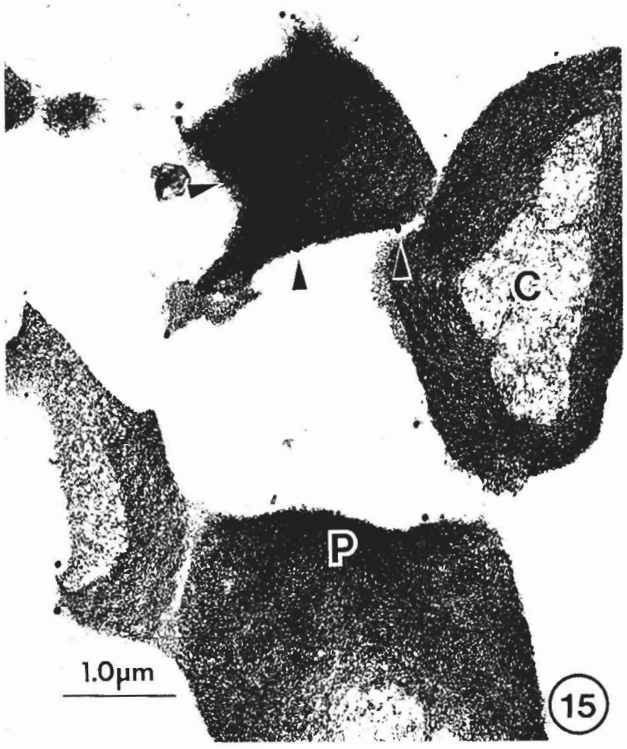
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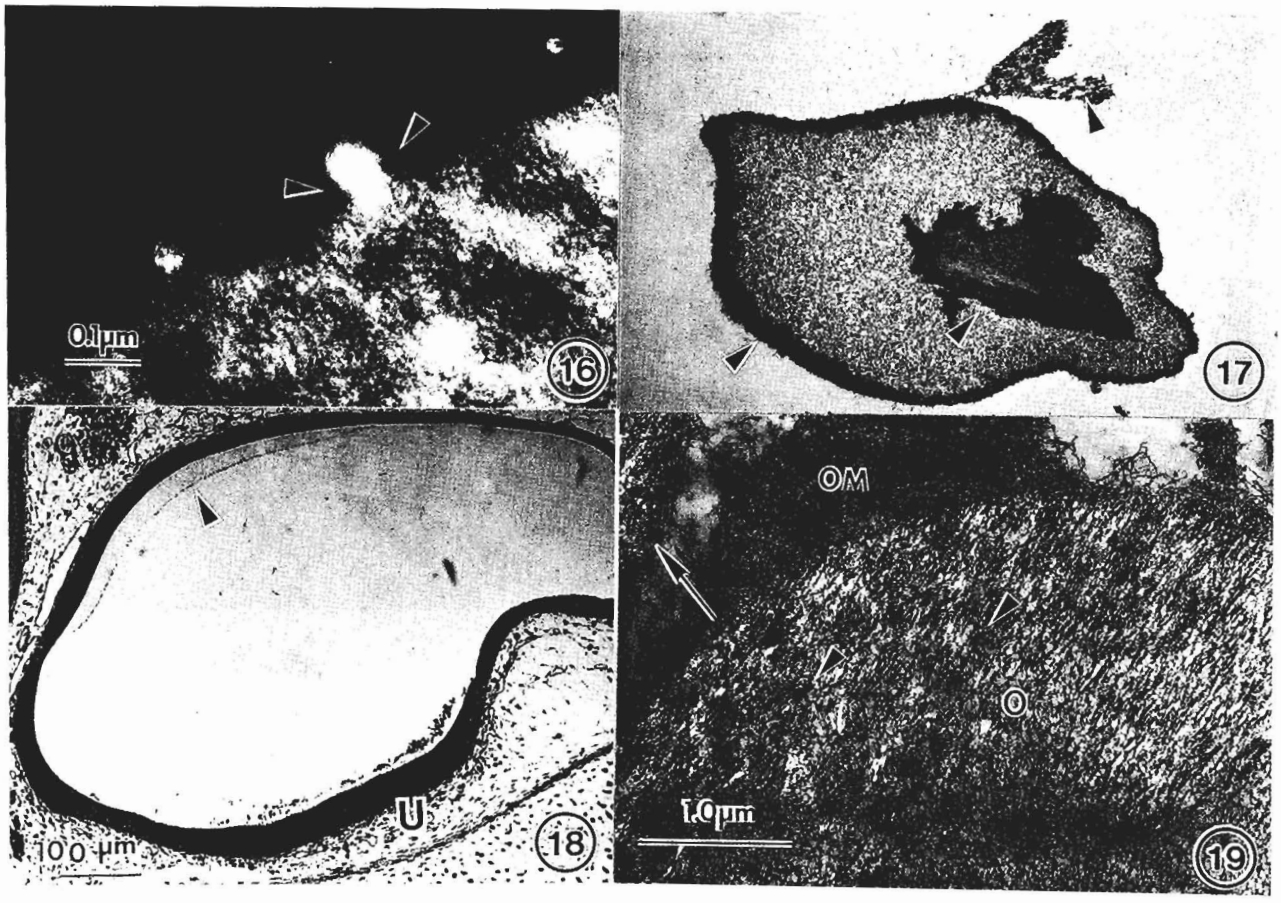


Fig. 16. The granules may provide a source of calcium molecules that are subsequently intermingled between the organic fibrils seen here at this high magnification. Note that the space vacated by chelation of the granules correspond more or less to some of the clear areas seen inside the otoconia crystals.

Fig. 17. When the otoconia are reacted in situ (in block) with Alcian blue which is known to react specifically with glycoproteins the central core is more heavily stained than the remaining otoconia, except for the very outer periphery, suggesting that different portions of the otoconia contain proportions of glycoproteins and glycosaminoglycans as demonstrated in our previous studies (Fermin, 1993; Fermin et al., 1995b).

Fig. 18. Calcific components needed for the turnover of calcium may also be sequestered to other areas of the macula away from the otoconia matrix, and retained as a reservoir that can be mobilized faster than if a secretion was required (arrowhead).

Fig. 19. In potassium pyroantimonate stained otoconia (O), the organic matrix (OM) that cements otoconia together remain largely unreactive. On the other hand, the fibrilles of the otoconia are intercollated with large amounts of dark material that suggests the presence of calcium, some of which are in between the organic matrix component (arrowheads). This is also seen at the periphery of individual otoconia (arrow).

Fig. 20. The epithelia (E) of the macula remain largely unreactive with histochemical stains such as Ruthenium Red. The central core of the otoconia is also unreactive, but the periphery is highly reactive suggestive of glycosaminoglycans which are highly negatively charged molecules.

Fig. 21. When the organic components around the otoconia are removed the perfect hexagonal shape of the otoconia is revealed with the angle faces (arrowheads) that provide the characteristic shape of the crystals.

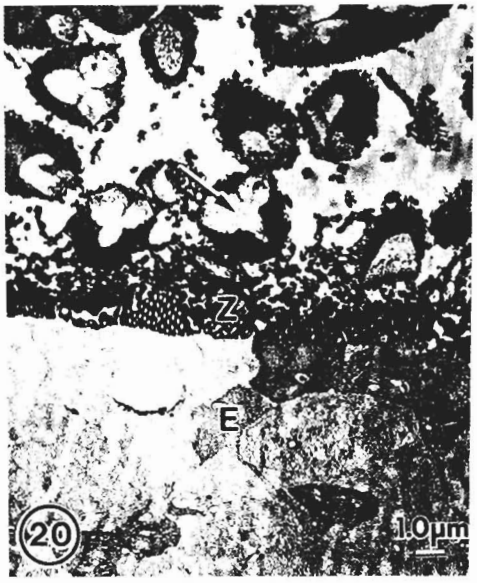
Fig. 22. Subunits of otoconia retained the hexagonal shape even at the ultrastructural level shown on this micrograph (arrowheads). These subunits organization suggests that crystallographic properties of the calcific components remain intact in vivo, but are disturbed by chemical fixation.

Fig. 23. Pitts created by sonication after exposure to chlorox also illustrates the angle face of the otoconia seen in cross sections and at the scanning microscope level. The stratification corresponds to pattern shown from cleaved calcite (Mann, 1986, 1988; Lima-de-Faria, 1995a).

Fig. 24. Small hexagonal subunit can be seen at very high magnifications of the organic matrix. The subunits (circles) may represent some of the smallest seeding nuclei for the formation of the calcite crystals that characterize the otoconia.

Fig. 25. Elemental analysis calcium content of dry otoconia showing that calcium is the main inorganic component of birds otoliths.

Fig. 26. Elemental calcium content of wet otoconia fixed in liquid propane. In wet otoconia (no chemical processing) calcium is the main inorganic component.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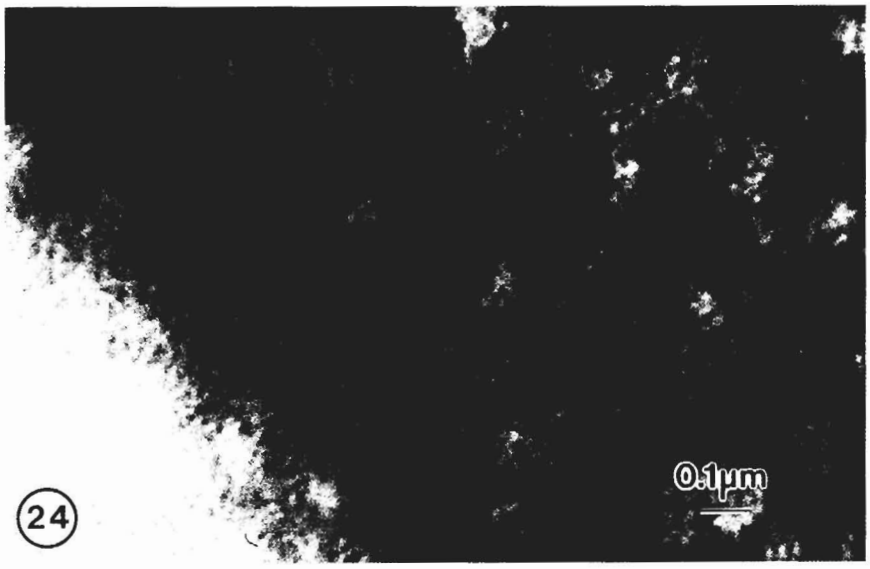
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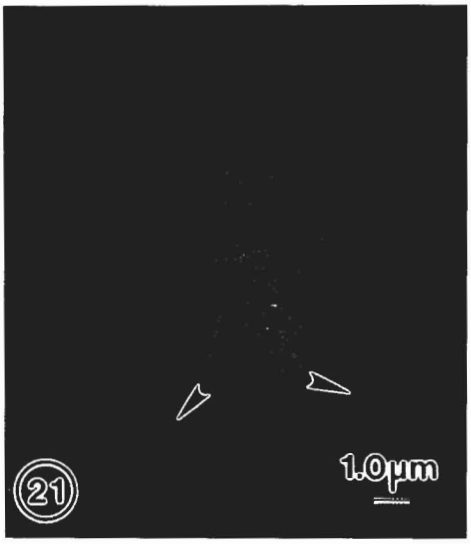
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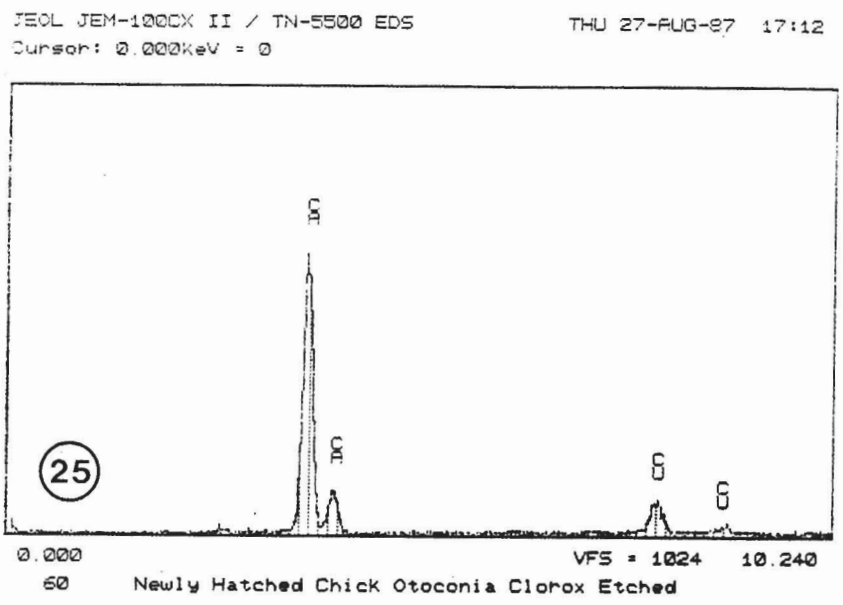
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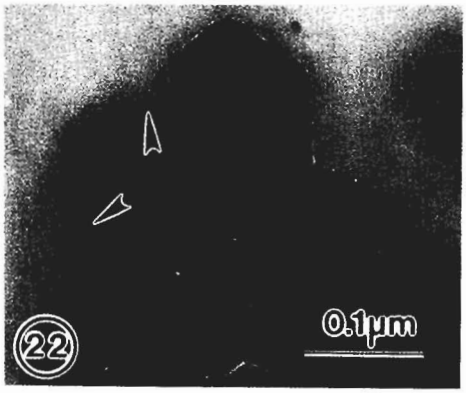
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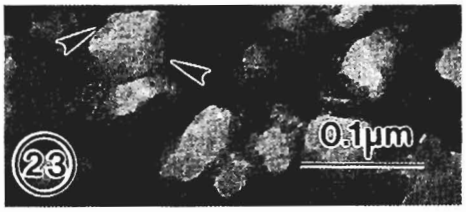
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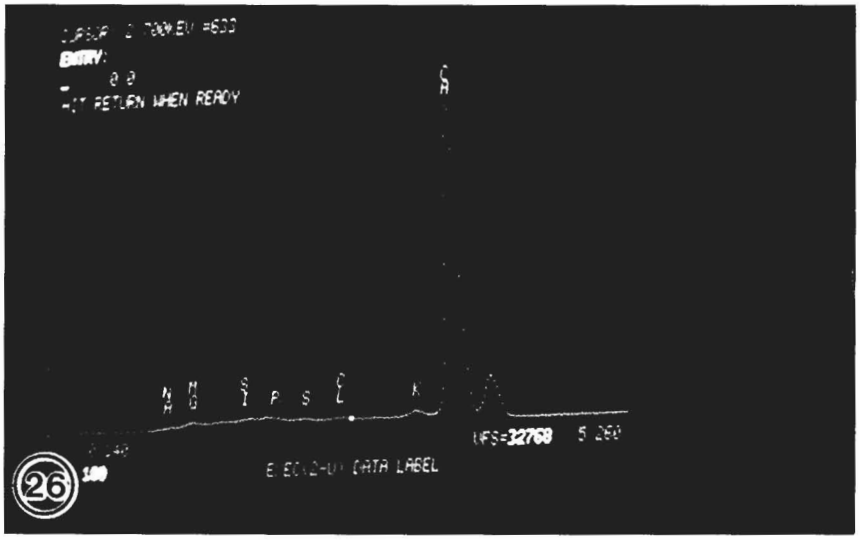
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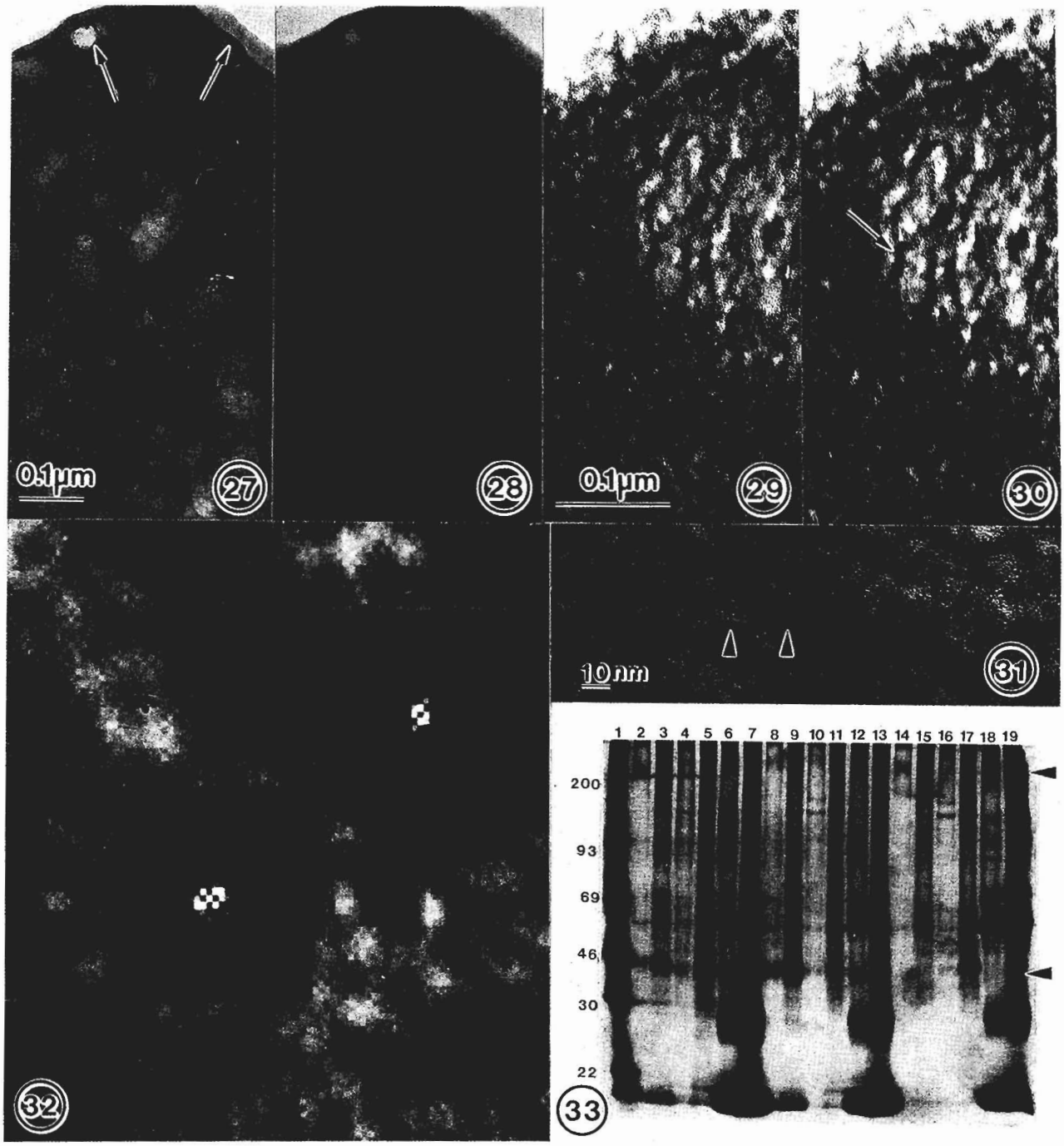


Figs. 27-28. As illustrated in Fig. 22, it appears that there is superimposition of the organic matrix and possibly of the inorganic components that calcify otoconia and can be appreciated in this stereopair (arrows).

Figs. 29-30. Stereopair. In addition to the superimposition of layers, otoconia may also contain intercollated intersecting subunits (arrows) that may contribute to the sharp edged-angled face of the hexagonal crystals.

Fig. 31. Newly hatched otoconia reacted with phosphotungstic acid (PTA) and fixed in aldehyde show electron dense areas with clear areas that may correspond to the places where the seeding of the calcium takes place. Forward Forrier Transform (FFT) analysis of this area with the imaging program Optimas from BioScan shows very distinct patterns for these areas of the organic matrix, suggesting that they indeed run in different directions. At least, the molecular components are arranged in different patterns.

Fig. 32. When high resolution transmission electron microscopic images such as shown in Figure 31 are digitized and pixelized to higher magnifications, the alternating dark and light patterns of the organic fibrils illustrated also in Figure 19, is enhanced. FFT analysis of dark (top) and light (bottom) areas yield two distinct FFT with non overlapping geometries, suggesting that the arrangements of the fibrils at those points is different. This type of arrangement may contribute to the turning of the fibrils and to the central core formation as discussed earlier by one of us (Fermin, 1993; Fermin et al., 1995b), and further explain part of the paradox between the perfect crystallographic projections of the crystals and the disorganized patterns of fixed fibrils (Fermin and Martin, 1994).

Fig. 33. Otoconia contains little protein. They contain, however, large amounts of polyglycosaminoglycans and glycoproteins. Most of the proteinic components found in otoconia preparations are contributed by contamination from tissue and/or blood (arrowheads). In this SDS page separation the following is illustrated. All samples were run in duplicate. Lane 1 is for the markers; Lanes 2 and 3 are otoconia; Lanes 4 and 4 are tissue; and Lanes 6 and 7 are blood. There is no difference in the profile of the components between 14 days of incubation and 5 days after hatching, as illustrated by this gel. Lanes 2-7 are shown at 14 days of incubation; Lanes 8-13 are shown at 16 days of incubation, and Lanes 14-19 are shown 5 days after hatching.

bending may be the result of intercepts of various planes at different angles (Figs. 29, 30). The intersection of various planes of organic matrix yield dense and light areas of electron density (Fig. 31) that yield very distinct Forward Fourier Transformed (FFT) images (Fig. 32).

The majority of the components in the organic matrix are glycoproteins and polylactosominoglycans (Fig. 33). They react strongly with cationic stains such as PTA (Fig. 34), and in not fully mature otoconia, phosphotungstic acid stained the organic matrix and the periphery of the otoconia intensively, but failed to stain the central core. The organization of the organic materials in the crystals of phosphotungstic acid fixed otoconia is very different from the counterpart matured (newly hatched) otoconia. Spherical granules (Fig. 35) that might correspond to calcific components are seen attached to the otoconia and within the otoconia body themselves. A stereopair of otoconia at this early stage

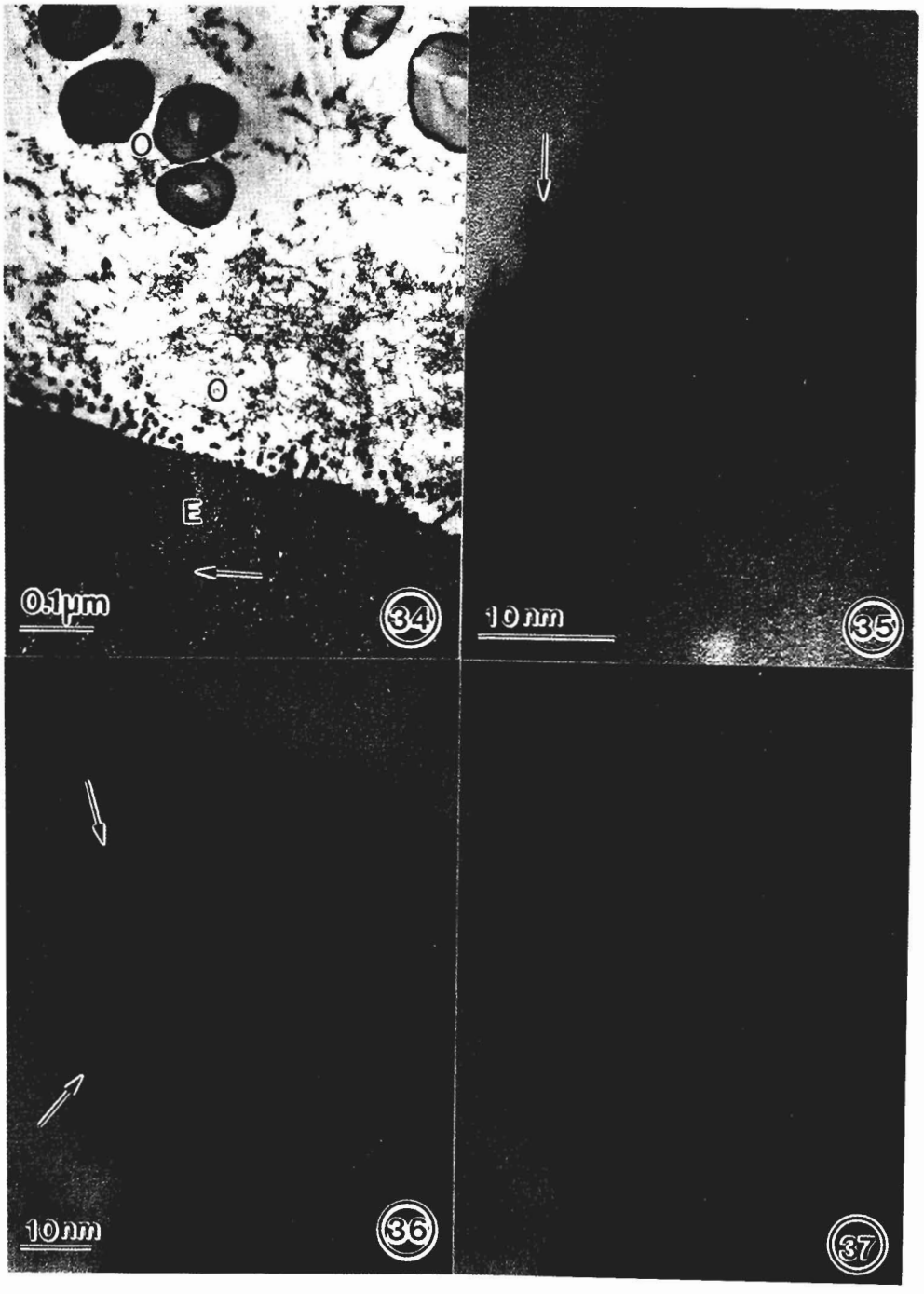


Fig. 34. 13-day-old embryos (stage 39) reacted with PTA during fixation shows a strong reaction of the glycoprotein rich organic matrix (O), immediately above the epithelium (E). Note the ultrastrural preservation of mitochondria (arrow) indicating that fixation was not a problem. The central core remains as a visible and reactive entity. Also note that otoconia shape is not perfectly hexagonal. Please contrast peripheral versus central core stains in this and Figure 43 stained with PTA, Figure 17 which was stained with Alcian blue, Figure 20 with tannic acid, Figure 44 reacted with glutaraldehyde and osmium without histochemical decoration.

Fig. 35. Granules that may be calcific in nature are also seen in PTA, both at the periphery and inside the otoconia.

Figs. 36-37. This stereopair demonstrates the presence of the granules at different levels of the otoconia crystal, at this early stage 39 (E13) in development. These granules (shown above magnification bar) were also observed in known PTA reactive otoconia.

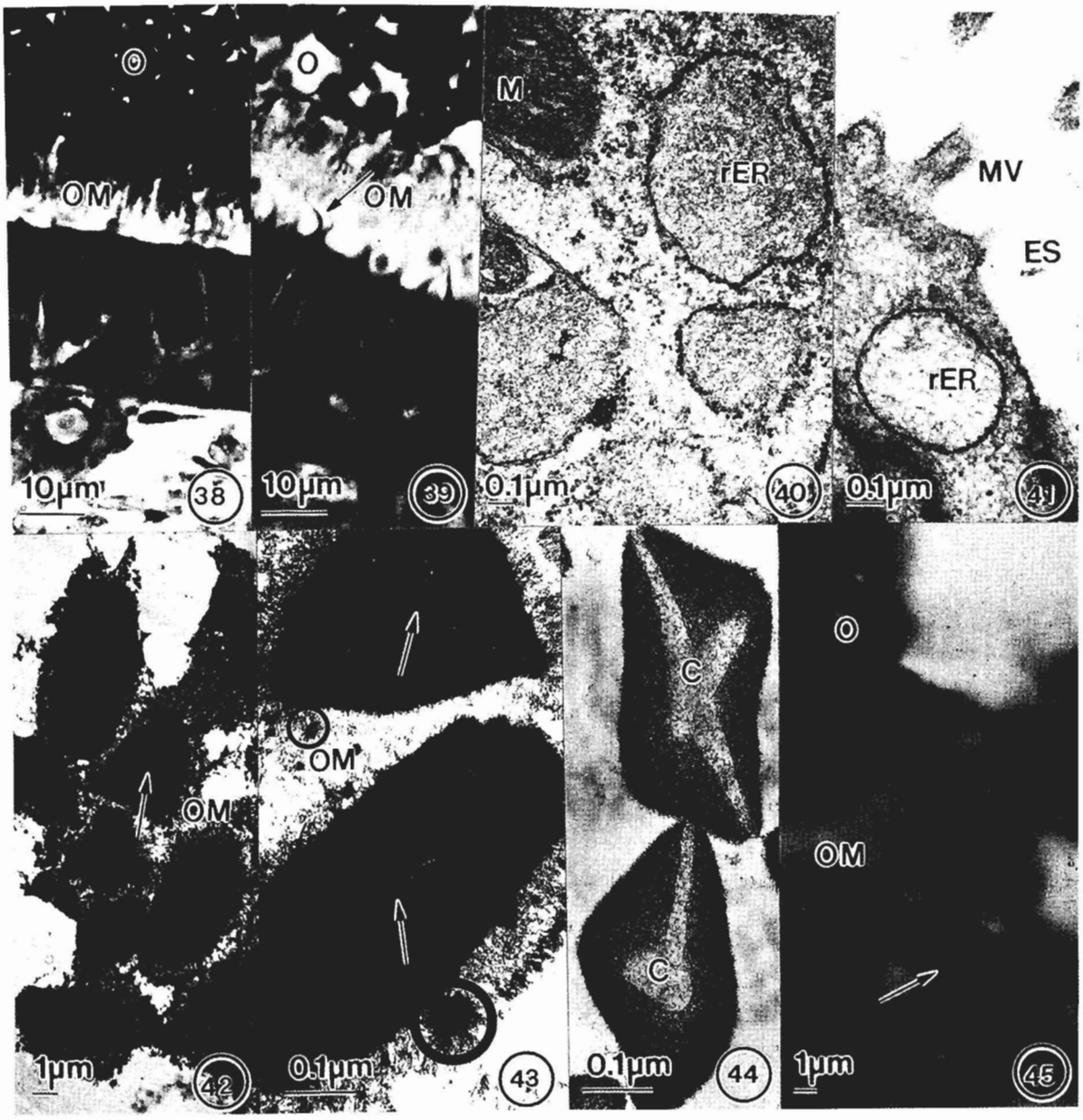


Fig. 38. Methacrylate plastic fine micron thick sections stained with toluidine blue and basic fuchsin. The large afferent fibers are clearly seen crossing the habenula perforata and surrounding two type I hair cells, and three type I hair cells on the left. The otoconial membrane (OM), for instance appears as a clear area between the epithelia and the otoconia themselves (O).

Fig. 39. Methacrylate plastic 5 μm thick sections stained with toluidine blue and paraphenylene-diamine after osmium tetroxide oxidation to enhance the density of the epithelia including the cilliary bundle (arrow), the otoconial membrane (OM) and the otoconia (O). When the tissue is ossmicated the stereociliary bundle becomes more apparent, and bending of the bundles due to the load imposed by the otoconia (O) over the otolithic membrane (OM) is clear at the location of the arrow.

Fig. 40. TEM of a supporting cell in the utricular macula with distended rough endoplasmic reticulum (rER) cistern filled with fibrillar material similar to that forming the otoconial membrane. A mitochondrion with intact crista (M) attesting to the quality of fixation is shown.

Fig. 41. The rER cisterns move close to the endolymphatic space (ES) where the material is often seen attached to the microvilli (MV). Similar type of secretory features were described for the auditory portion of the chick inner ear (Cohen and Fermin, 1978, 1985), and are probably present in the vestibule too during active secretory stages 34-40 (E8-E14) of incubation.

Fig. 42. Otoconia reacted with phosphotungstic acid showing the characteristic cat's eye appearance of the central core and its branches (arrow), the less dense otoconial matrix fibrils that cement otoconia (OM) and numerous dense deposits thoughout.

Fig. 43. Higher magnification phosphotungstic acid stained material near the hatching stage 46 (E29) showing the typical cat's eye appearance of the central core (arrow) and two dense deposits (circles) near or in contact with the crystals. They may represent calcium deposits similar to those shown before by (Salamat et al., 1980) in the rat (Campos et al., 1984) and older species (Lychakov, 1988a,b, 1997).

Fig. 44. In standard lead-uranyl staining after glutaraldehyde and OsO_4 fixation the central core (C) is more pronounced probably due to extraction and/or weaker cross linking of the macromolecules that are fixed in place by phosphotungstic acid. The central core may contribute to buoyancy of crystals in the endolymph.

Fig. 45. Regardless of the technique used, the central core (arrow) is apparent in otoconia (O) of the otoconial membrane (OM) as illustrated here in a sample that was quickly frozen in liquid propane at -212 °C.

of development demonstrates the intermixing of the granules within the organic matrix (Figs. 36, 37).

Relationship between the epithelia and the otoconial membrane

Newly hatched otoconia (mature) are intensively stained with the standard histological stains. When the inner ear is embedded in methacrylate plastic and sectioned at 5 μm of thickness, the structures of the entire macula are appreciated. The afferent fibers penetrate into the habenula perforata and wrap around several hair cells Type I clearly seen as the hair cells emerge with the stereociliary bundles at the edge of otoconial membrane (Fig. 38). At the places where the otoconia is heaviest, bending of the stereociliary bundle by the way of the otoconia is apparent in osmicated material (Fig. 39). The most likely contributor to the organic material that forms the lower layer of the otoconial membrane and cements the otoconia together are rough endo-plasmic reticulum cisterns that contain proteinaceous secretory materials (Fig. 40). The rough endoplasmic reticulum cistern can also be seen at the apex of the supporting cells and often discharges its

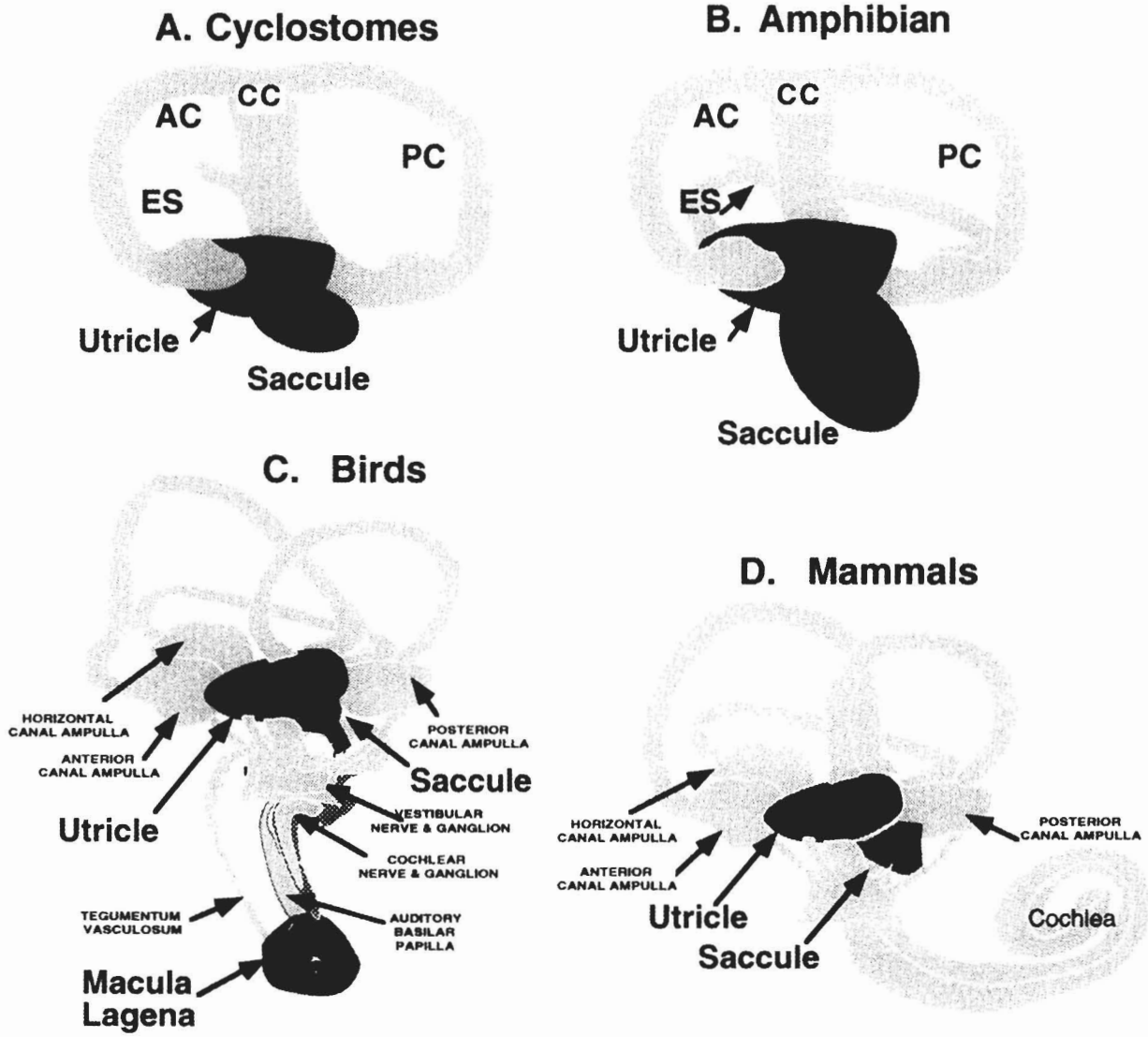


Fig. 46. Summary diagram showing transition in structure forming the membranous labyrinth from cyclostomes to mammals. From the absence of horizontal semicircular canal in cyclostomes to the complex cochlea of mammals, the vertebrate inner ear retains the saccule and the utricle. The saccule is prominent and bulky up to amphibian, but in birds and mammals, the utricle is larger. In some lower vertebrate species, the saccule serves an auditory function and it is possible that its size reduction in higher vertebrates be related to the more sophisticated means of detecting sound waves through a cochlea acquired by birds and mammals. The prominent macula lagena (Jorgensen, 1970; Lavigne-Rebillard et al., 1985) which is a vestibular structure and probably developed from the saccule, is difficult to reconcile when the mammalian and the avian membranous structures are compared side by side.

contents into the endolymphatic space (Fig. 41). Contrary to the appearance of the organic matrix of younger otoconia, newly hatched otoconia processed in a similar manner to those illustrated in Figs. 35-37 show a very distinct appearance. The organic matrix is denser, and the cementing organic material around each otoconia is very heavily stained (Fig. 42). Nonetheless, the central core with characteristic "cat eye" appearance is clearly visible, despite the intensity of the stain's reaction. In addition, granules similar to those illustrated previously in Figs. 16, 24, and Figs. 35-37 are clearly visible in PTA stained material (Fig. 43). The central core of each otoconia remains as a very characteristic marker of the crystal, in non-histochemical stained specimens (Fig. 44). The central core is also visible in otoconia quickly frozen at -212 °C in liquid propane, suggesting that the central core is an integral portion of the otoconia and may serve an important function.

Evolution of the otoliths

The vertebrate otolithic membrane is formed by: 1) a gelatinous layer, 2) a subcupular meshwork, and 3) an otolithic apparatus proper (Fig. 46). The otolithic apparatus (also called otolith) may contain numerous small crystalline otoconia, a single large crystallite otolith, or a combination of the two. Despite otoliths' similar function across species, their mode of formation, shape, size, mass, growth, consistency, chemical and crystallographic properties vary considerably (Carlstrom, 1963; Lim, 1974b; Popper and Hoxter, 1987; Lychakov, 1988b, 1995b; Pote and Ross, 1991). The structural variability of the otolithic membrane follows certain evolutionary trends (Carlstrom, 1963; Lychakov, 1988b, 1995b) from the old to most recent species. In modern species the process of otoconia formation seem to recapitulate the morphological characteristics of older species.

The appearance of the otoliths during evolution is connected with the need of vertebrates to have a constant growing and load mass in the otolithic organs to stimulate the receptor hair cells of the macular epithelia (Lychakov, 1995b). In living nontetrapopds there are three types of otoliths: a) the single amorphous otolith which arises by fusion of otoconia; b) the composition otolith which is essentially a conglomerate of otoconia, and c) the single polycrystalline otolith (Lychakov, 1994a,b).

a) Single amorphous otoliths (Figs. 47, 48) are found only in the otolithic membrane of lampreys (Tret'yakov, 1915; Carlstrom, 1963; Volk, 1986; Lychakov, 1988b, 1995b). The lamprey otoliths are formed by fusion of a large number of spherical otoconia. There are no boundaries between the fused otoconia of the lamprey otolith (Fig. 49). The lamprey otoliths grow by addition of the newly-formed spherical otoconia to the base of the otolith (Carlstrom, 1963; Volk, 1986; Lychakov, 1995b). The otoliths and the spherical otoconia of the lamprey consists predominantly of calcium phosphate in its non-crystalline form (Carlstrom, 1963). Considering the phylogenetic position of the *Cyclostomata*, the lamprey otoliths may be the nontetrapod ancestral otoliths.

During nontetrapod evolution some modification of the chemical composition of the otolithic apparatus occurred. In contrast to the otolithic apparatus of the *Cyclostomata*, all otoliths and otoconia of the *Gnathostomata* consist primarily of calcium carbonate in various crystalline forms: calcite, aragonite, vaterite and calcium carbonate monohydrate (Carlstrom, 1963). The

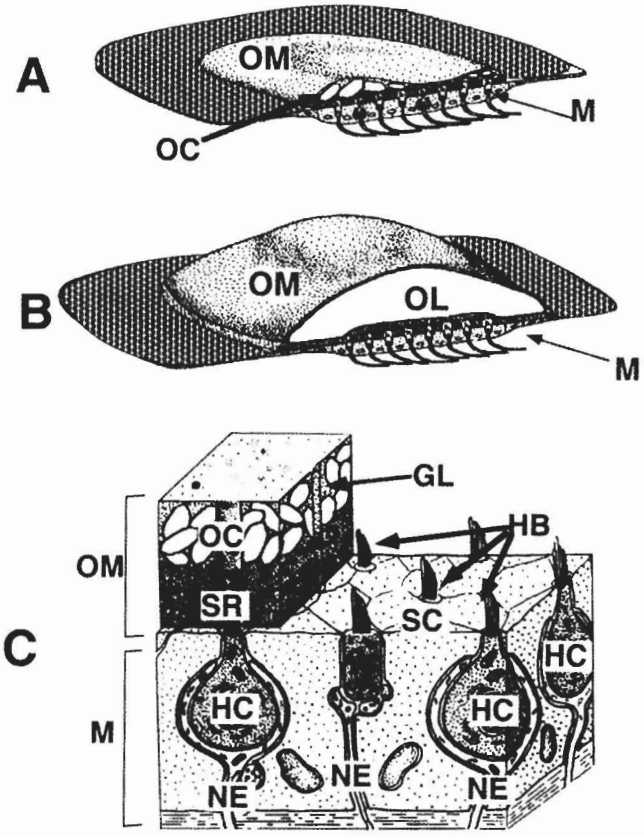
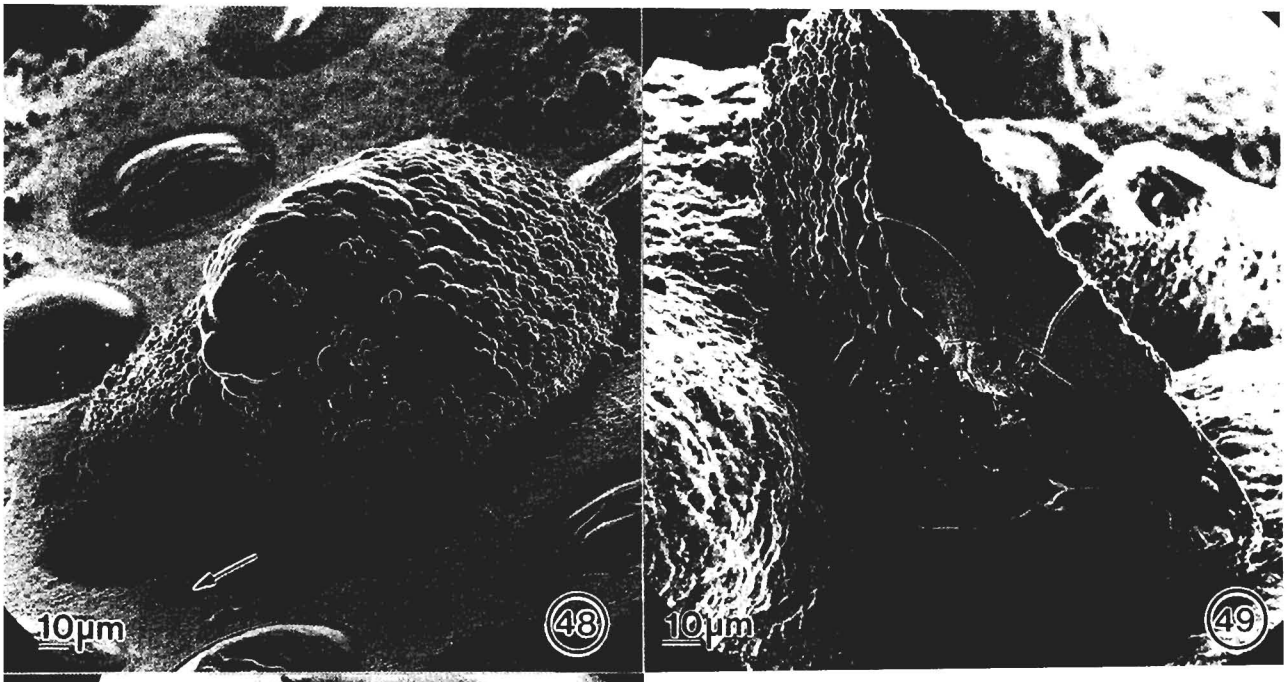


Fig. 47. Diagrams showing structure of the otolithic membrane in mammals **(A, C)** and fishes **(B)**. The otolithic membrane (OM) covers the macula (M) which consists of receptor hair cells (HC) and supporting cells (SC). The otolithic membrane consists of an otolithic apparatus, represented usually by a set of small crystalline otoconia (OC) or by a single large crystalline otolith (OL). A gelatinous layer (GL), on which the otolithic apparatus lies and which joins the otoconia together and covers them (or the otolith) on the side of the endolymphatic space, the subcupular reticulum (sr) the principal structural components of which are thin filaments connecting the gelatinous layer with the surface of the macula. Because of density differences, the displacement of the otolithic membrane under the influence of linear accelerations, including gravity, differs in amplitude and phase from that of surrounding tissues and macula. Displacement of the otolithic membrane relative to the macular epithelia results in the inclination of hair bundles (hb) of receptor cells. Inclination of hair bundles produce physiological response of receptor cells and, thus, the information about movements, vibrations, or head inclinations is transformed into excitation of receptor cells and nerve endings (ne) (Lychakov, 1988b).

probable explanation for this difference is that in *Cyclostomata*, unlike in other vertebrates, bony tissue and associated mineralized formations containing calcium phosphate are absent (Lychakov, 1988b). The use of calcium phosphate as the main substrate in fishes and tetrapods suggests that humoral factors that normally affect the bony tissue and formations would also affect the otolithic apparatus as well. Instead of creating an additional system of internal homeostatic balance these animals followed and acquired an independent otolithic system, with calcium carbonate as substrate. The occurrence of carbonate in lamprey otoliths (Carlstrom, 1963) could has been considered as a contributory factor for the evolution of chemical composition of the otolithic apparatus. Changing of the inorganic component and appropriate changing of the organic components produced better physical properties of the otolithic apparatus. Calculations of river lampreys otolith composition showed that the calcium phosphate content is 53.7%, whereas otoliths of fishes have



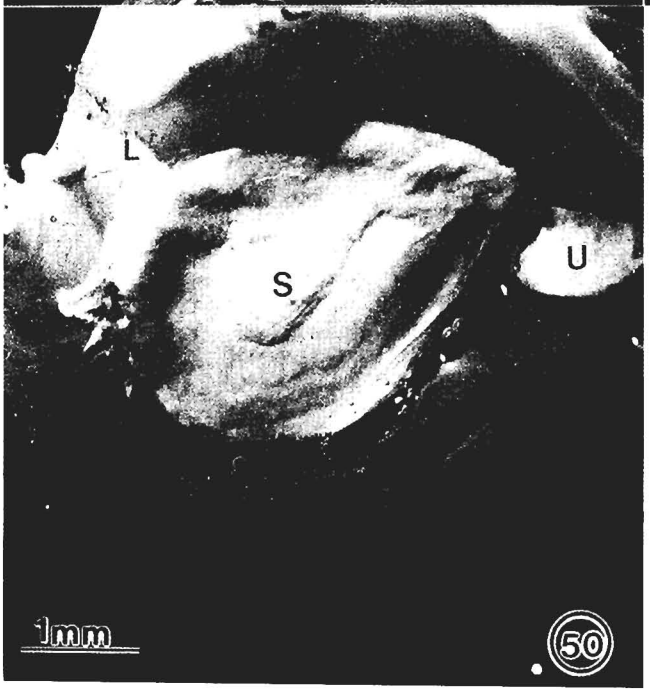


Fig. 48. Large utricular otolith of river lamprey *Lampetra fluviatilis*. The surface fused otoconia differ in size. The largest ones are situated on top of the otolith. The smallest otoconia are situated in the otolith base, facing the macula. Arrow marks rostral direction (Lychakov, 1995b). The organization resembles that seen in forming avian otoconia (Figs. 8-10).

Fig. 49. Utricular otolith of river lamprey *Lampetra fluviatilis* split in the middle. The otolith is homogeneous inside and there are no boundaries between single fused otoconia (Lychakov, 1995b). This arrangement resembles the granular incorporation of calcium positive granules we observed in avian otoconia (Figs. 12-16) and reported previously (Fermin and Igarashi, 1985b, 1986). The difference between the old and the new species (Lamprey and Birds) is that in the bird accumulation occurs at the single crystal level whereas in older species accumulation (accretion) occurs onto a single large otolith.

Fig. 50. Opened right labyrinth of *Raja clavata* (light microscopy). After dissection of auditory capsule and removal of the lateral wall of the membranous labyrinth, the large compositional otoliths could be seen with the naked eye. The saccular (S) and utricular (U) compositional otoliths look like a white dense lamina. The lagenar (L) otolith departs as a small process from saccular compositional otolith (Lychakov et al., 1985).

calcium carbonate content of between 90.9% and 96.3% (Lychakov and Lavrova, 1994b). Consequently, the density of the otoliths of the *Gnathostomata* is higher than that of the *Cyclostomata*. It is probable that the change in the chemical composition of the otolithic apparatus in a common ancestor of fishes permitted otoconia to remain unfused. However, the need for a large continually growing load mass in the otolithic organs was retained along the evolutionary ladder (Lychakov, 1988a). The solution to the need of continuously adding more weight to the otolith was the creation of the composition otolith.

b) The composition otolith is a conglomerate of individual otoconia. Gelatinous material joins the otoconia together. The otoconia in the composition otoliths are joined together to varying degrees among

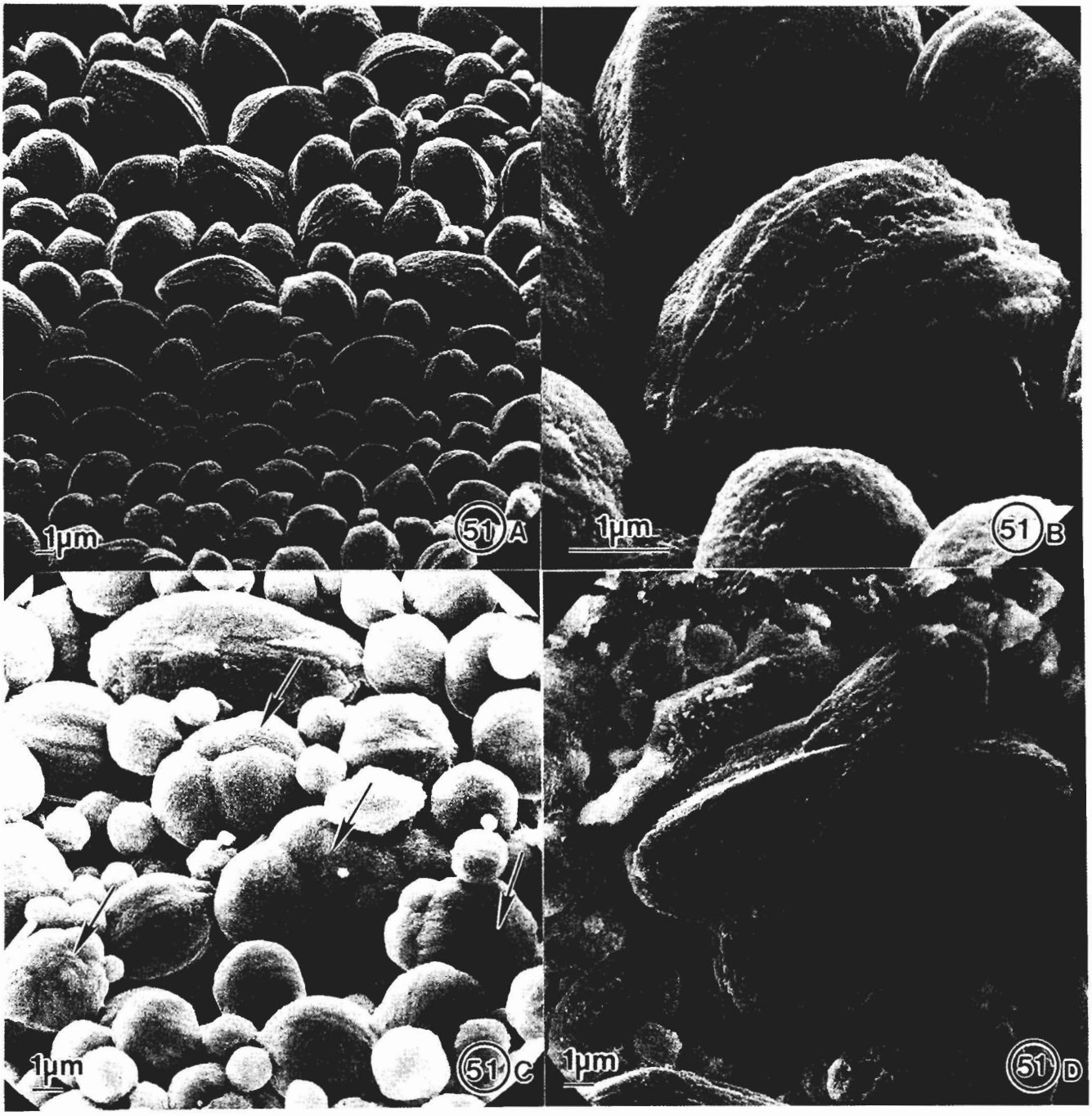


Fig. 51. Otoconia of saccular compositional otolith in *Raja clavata*. As a rule, the otoconia are almond-shaped or spherical **(A-B)**. Some otoconia look like fused spherules (arrow) **(C)** or have cross-shape **(D)** features demonstrated previously by Lychakov et al. (1985).

different species (Carlstrom, 1963; Lim, 1974b; Barber and Emerson, 1980; Lychakov et al., 1985, 1988b; Gauldie et al., 1986; Bulog, 1989; Muligan et al., 1989; Mulligan and Gauldie, 1989; Hanson et al., 1990; Lychakov, 1995a, 1997). In some type of otoliths, otoconia are often packed layer by layer into a large amorphous mass. The composition otoliths usually have the form of a thick lamina or agglomeration, spherical or irregular in shape (Figs. 50-54). Some cartilaginous fishes can increase the otolithic mass by allowing small grains of sand to enter the labyrinth and join into the otolithic membrane (Fig. 53). For instance, the endolymphatic canal of *Elasmobranches* is connected to the body surface, and opens externally in some sharks and rays (Carlstrom, 1963; Lim, 1974b; Lychakov et al., 1985; Hanson et al., 1990). The composition otoliths are typical in the otolithic organs of the *Chondrichthyes*, the *Ceratodiformes,* in the utriculus and lagena of the acipensers, and also in the sacculus of amphibians and reptiles (Carlstrom, 1963; Lim, 1974b; Barber and Emerson, 1980; Gauldie et al., 1986; Lychakov, 1988b; Bulog, 1989; Muligan et al., 1989; Mulligan and Gauldie, 1989; Hanson et al., 1990; Lychakov, 1995a, 1997).

In the course of otolithic evolution, bony fishes developed a new mechanism of otolith embryonic development and maturation, which coincided with the appearance of single polycrystalline otoliths. The single polycrystalline otolith (Figs. 55, 56) is a solid crystal that varies among species (Carlstrom, 1963; Lim, 1974b; Lychakov, 1988b) but generally consists of calcium carbonate in its aragonite form (Carlstrom, 1963).

c) The single polycrystalline otolith possesses several newer characteristics than the amorphous and compositional otoliths (Lychakov, 1988b, 1995b). First, the density of the single polycrystalline otoliths is higher than that of the other two types of the otoliths. Second, since the otolithic organs in fishes are organs of hearing, the use of a homogenous, single otolith as an oscillatory system is preferable to an otolithic apparatus consisting of multiple otoconia. Third, in contrast to the single amorphous and composition otoliths, the single polycrystalline otoliths typically have irregular shapes with blades, swellings, body twists and other irregularities (Figs. 55, 56). The single polycrystalline otolith can preserve its shape during growth. Previous mathematical calculation of the fish otoliths shape and size suggests that their shape contributes to directional hearing (Lychakov and Rebane, 1993).

At the same time, it is necessary to note that the ability to form otoconia-like structures is also preserved in fishes which have single polycrystalline otoliths, and it is used in the process of embryonic development of the otoliths. For example, the initial stage of otoliths formation in *Polypterus senegalus* is the emergence of spherule-otoconia with the diameter 2.5 μm (Thomot and Bauchot, 1987). These otoconia form the nucleus of the future otolith. Other units attach to this first seeding nucleus and form larger units (Thomot and Bauchot, 1987), a process that was previously observed in toadfishes (Sokolowski and Popper, 1987). The primary spherule-otoconia are described in the central part of the otolith of a number of bony fishes (Tanaka et al., 1981; Vinnikov et al., 1981a; Radtke and Dean, 1982; Neilson et al., 1985). The small spherule shaped otoconia probably form the seeding nucleus that permits accumulation of organic and inorganic materials to form larger but single polycrystalline otolith.

Single otoliths probably evolved independently several times; once in *Cyclostomata* (single amorphous otolith) and later in *Osteichthyes* (single polycrystalline otolith). However, there is a good reason to believe that the single polycrystalline otolith also developed independently in such groups as *Acipenseriformes* and *Teleoste* and other groups of bony fishes as well. Single otolith fish growth was shown to occur by accretion of individual layer to a seeding nuclei (Pannella, 1971).

The single otolith of teleosts and acipensers differ in their crystal lattice and crystal shape, differences that are probably reflected in mode of formation of the whole otolith (Carlstrom, 1963; Lychakov, 1995a,b). One of us showed previously that the saccular otoliths of the

Fig. 52. Utricular compositional otolith of *Acipenser nudiventris*. Utricular otolith represents a white dense lamina, which can be pulled out from the labyrinth as a whole unit without much damage. In all specimens examined, the utricular compositional otoliths contain one microotoliths (MO), about 300 μm and other smaller otoconia (Lychakov, 1995a).

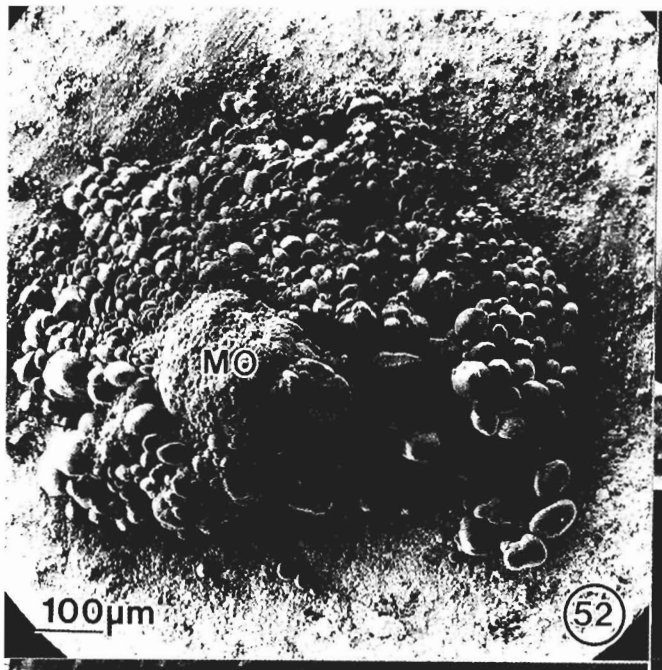
Fig. 53. Otoconia of saccular compositional otolith in *Squalus acanthias*. The saccular compositional otolith consists of the endogenous otoconia (EO) mostly cubical and exogenous sand grains (SG) of irregular shape (Lychakov et al., 1985).

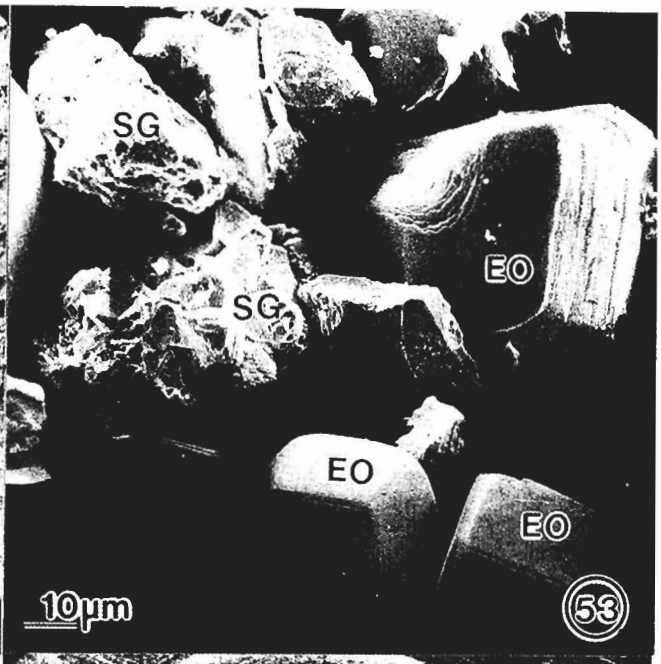
Fig. 54. Surface view of saccular compositional otolith of *Teratoscincus scincus* shows the irregular surface of the stone. Note lack of any known crystallographic planes among the six forms described in the text (Lychakov, 1997).

Fig. 55. Left saccular otolith of *Engraulis encrasicholus* (Lychakov and Rebane, 1993) in which the surface of one stone suggests uneven aggregation of material during formation.

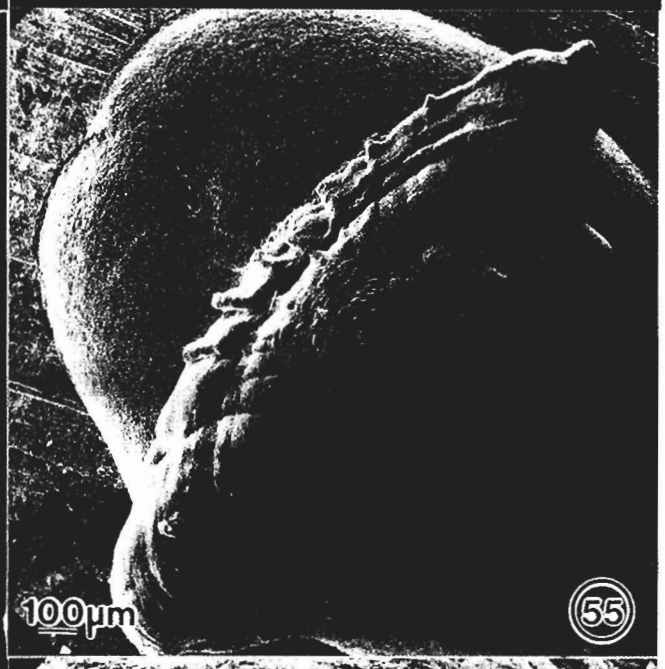
Fig. 56. Left saccular otolith of *Merlangus merlangus* (Lychakov and Rebane, 1993) and as in the previous spcies, this stone shows none of the known crystaollographic forms described in the text.

Fig. 57. Saccular otolith of *Acipenser nudiventris*. General view of otolith from the endolymphatic side. The otolith is a conglomerate of different parts with low and high elevations. The wide, blade-like stones (ob) consists of segments with a small-nodular surface. Each part surface displays large layers running parallel to each other (arrows). The otolith apex (oa) looks like an aggregate of fused concretions (Lychakov, 1995a).


MO
100μm
52


SG
SG
EO
EO
EO
10μm
53


100μm
54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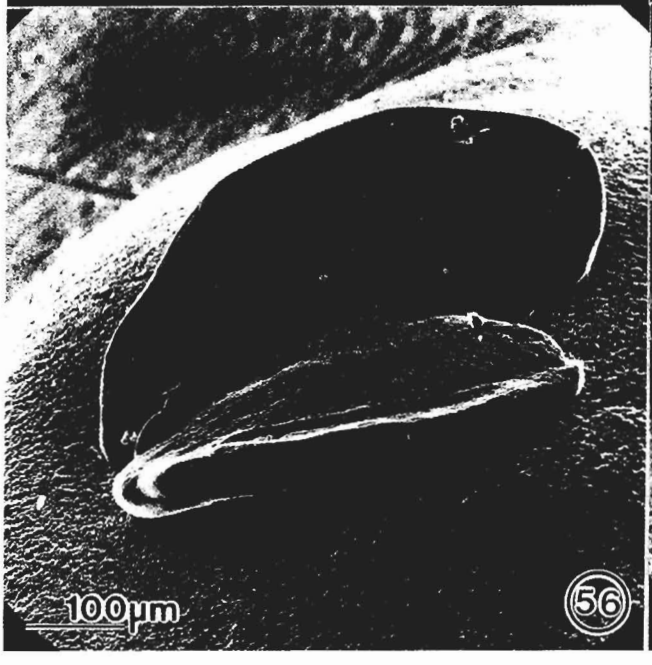
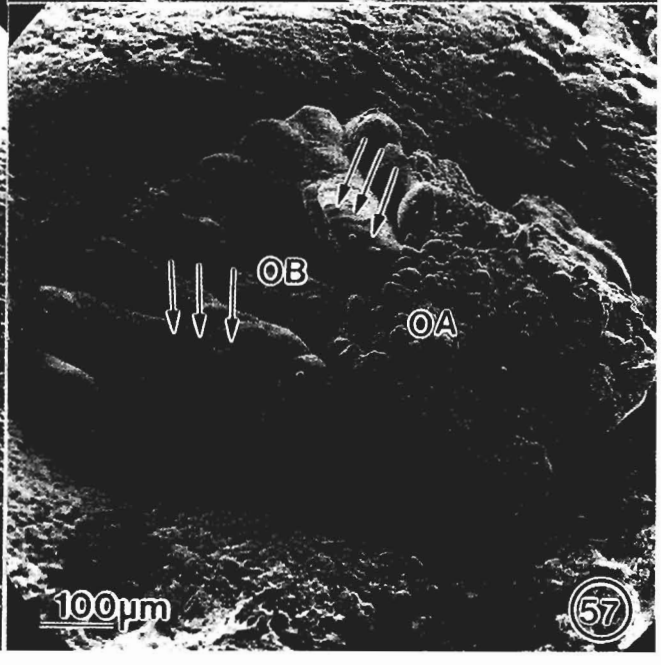

100μm
56


OB
OA
100μm
57

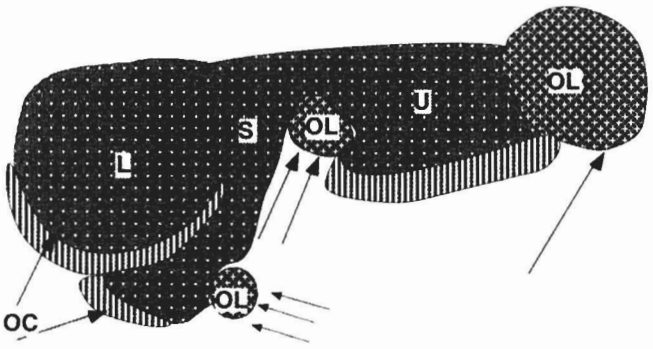
Acipenser fry consist of two parts: the base with the blade like unit, and an apex (Fig. 57), resembling an aggregate of fused concretions (Lychakov, 1995a). The base of the aggregation has the typical pattern and the usual lamellar structure of teleosts' otolith of (Lychakov, 1995a). In contrast to the typical composition otolith, the otoconia in the apex of the single acipenser otolith are not joined by organic matrix, but rather fused. This fusion is presumably influenced by chemicals and/or crystallographic growth planes in the otolithic apparatus of acipensers. Some otoconia lay unattached on the upper surface of the otolithic apparatus. The composite structure and vateritic constitution of single acipenser otoliths suggest that the single polycrystalline otoliths of the acipensers belong to the single polycrystalline otoliths of bony fishes. Besides, it is tempting to speculate that the composition otolith of the *Ceratodiformes* originated from a composition otolith of the ancestral bony fishes; and that the single polycrystalline otolith of the *Coelacanthini* evolved independently from the otolith (Fig. 58) of the *Actinopterygii* (Lychakov, 1995a,b). Future studies of nontetrapod otoliths will help to clarify the suggested scheme of evolution of the otoliths (Fig. 58).

Evolution of the Otoconia

The comparative morphological and embryological data suggests that otoconia are probably one of the earliest acquisition of the equilibrium organs in vertebrates (Tret'yakov, 1915). It seems that the ancestral vertebrate otolithic apparatus appeared first as thin secretion layer over the sensory cells. A thin layer of uncalcified organic matrix is first secreted in the vertebrate otolithic membrane of newer vertebrates (Fermin and Igarashi, 1985b, 1986; Fermin, 1993).

In the living vertebrate otoconia can serve as substrate for forming otoconia-like structures during embryonic development of the single polycrystalline otoliths. Otoconia can also participate in the enlargement of single amorphous and compositional otoliths. Finally, otoconia can contibute thin, flexible, delicate layers that deflect the stereocilia of the hair cells and initiate the transduction process that leads to detection of linear accerelation. This layer appeared for the first time in *Cyclostomata* and it is the basic structural component of the tetrapode utricle. In higher tetrapods the thin otoconial layer is structurally differentiated (Lychakov, 1988b). However, in the older vertebrates (for instance the lamprey) the otoconial layer is not associated with any specialized striola-like region which characterizes higher vertebrates. Previous works were unable to show any evidence of an striola region, nor have those works demonstrated any heterogeneity in the otoconia of the otolithic membrane of river or sea lamprey (Tret'yakov, 1915; Popper and Hoxter, 1987).

The changing patterns of otoconia formation along the phylogenetic tree are recapitulated during develop-



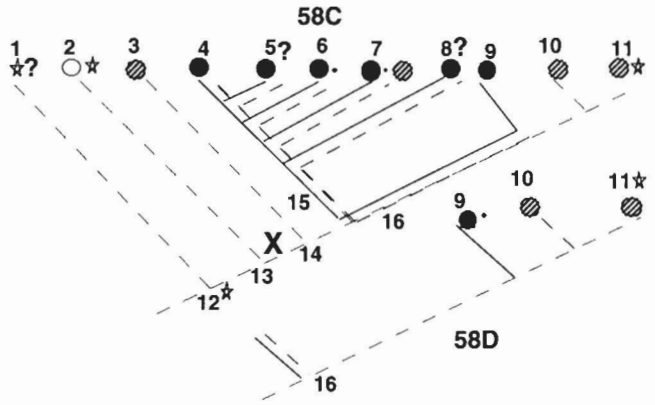


Fig. 58. A. Diagram of the right otolithic apparatus in adult river lamprey *Lampetra fluviatilis*. The otolithic membrane of adult river lamprey consists of three parts: anterior horizontal or utricular part (U), vertical or saccular part (S), and posterior horizontal or lagenar part (L). The otolithic membrane contains a thin structurally undifferentiated layer of spherical otoconia (OC) and otoliths (OL), formed as a result of otoconia fusion. The utricular part of the common otolithic membrane contains two otoliths. A larger otolith (arrow) is situated rostrally and weights 12.0-13.5 gr, while a small otolith (double arrow), with a mass ten times smaller (Lychakov and Lavrova, 1994b), occupies the caudal part of the utricular part. The saccular otolith (triple arrow) is small in size and lies in ventrorostral corner of the saccular part of the otolithic membrane. There are no otoliths in the lagenar part of otolithic membrane (Lychakov, 1995b). **C, D.** Diagram showing the relations of non-tetrapods presently living with each other and amphibians, and the structure of their otolithic apparatus, cladogram form (Burkhardt et al., 1983). Cladogram shows the relative closeness and relationship among the groups marked with numbers. Temporal relations between groups are not indicated. 1: *Myxin*; 2: *Petromyzones*; 3: *Chondrichtyes*; 4: *Teleostei*; 5: *Amiiformes*, 6: *Lepisosteiformes*; 7:*Acipenseriformes*; 8: *Polypteriformes*; 9: *Coelacanthiformes*; 10:*Ceratodiformes*; 11: Amphibians; 12: Vertebrate ancestors; 13: *Gnathostomata*; 14: Bony fishes; 15: *Actinopterygii*; 16: *Sarcopterygii*. The presence of otoconia is marked with dash lines, the presence of polycrystalline otoliths is marked with solid line, the presence of a thin pliable otoconia layer is marked with a star, the presence of a single amorphous otolith is marked with an open circle, the presence of compositional otolith is marked with a hatched circle, the presence of a single polycrystalline otolith is marked with filled circle, the presence of many otoconia together with a whole polycrystalline otolith is marked with a dot. Question-mark indicate that the precise organization of otoconia in otolithic membrane is not clarified. The X between 12 and 13 indicates a change from calcium phosphate to calcium carbonate in the otolithic apparatus. Fig. 58B shows a monophyletic way of formation of single polycrystalline otoliths in bony fishes, whereas Fig. 58C shows a polyphyletic way of formation of single polycrystalline otoliths in bony fishes from (Lychakov, 1995a,b)

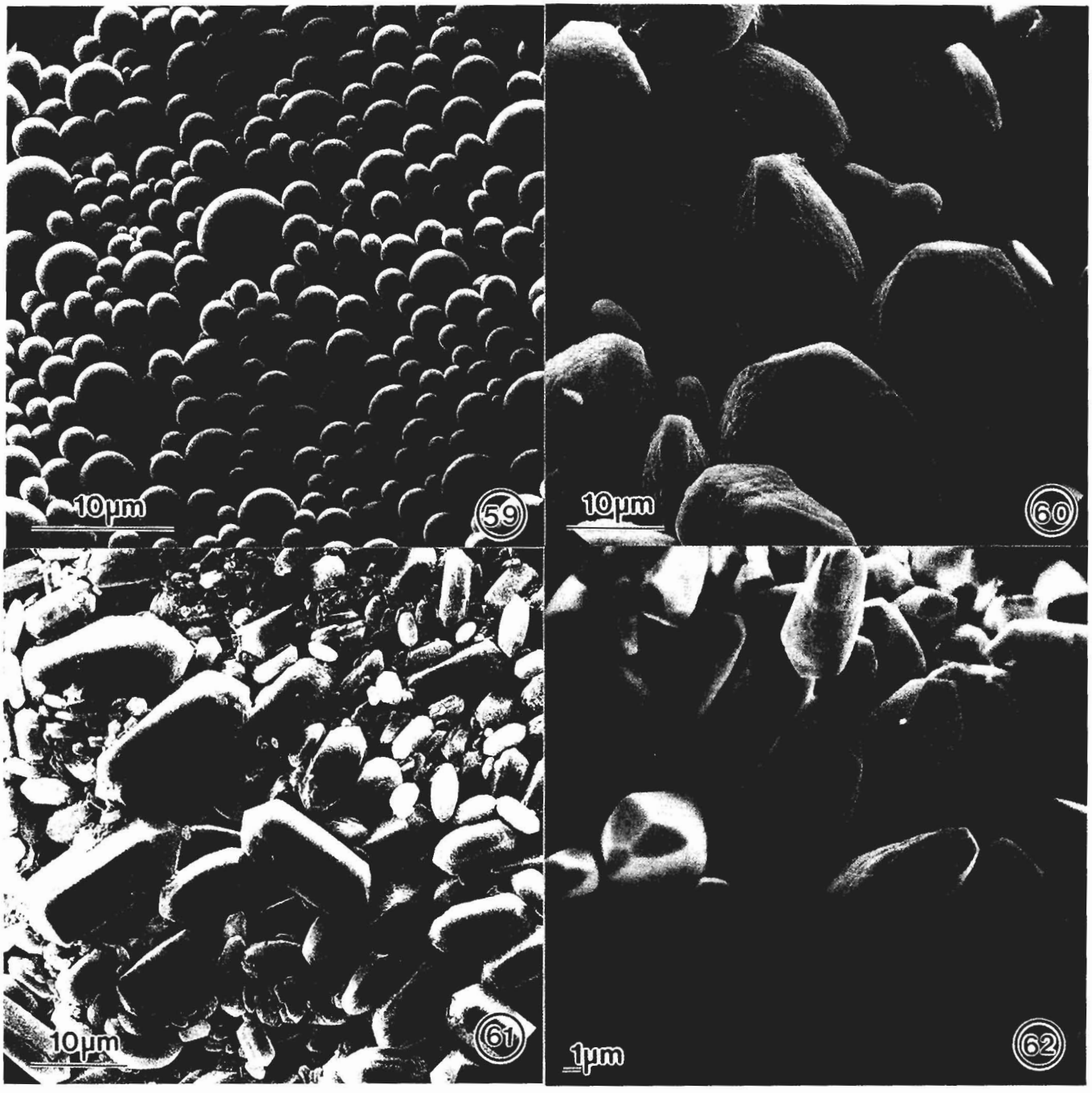


Fig. 59. Spherical otoconia in the otolithic membrane of *Lampetra fluviatilis*. Otoconia are 2-25 μm in diameter (Lychakov, 1995b). Similar to hexagonal otoconia of higher species (Figs. 63-70) these otoconia are also of different sizes.

Fig. 60. Lens-like otoconia of utricular compositional otolith in *Acipenser nudiventri* (Lychakov, 1995a) with a close resemblance for those shown in Fig. 51 of the *Raja clavata*.

Fig. 61. The slab-shaped otoconia of saccular compositional otolith in *Bufo bufo* (Lychakov, 1997) with primastic ends and rectangular bodies without the typical hexagonal shape of avian and mammalian otoconia.

Fig. 62. Guinea pigs utricular otoconia with a rounded body which terminates on both sides in three faces that converge at a single point (Vinnikov et al., 1982), illustrating the typical morphology and crystalline form of hexagonal otoconia found in birds in mammals.

ment in newer vertebrate species. For instance, the otoconial membrane of birds starts out as thin secretion layer over the sensory epithelia (Fermin and Igarashi, 1985b, 1986; Fermin, 1993). Gradually more organic substance is laid down and progressively the upper portions of the layer acquire segmentation like-lines that form the template of mature hexagonal otoconia. Individual hexagonal otoconia appear first on the upper portion of the gelatinous thin secretion away from the stereociliary bundle where histochemical and immunohistochemical stains demonstrated glycosaminoglycans (Fermin and Martin, 1994; Fermin et al., 1995b) and glycoproteins (Fermin et al., 1990). An amorphous gelatinous layer (reminescent of the lamprey organic layer), the gradual accumulation and enlargement of the gelatinous layer and the gradual calcification of the organic matrix sequence of events probably exist in all mammals, and published data suggest that the inorganic and organic matrix are integral components of the mature vertebrate otoliths (Lyon, 1955a; Balsamo et al., 1969; Lim, 1973; Ross and Peacor, 1975; Nakahara and Bevalander, 1979; Anniko, 1980; Salamat et al., 1980; Wright and Hubbard, 1981; Pote and Ross, 1986; Kawamata, 1990b; Pote et al., 1993a; Kido, 1997).

Changes in the shape of otoconia resulted from new mineral composition and mechanisms of formations. Otoconia in *Cyclostomata* are spherical (Fig. 59) (Tret'yakov, 1915; Carlstrom, 1963; Lychakov, 1995b), and round otoconia were described in some phylogenetically older vertebrates (Carlstrom, 1963; Muligan et al., 1989; Mulligan and Gauldie, 1989). Spherule-like otoconia serving as nuclei of single polycrystalline otoliths were described (Neilson et al., 1985; Thomot and Bauchot, 1987). Otoconia can be cubical (shark) (Fig. 53), lemon-like (shark, ray) (Fig. 51), lens-like (sturgeon) (Fig. 60), spindle-like (amphibians) (Fig. 61) or barrel-like with sharpened ends (amphibians, reptiles, birds, mammals) shape (Fig. 62). Crenelated or sponge-like otoconia of spherical shape, described in otolithic membranes of some vertebrates (Harada, 1979; Hunter-Duvar, 1983; Gauldie et al., 1986; Lychakov, 1997), may not function as otoconia, but serve as carriers for calcium salts from the macula to the otoconial layer. Irregularly shape otoliths such as sponge-like otoconia could also represent artifacts of fixation due to compression of the gelatinous layer of the otolithic membrane rather than real differences attributable to evolutionary traits. Nonetheless, fixation by different concentration of aldehydes or under control extremely cold conditions suggests that the shapes obtained in histological preparations are real including the central core of avian and mammalian otoconia (Figs. 42-45).

Thus, during the process of evolution, a transfer from spherical to other otoconia shapes took place. The first question that comes to mind is what is the functional significance of this shape transfer? Certain irregularites of otoconia cause by diseases in older vertebrates produce round and/or spherical otoconia (Wright et al., 1979, 1982) suggesting that the transfer of shape change was forced by factors that are as yet not completely understood. From the functional point of view most animals need to increase the otoconia loading mass with increase body volume. To accomplish this, the molecules participating in the formation of otoconia should acquire the proper density (g/cm^3) and otoconia proper should be packed in the otolithic membrane with required density (number of otoconia/cm^3). Otoconia should also offer resistance to dissolution in the unsaturated endolymph, but remain attached to the organic matrix of the otoconial membrane. In addition, packing of the fibrils should allow exchange of macromolecules such as glycoproteins and glycosaminoglycans previously demonstrated as important indicators of otoconia calcification status (Fermin et al., 1995b). Calcification of cartilage is also accompanied by a change of the glycosaminoglycan content in the otoliths. Glycoproteins and polylactosaminoglycan on or near the maculae may influence mineralization of the otoliths. Previous studies showed that the concentration of glycans is higher in the utricular OM than in adjacent cupular masses. Analyses of teeth's proteoglycans indicate that mineralizing matrices (like the OM) may have a differential distribution of certain macromolecules and that such distribution could affect mineralization (Fermin et al., 1995b). In teeth, where an organic matrix is secreted and then mineralized, proteoglycan concentration was found to change during mineralization (Bartold et al., 1988; Bartold, 1990), and in bone, proteoglycans are part of organic matrix in vivo and in vitro. In the inner ear, sequestration of calcium into the maculae may be facilitated by high proteoglycan concentration in the maculae and lower in the cupulae (Fermin et al., 1990), and/or other elements (López-Escámez et al., 1993c).

Unfortunately, presently we do not have enough data to evaluate functional role of the otoconia shape in different animals. We can only speculate that otoconia shape can influence the intensity of the ion interchange between otoconia and endolymph. Consequently, affecting the stability and the life span of otoconia which in turn would influence their density and packing in otolithic membrane. In the last case, the role of the otoconia shape can be important only when otoconia are tightly packed.

The physico-chemical mechanisms providing the variety of the shape of otoconia are not presently known either (Pote and Ross, 1991). It is evident, however, that the shape of otoconia is determined by the qualities of proteins forming their organic matrix (Pote and Ross, 1991) and, thus, it is a genetically determinate parameter.

As for lamprey otoconia shape (Lychakov, 1995b), we can note that the regular shape of otoconia is probably influenced by the packing of amorphous calcium phosphate into spheres (Figs. 52, 59), spherical (granular electron dense) deposits were demonstrated in otoconial of chick embryos (Fernández and Goldberg, 1971; Fermin and Igarashi, 1985b, 1986) and are

illustrated here (Figs. 12-16). This is a very economical means of packing macromolecules as far as surface area and volume are concerned. That is, spheres possess the smallest surface energy. The spherical shape is also advantageous because it allows the reduction of the ion interchange between otoconia and endolymph as much as possible. The rejection of the perfect spherical shape seems to be connected with the change of the composition of the otoconia. In particular, the protein of the organic matrix and transition to crystalline calcium carbonate form (Igarashi and Alford, 1969; Pote and Ross, 1986; Ross and Donovan, 1986; Ross et al., 1987b; Fermin, 1993; Pote et al., 1993a,b; Fermin et al., 1995b; Kido, 1997). Thus, energy saving mechanisms as discussed later in the concluding remarks are favored over energy wasting mechinisms.

Ancestral Otolithic Apparatus

Previous work (Tret'yakov, 1915; Carlstrom, 1963; Lychakov, 1995b) indicates that the otolithic membrane of river lamprey probably represents the oldest otolithic membrane of living vertebrates. This is derived first from the philogenetic antiquity of the otolithic membrane of river lamprey; second, from structural association between the organic and inorganic components of the otolithic membrane (Fig. 47), and third from the utilization of amorphous calcium phosphate as the inorganic substrate of otoconia.

Some structural features of the lamprey otolithic membrane are found along the phylogenetic tree from the most primitive to the newer species. These are: 1) the capacity to form otoconia rather than just a single otolith, 2) the use of calcium as the main cation of the inorganic component (Ross, 1984), 3) the use of sodium ions during the growth of otoconia and otoliths (Vinnikov et al., 1983; Lychakov and Lavrova, 1994b), and 4) use of macromolecules assembled into an organic substrate that have affinity for acid histochemical stains. Assembly of an organic matrix for growing otoconia seen as a thin layer over the macular epithelium of river lamprey (Tret'yakov, 1915), the primary spherule-otoconia of the otoliths in *Polypterus senegalus* (Thomot and Bauchot, 1987), the periphery of growing slightly calcified otolith of trout (Mugiya, 1968) all stain with acid histochemical stains (Igarashi and Alford, 1969). A sharp temporary reduction of affinity to the basic stains and appearance of PAS positive staining takes place in mice embryos during the period of active formation of otoconia (Veenhof, 1969a), suggesting that the phylogenetic changes observed between the otoconia of old and new species may be recapitulated in the development of newer ones. A differential display of different macromolecules with distinct properties for attracting negatively and positively charged molecules was previously related in the otoliths to the capacity to control microenvironments in the calcifying macular components and the non calcifying ampular components (Fermin, 1993; Fermin et al., 1995b). Differential attractions of ions to macromolecules were demonstrated in calcifying cartilage as the determining factor.

Finally, some structural features of the lamprey otolithic membrane develop in the most evolutionary advanced animals. The otolithic membrane of river lamprey contains several otoliths and a thin otoconia layer, i.e. exactly the two main structures which later evolved in fishes and tetrapods (Lychakov, 1988b, 1995b). But, the otolithic membrane of river lamprey is not just the result of the simple mechanical combination of these two structures, rather it seems that during the process of lamprey otoconia formation an increase of the otoconia mass at the expense of new formation of spherical otoconia took place (Tret'yakov, 1915; Volk, 1986). Thus, the otolithic membrane of river lamprey exemplifies otolithic membranes of water animals and in non-tetrapods the ability to increase their mass to serve as loading devices to stimulate the hair cells in the epithelia (Lychakov, 1988b, 1995b). On the other hand, the fact that the otoliths of river lamprey have an extremely small mass (Lychakov and Lavrova, 1994b) makes them a peculiar prototype of future structurally differentiated otolithic membrane. The differences in the mass of different parts of the otolithic membrane may provide tuning for the corresponding parts of macula to detect accelerations of different intensity (Lychakov, 1988b, 1995b).

Evolution of the otolithic apparatus

The comparative morphological data gives us an overall picture of evolution of the otolithic apparatus (Fig. 58) (Lychakov, 1988b, 1995b). It is highly likely that otoconia are the oldest components to be acquired by the vertebrate equilibrium organs. During evolution, the transfer from a thin undifferentiated gelatinous layer to a conglomerate of otoconia, with the formation of a single amorphous otolith in lamprey and compositional otolith in philogenetically oldest fishes, took place. It is very interesting that the otolithic membrane of river lamprey contains, both components found in newer species, i.e. amorphous otoliths and a thin undifferentiated otoconial layer. These two structures which later evolved in fishes and tetrapods (Lychakov, 1988b, 1995b), are both primitive in their chemical and morphological organization.

The otolithic membranes of *Elasmobranchs* and other philogenetically older fishes do not contain a thin otoconial layer. In the labyrinth of these fishes, only compositional otoliths are present. During evolution, bony fishes developed new mechanisms of otoconial membrane and crystal growth. But the labyrinth of some phylogenetically older bony fishes contains compositional otoliths, the philogenetically new single polycrystalline otoliths and single otoconia as well. Only in teleosts there is not a compositional otolith or the thin otoconial layer. There are no free otoconia in the otolithic organs of the true teleosts at all. The only known exception among teleosts is the sunfish *Mola*

mola. The otolith of this fish contains a conglomerate of otoconia cemented together (Gauldie, 1990). It may be assumed that in the sunfish the process of embryonic labyrinth development is accompanied by an evolutionary regression in the formation of a single otolith. It is likely that the simultaneous hypertrophy of primary embryonic otoconia also occurred. And, in fact atrophy is an inherent feature of otolith development in *Mola mola*. In the sunfish the swimbladder, tail, posterior part of vertebral column, and other structures disappear in the course of ontogeny (Makuschok, 1971) and these changes may also be related to the animal ability to sense its environment.

In tetrapods we can trace not only the return to the thin otoconial layer but also its refined design or the appearance among components integrated into a single unit. But in the labyrinth of the lower tetrapods (amphibians and reptiles), there are simultaneously the compositional otolith (in sacculus) and the thin undifferentiated otoconial layer (in utriculus). In the labyrinth of high tetrapods such as mammals, there are only thin differentiated otolithic membranes (otolithic apparatus plus organic layers). Possible functional advantages for the structural differentiation of the otolithic membrane in higher vertebrates was the ability to utilize single crystals for longer periods of time without the need of a continuously growing phase (Lychakov, 1988a).

At the present time, we have no precise data about the packing of otoconia in the otolithic organs of philogenetically older fishes such as *Amiiformes*, *Polypteriformes* and *Sarcopterygii*, one of the reasons why we can not follow the complete evolutionary transfers of the otolithic membrane from fishes to tetrapods.

Effects of long-term hypergravity on the otoconia of the vestibular system in hamsters

The utricle and saccule are otolithic organs of higher vertebrates and are susceptible to changes in G-load during embryonic development. A change in G-load can be induced by hypergravity. As a result of substained exposure to altered gravity during development of the utricule and the saccule of rodents, the vestibular sensory input becomes functionally appropriate for the increased G-load and animals adapt to the change in gravity load. There are modifications of the sensory elements and of the loading masses that stimulate the hair cells.

Alterations in the size or shape of the otoconia from the utricle or saccule after pre- or postnatal exposure to hypergravity have been reported, although these findings are often contradictory (Lim et al., 1974; Krasnov, 1991; Pedrozo and Wiederhold, 1994; Hara et al., 1995). If long term exposure to an altered gravity environment (Lim, 1974a; Krasnov, 1991; Pedrozo and Wiederhold, 1994; Hara et al., 1995) alters the vestibular input to the central nervous system (CNS), then disruption of vestibular controlled behavior is expected.

In our experiments, we investigated the effect of long-term hypergravity (2.5 G) on the morphology of the otoconia in the otolith organs and the function of the peripheral vestibular system in general. By means of a centrifuge, at a rotation speed of 34.3 rpm (arm length = 2 m), an increased G-load condition (hypergravity or HG) was created to which golden hamsters *Mesocricetus auratus* were subjected. The hamsters were conceived and born in hypergravity (HG hamsters) or in normal gravity (CON hamsters). Their perceptive-motor skills were assessed when the HG hamsters lived in hypergravity (4 months) and afterwards during 4 months in normal gravity. We found that the HG hamsters showed persistent disturbances in vestibular behavior, such as swimming and air-righting, whereas control (CON) hamsters (born and living in normal gravity) showed no disturbances (unpublished data). These disturbances were more severe in HG developed hamsters than in hamsters subjected to hypergravity after birth (Sondag et al., 1995a,b, 1996b) (Table 1). The animals had fewer problems with tasks in which proprioceptive information was available, as during normal locomotion and balancing.

The results of the EDAX element analysis revealed that hypergravity during the embryonal development does not alter the Ca^{2+} content of utricular or saccular otoconia. Scanning electron microscopy showed that hypergravity did not change the size or shape of the otoconia. Small, medium-sized and large otoconia were present in 2.5G and 1G developed hamsters (Figs. 63, 64). However, the relative size of utricular areas with large, medium-sized or small otoconia was altered in HG developed hamsters when compared to hamsters developed in 1G (Sondag et al., 1996a). The area containing the large otoconia was decreased in HG

Table 1. Summary of all the behavioral tests to evaluate the performance of the peceptive motor skills.

GROUP	PERCEPTIVE - MOTORS TASKS				
	Gait/Strie	Balanceing on rail	Swimming speed	Swimming orientation	Air-Righting
Young: born in 1 G, exposed to 2.5 G	#	X	0	X	#
Adult: born in 1 G, exposed to 2.5 G	0	XX	0	0	X
Young: born in 2.5 G, exposed to 2.5 G	0	X	XX	XX	XX
Young: born in 1 G, exposed to rotation (1G, 34.3rpm)	0	X	0	0	0

#: not tested in this group; 0: not different from controls; X: worse or decreased performance when compared to controls; XX: task for 50% of the group too difficult.

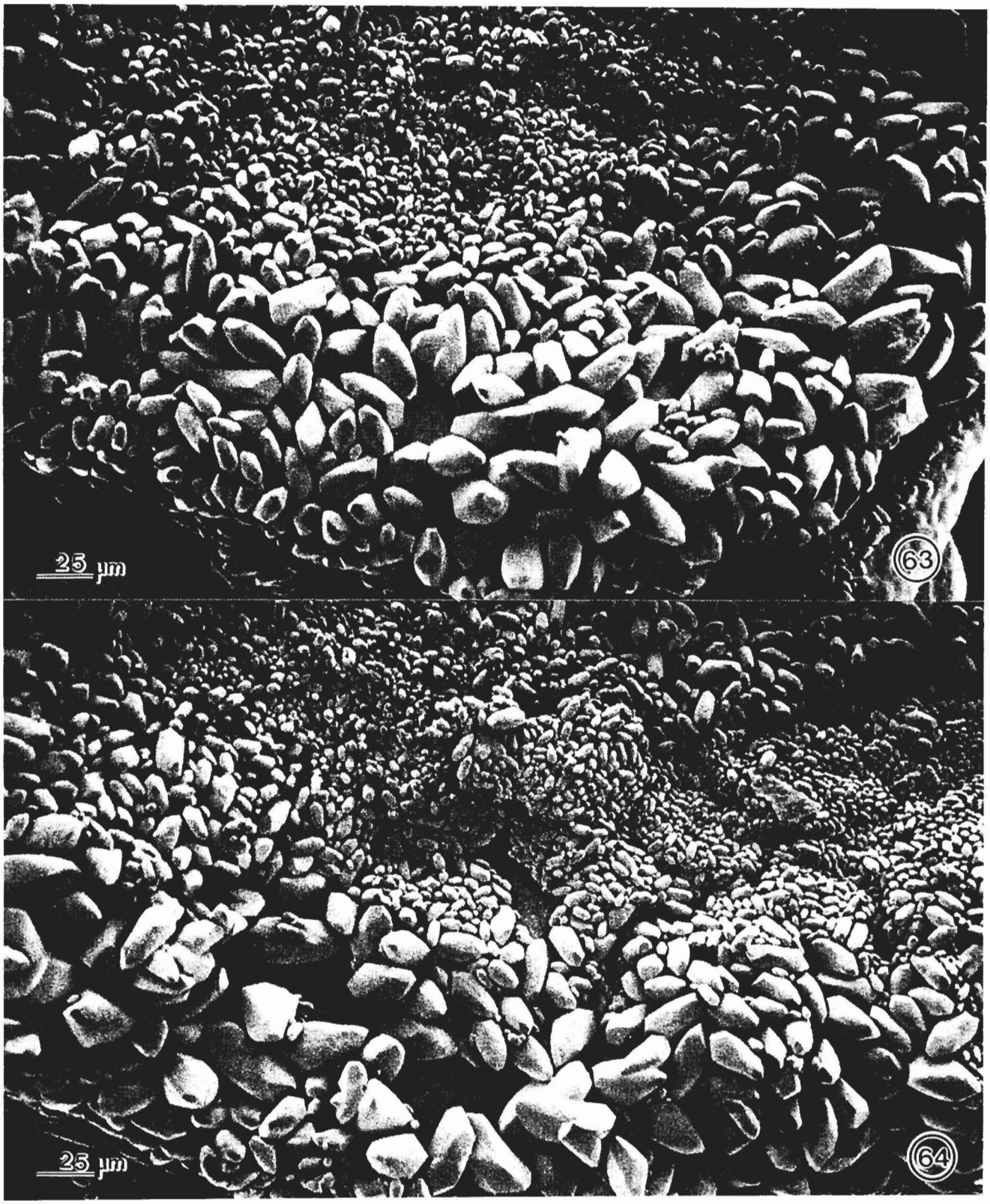


Fig. 63. Otoconial membrane from the utricle of a 2G exposed hamster. Crystals of varying size and shapes are observed with a gradation from large to small otoconia but each of hexagonal shape.

Fig. 64. Otoconial membrane from the utricle of a 1G (control) exposed hamster. There is no difference between these crystals and those of the 2G rodent exposed otoconia. This is in contrast to a marked difference between the otoconia of 1G and 2G exposed chickens (please see Figure 70b).

developed hamsters, while the area containing the medium-sized otoconia was increased (Fig. 65) (Krasnov, 1991) suggesting that the peripheral utricular area, in which the large otoconia are located, is especially susceptible to changes in gravity. The observed changes in the distribution of the otoconial areas only appeared during embryonal development under HG conditions; hamsters conceived and born under 1G and subjected to HG for 6 months after weaning showed no differences with controls (Sondag et al., 1995a,b, 1996a,b). Furthermore, the growth of some areas of the utricular otoconial patch seems to be delayed in the HG hamster. Differences in otoconial distribution still exists after 8 months of normal gravity, suggesting that this alteration is irreversible. We concluded that severity of disturbances in spatial orientation is dependent on the environment in which embryonal development takes place and on the age of the animals when subjected to altered gravity. An alteration in the distribution of the otoconia of the utricles examined is one of the causes for the disturbances in vestibular behavior in HG developed hamsters. Moreover, hypergravity and rotation affect vestibular behavior differently.

Morphological changes in utricular otoconia of developing chick embryo exposed to 2G gravity

The otolithic organs are gravity sensors that may be affected by changes of gravity. The effect of altered gravity on the vestibular system was previously investigated in mammals (Igarashi and Nagaba, 1967; Parker, 1991) and birds (Howland and Ballarino, 1981; Ballarino and Howland, 1984; Hara et al., 1995) with transmission and electron microscopy, and during embryonic development in mammals (Harada, 1978; Nakahara and Bevalander, 1979; Anniko, 1980; Salamat et al., 1980; Nakahara et al., 1981; Sánchez-Fernández and Rivera-Pomar, 1983; Takumida and Harada, 1984; Ciges et al., 1985; Sondag et al., 1996a). This portion of the review reports our results from scanning electron microscopic observations of the chick utricle from embryos exposed to prolonged (0-18 embryonic days) hypergravity with centrifugation.

By the 11th day of incubation (stage 37), otoconia in the control chick embryos appeared normal (Fig. 66) of even distributed sizes and shapes. In addition, the cementing organic matrix was easily detached. Otoconia of the 2G centrifuged chick embryos were smaller, appeared immature and were covered at both ends of the hexagons with spongy material that resembled disorganized organic matrix (Fig. 67). At the marginal zones of the utricle, otoconia were generally larger than in control and the cementing organic matrix did not separate easily (Figs. 68) from the crystals. Giant otoconia become more prominent by the 15th day of incubation at 2G (Figs. 69, 70a) and could be seen at low magnification. The giant otoconia were located on the utricular otolithic membrane along the marginal zone and all the giant otoconia exceeded 40 μm, reaching up to 100 μm in length (Fig. 70b-d). About 60% of the 2G centrifuged chick embryos had giant otoconia, whereas less than 3% of the control embryos had giant otoconia.

Exposure of chick embryos to hypergravity due to centrifugation: i) delays formation of otoconia, and ii) induces the formation of giant otoconia along the marginal zone of the utricular macula. In normal developing chick embyos only one out of approximately 200 dissections showed giant otoconia (Ballarino and Howland, 1984). Chick centrifuged for prolonged periods of time during development undergo changes of the otoconial membrane that include the formation of giant otoconia (Hara et al., 1995). We compared the embryos of the same hatching day or at the same embryonic stages of Lillie's classification (Hamburger and Hamilton, 1951). The delay in the process of otoconia formation may be caused by either inhibition of Ca^{++} deposits or to a change of otoconial precursor macromolecules. A differential depostion of various macromolecules was proposed as crucial for the mineralization of the inner ear organic matrix (Fermin et al., 1995b). The increased gravity vector force to which the otoconia are subjected during centrifugation may interfere with aggregation of macromolecules within and

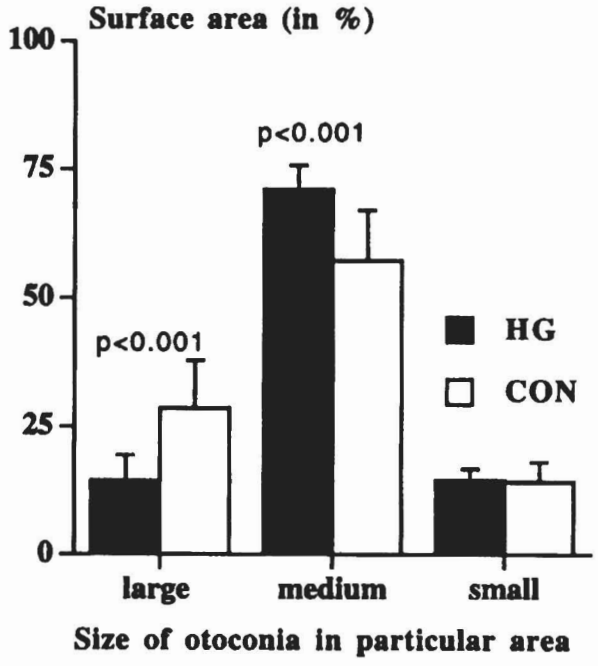


Fig. 65. Comparison otoconial size between control (9 months at 1G) and experimental (5 months at 2G followed by 4 months at 1G). There was no difference in the size of small otoconia between hypergravity and normal hamsters, possibly because smaller otoconia are formed anew a supposition that needs corroboration. A significant difference between medium and large otoconial size was estimated between hypergravity and control hamsters and one may speculate that such an increase resulted from aggregation of salts to already formed crystals particularly at the upper surface of the otoliths but this notion is too unproven.

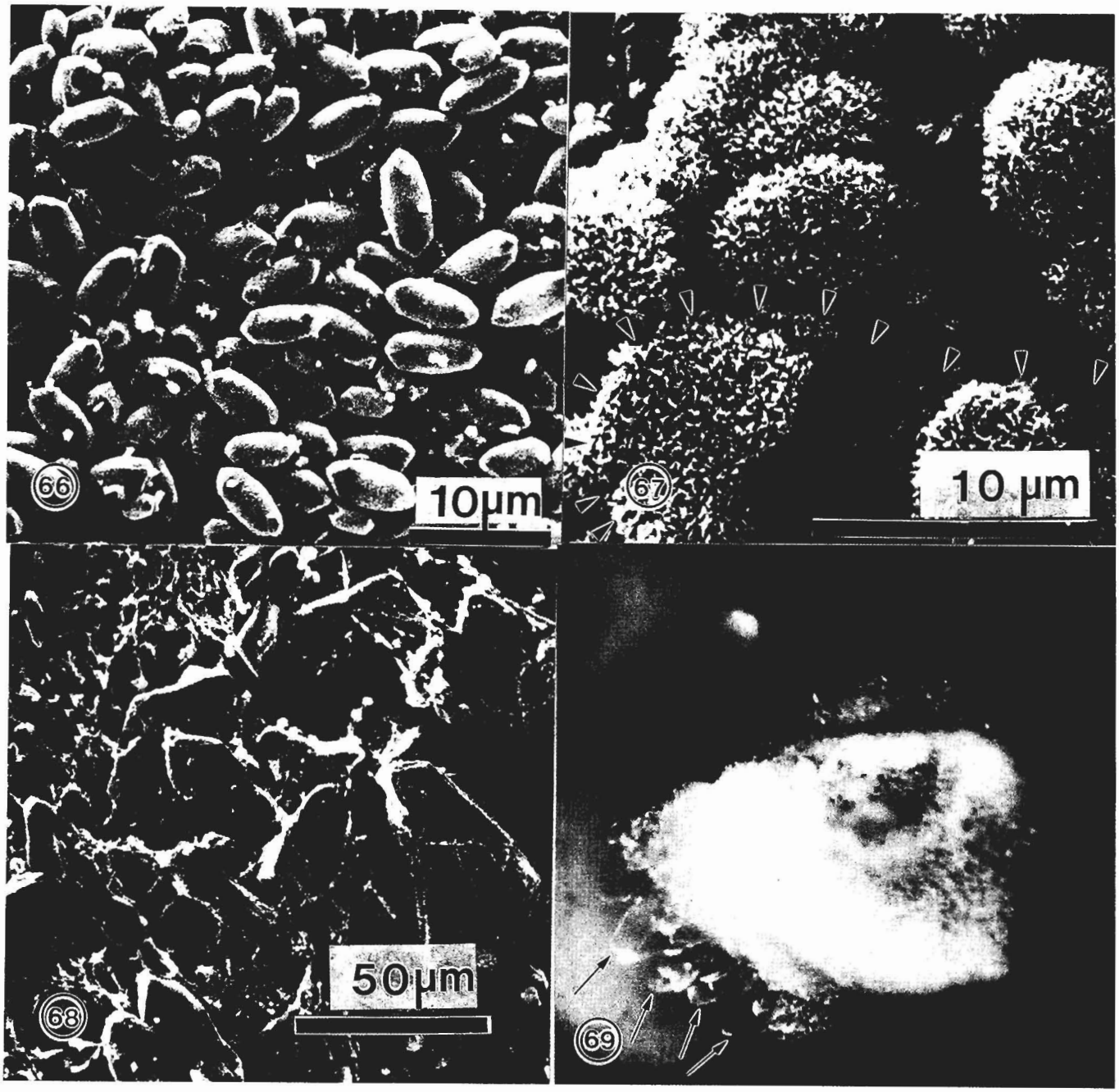


Fig. 66. Otoconia from a normal 1G incubated embryo with hexagonal crystals. The typical hexagonal shape and size of avian otoconia is evident.

Fig. 67. Otoconia from a 2G incubated embryo showed otoconia that had spongy material at either end of the hexagon (arrowheads). It should be noted though that the chicks above were exposed continuously to the 2G force during development as oppose to the hamster which were not.

Fig. 68. Otoconia from a 2G incubated embryo at the marginal zone of the utricle with giant otoconia of varying size and shapes. Organic material is seen attached to otoconia.

Fig. 69. Surface view of the utricle from a 2G chick showing giant otoconia at the marginal zones (arrows). Giant otoconia are generally seen at the marginal zones of the saccule of chicks and seldom the utricle, as induced by the 2G continued force from 1-18 days of embryonic development.

outside the otoconial organic phase. Molecules with varying histochemical properties and electronical charges were identified in the developing and mature chick otolith (Fermin, 1993). Glycosaminoglycans are important components of the mineralized and unmineralized gelatinous masses of the inner ear (Fermin et al., 1990). Unfortunately, the mechanism that interferes with otoconial formation leading to giant otoconia aggregation is not known. Among the mechanisms inducing giant otoconia formation are those affecting carbonic anhydrase (Drescher, 1977) and/or alteration of the pH with subsequent changes in the affinity of highly charged macromolecules (Prieto and Merchan, 1986; Ross et al., 1987b; Lim and Rueda, 1990; Prieto et al.,

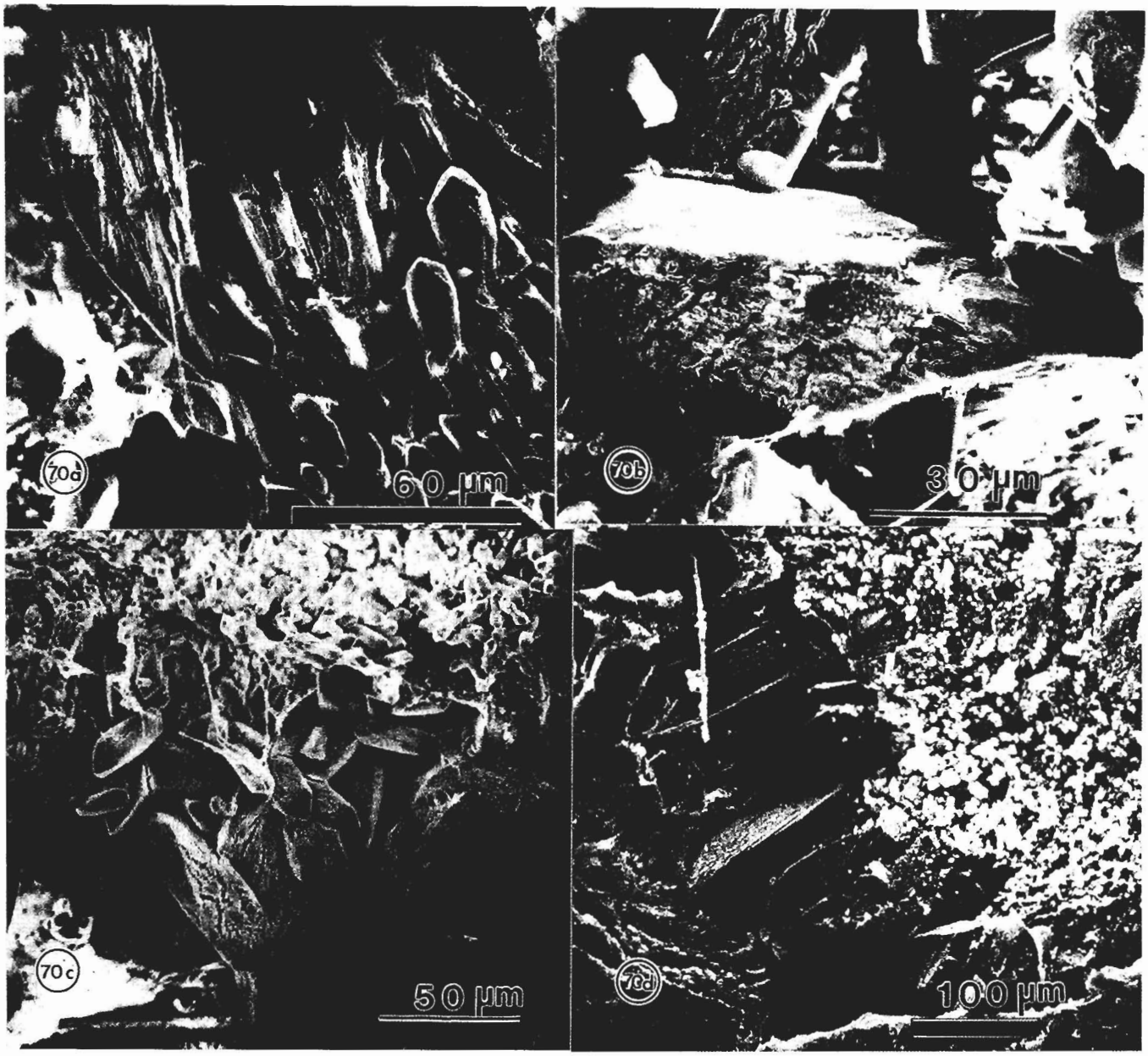


Fig. 70. Details of the utricle from a 2G chick showing the transitional zone **(a)** between the regular size and giant otoconia at the marginal zone where giant otoconia can reach up to 100 μm and had irregular shape face angles not resembling a hexagon **(b)**. In other regions of the marginal zone otoconia retain their hexagonal shapes and reach sizes generally only seen at the marginal zones of the saccule **(c)**. In other areas, the otoconia seem to remain attached **(d)** to the epithelia as shown previously by (Fermin et al., 1987) during the early stages of genesis. Otoconia in Figure 70d also display trapazoid rather than hexagonal shapes. Such shapes were previously reported in amphibian otoconia (Pote and Ross, 1993) and in diseased otoconia of mammals (Purichia and Erway, 1972; Harada and Sugimoto, 1977; Harada et al., 1978; Wright and Hubbard, 1981; Wright et al., 1982; Anniko et al., 1984; Lim, 1984; Igarashi et al., 1993a).

1990; Santi et al., 1990). Finally, the normal fragmentation process of the uncalcified otoconial membrane (Fermin et al., 1987) may be affected by hypergravity through centrifugation. Nonetheless, the above speculations require further analaysis.

Rodent macular damage by aminoglycosides

In previous studies morphological analyses of nerve endings following unilateral intraotic gentamicin (GM) administration to chinchillas (Tanyeri et al., 1995), streptomycin (STP) to guinea pigs (Lee and Kimura, 1994), incubation of explanted vestibular organs derived from adult guinea pigs, humans with neomycin, or short-term parenteral GM administration to guinea pigs (Forge et al., 1993) showed that hair cells are damaged by these aminoglycosides. It has also been postulated that recovery of vestibular function after termination of drug regimen, may be due to production of new hair cells that in aves was shown to derive from supporting cells (Rubel, 1992). If indeed recovery of function is accompanied by production of hair cells in mammals as in birds, remodeling of the sensory epithelia and otoliths of the maculae should occur concomitantly with recovery of function.

Chronic 49 day streptomycin (STP) administration to young rats specifically damaged the otolith organ-related motor abilities such as swimming behavior in the pigmented rat. When STP treatment was discontinued, the swimming behavior gradually returned to normal, after a period of 9 week recovery. Ten, twenty-day-old male Long Evans rats were injected daily intramuscularly in the thigh in alternated hindlegs for 48-57 days with 400 mg/kg body weight of STP dissolved in phosphate buffered saline (PBS). After completion of each experimental manipulation animals were deeply anesthetized with 475 mg/kg body weight chloral hydrate (Sigma) and transcardially perfused with 300 ml of PBS followed by 350-400 ml of 4% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4 at 4 °C, over a period of 15 min.

We chose STP as a tool to investigate the contribution of each vestibular organ to the overall vestibular function, and have described that specific damage to guinea pig vestibulary cristae HC caused by continuous administration of STP produces a concomitant loss of both semicircular canal function and of the enzyme of synthesis of GABA (glutamate decarboxylase, GAD (Meza et al., 1989)), of HC localization in the guinea pig (López et al., 1992; Meza et al., 1992b). This multidisciplinary approach proved very useful because it led to the description, for the first time in a mammal, of a recuperation of semicircular canal function and reinstallation of the activity of GAD when STP treatment was interrupted (Meza et al., 1992a). We found that semicircular canal function, as determined by post-rotatory nystagmus recording, was intact, but some motor disabilities were observed when swimming behavior was assessed (Meza et al., 1996).

Electrophysiological recording of auditory evoked potentials demonstrated that no damage was inflicted to auditory function. These observations allowed us to postulate that in the rat, STP causes a specific damage to otolith-related functions, sparing those of semicircular canal and auditory elements. Furthermore, when STP treatment was discontinued, motor impairments displayed by the rat in the swimming test gradually returned to normality, indicating that STP affected rats, as guinea pigs, could recover following interruption of STP administration (Meza et al., 1996).

Morphologically, there was a correspondence between the abnormal behavior of injected and normal rats. The utricular macula of saline injected rat (Fig. 71) displayed normal organization of supporting and sensory epithelia with intact overlaying otoconial membrane. The epithelia appeared pseudostratified with multiple supporting cells nuclei near the basement membrane (Fig. 72) and hexagonal otoconia with central prominent central cores. The utricular macular epithelia of the non-recovered rats (Fig. 73) was 40% thinner from the endolymphatic lumen to the basement membrane, than the saline control injected and the STP injected recovered rats. In addition, the nuclei of the supporting cells were less numerous and not pseudostratified, but rather were lined on single file next to the basement membrane. The otoconia were smaller, not perfect hexagons and the cementing substance between otoconia was sparse (Fig. 74). This condition contrasted with the epithelia of the recovered rats in which the thickness of the utricular epithelia was only about 20% thinner and the hyaline cartilagenous plate was less cellular than unrecovered rats (Fig. 75). The nuclei of supporting cells showed signs of pseudostratification, but most nuclei were still arranged in single file and the otoconia were hexagonal with prominent central cores (Fig. 76).

The above results suggest that following administration of streptomycin under the regime we used, some animals recovered with concomitant recovery of function that is paralleled by reorganization of the sensory and secretory epithelia. In addition, animals that showed full recovery of function also showed normal otolithic membrane with hexagonal otoconia and prominent central cores. It remains to be determined whether hair cells type I and type II recover at the same time or type II hair cell precedes the reappearance of type I hair cell (Maseto and Correia, 1997a,b). The results presented here from STP injected rats and data from the above publications suggest that hair cells repopulate the same area of the macula they occupy prior to damage, after recovering from aminoglycoside damage. However how the hair cells repopulate those areas is not clear. Correia's group suggested that in the pigeon type II hair cells may derive from supporting cells, a situation that may not hold true for type II hair cells. Their evidence suggests that type II hair cell appear first and then type II (Maseto and Correia, 1997b). The reappearance of both types of hair cells was linked to the activation of ionic channels on the cells

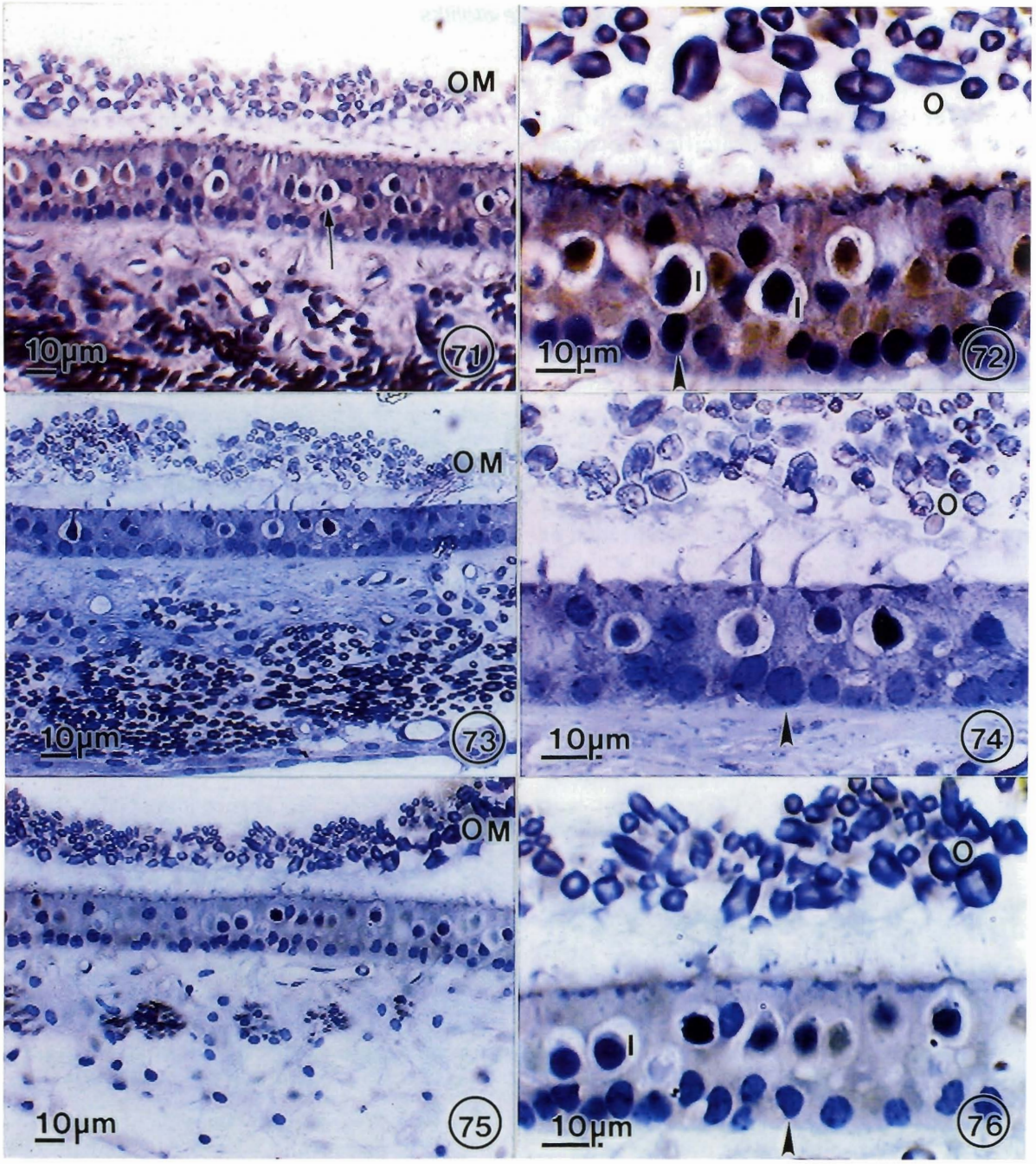


Fig. 71. Utricle from a saline injected rat with normal hair cells type I (arrow), supporting cells and hyaline cartilage. The otoconial membrane (OM) appears intact.

Fig. 72. Higher magnification from Figure 71 showing details of hair cells type I afferent nerve chalice (I), otoconia (O) and pseudostratified arrangement (arrowhead) of the supporting cells nuclei. Note thickness of the epithelia and central core of otoconia (O).

Fig. 73. Utricle from a streptomycin injected unrecovered rat with thin epithelia, single file of supporting cells nuclei and disorganized otoconial membrane (OM). Numerous myelinated fibers in cross section are seen in the hyline cartilage.

Fig. 74. Higher magnification from Figure 73 showing details of hair cells, otoconia and enlarged nuclei of supporting cells (arrowhead). Note thickness of the epithelia and central core of otoconia (O). The characteristic dense peripheral vs central lighter core has been lost.

Fig. 75. Utricle from a streptomycin injected recovered rat with intact otoconial membrane (OM), normal looking hair cells type I, but thinner epithelia than saline controls.

Fig. 76. Higher magnification from Figure 75 showing details of epithelia, with normal looking hair cells type I afferent nerve chalice (I) and otoconia (O). Otoconia shape and contrasting peripheral-central areas are retained. Note thickness of the epithelia and central core of otoconia and the pseudostratification of the supporting cells nuclei (arrowhead) when compared to those supporting cells nuclei of unrecovered rat above in Figure 74.

(Maseto and Correia, 1997a), but a transition of channel type in presumptive supporting cells and emerging hair cells was not corroborated. If hair cells type II are phylogenetically older than type I, the reappearance sequence of the two type of cells after chemically or physically induced damage may be important for our understanding of the evolutionary significance of hair cells in different species, different organs and different areas of those organs in the ear (Goldberg and Fernández, 1980; Highstein et al., 1987; Goldberg et al., 1990b; Ross et al., 1991), save that the macula of the older species do not have true type I hair cells (Highstein and Baker, 1985; Guth et al., 1994). Interestingly, the utricle of chicks exposed to 2G gravity during development hatch with few hair cells type I in the striolar region (data not shown), but the altered ratios of type I and II hair cells may be induced by factors other than hypergravity (Hara et al., 1995).

Elemental composition of otoliths

Vertebrate otoconia, located on the gelatinous membrane of the vestibular receptor in the utricle and saccule of mammals and the macula lagena of birds, are composed of three major forms of crystallized calcium carbonate: calcite, aragonite and vaterite. Each form is distinguished by its crystal structure, specific gravity, refractive index and hardness (Steyger and Wiederhold, 1995). Calcite is present in otoconia of mammals, birds and sharks; vaterite in otoconia of chondrostean fishes; aragonite in otoliths of holostean fishes; and both aragonite and calcite in the otoliths of reptiles and amphibians (Carlstrom, 1963; Lim, 1973; Marmo et al., 1983; Pote and Ross, 1991; Pote et al., 1993a).

Otoconia and the gelatinous membrane that hold them together constitute a dynamic system where Ca^{++} and other ions are exchanged during otoconial remodeling (Ross and Pote, 1984), but the exact nature of otoconial turnover has not been demonstrated. The development of electron probe X-ray microanalysis (EPMA) in recent decades has provided a powerful tool to investigate biomineralization in calcified tissues, mainly in cartilage, bone and teeth (Aly et al., 1977; Sánchez-Quevedo et al., 1989; Höhling et al., 1991; Boyce et al., 1992) and now we apply it to otoconia anaysis.

In the inner ear EPMA has been used to study the ionic composition of cochlear and vestibular structures (Ryan et al., 1979; Anniko and Wroblewski, 1981), with special attention to otoconial biomineralization under normal and pathological conditions. However, the results reported by different authors have been difficult to compare because of differences in the preparation of inner ear samples, the redistribution of diffusible ions, and the quality of morphological preservation. In addition, the relatively brief experience in quantitative EPMA for biological bulk specimens was overcome only recently, when absolute concentrations of the chemical elements were measured using a set of bulk microcrystalline standards (Campos et al., 1992; López-Escámez et al., 1992a, 1994). These standards were shown to be stable, homogeneous and reproducible over a range of voltages used to study otoconia (López-Escámez et al., 1993,b) providing for the first time a realistic estimate of the actual ionic values in those samples.

Elemental composition of developing and mature otoconia

It was reported by (Anniko et al., 1984) that human otoconia contained an extremely high concentration of calcium, and that sodium, Mg, P, S, Cl and potassium were also present. Non-calcium elements are more prominent in fetal and early postnatal life. In studies of normal mice the same authors found the same elemental composition as in humans, with trace amounts of non-calcium elements. Fetal otoconia in these animals also have higher concentrations of non-calcium elements than adult otoconia (Anniko et al., 1987). In otoconia of adult Wistar rats the non-calcium fraction was greater (9.71%) in otoliths smaller than 5 μm in diameter in comparison with otoliths larger than 15 μm in diameter (3.33%). Iron was detectable in the latter (Crespo et al., 1985). In some species, such as the guitarfish, Fe^{++} was found in magnetic particles that were intermingled with calcium carbonate otoliths (O'Leary et al., 1981). Micro-analytical studies in Wistar rats determined that otolithic levels of calcium decreased postnatally before reaching adult levels (Ciges et al., 1985).

When digitalized EPMA was used to study the topographical distribution of calcium content in the whole otolithic membrane, maximum calcium density was found along peripherical regions of the maculae (Fig. 77a,b). Variations in the groupings of otoliths, and thus in mineral density, are probably related to selective stimulation of different areas of the underlying sensory cells (Campos et al., 1985). Others have shown that striolar and non striolar regions of the maculae have different otolithic membrane arrangements, including sensory hair cells differential placement that is matched by specific innervation mixture of afferent and efferent nerve endings (Lindeman, 1969; Lim, 1979; Goldberg, 1981; Fernández et al., 1988, 1990a, 1995).

Elemental composition of altered otoconia

The elemental composition of otoconia can be altered by genetic factors, aging and drug induced ototoxicity. Electron probe microanalytical studies of the otoliths in mutant mice with inner ear alterations, which can potentially shed light on the influence of genetic factors, are unfortunately scarce. Others (Anniko and Wroblewski, 1983) found that in shaker-1 mutant mice the concentration of non-calcium elements was similar to that in normal fetal otoconia. Calcium levels in the otoconia of adult normal mice and shaker-2 mutants did not differ significantly. It is thus unlikely that the shaking-waltzing behavior of shaker-1 and shaker-2 mutants was derived from a minimal derangement of the

elemental composition of otoconia. Other studies indicate that manganese supplementation of pregnant pallid mice decreases the incidence of abnormal behavior and the severity of otoconial defects among the pallid mice progeny. Zinc supplementation of pregnant pallid mice and lactating mutants significantlly improved the density of the otoconial mass (Erway et al., 1986). Manganese and zinc may be related to glycosamino-

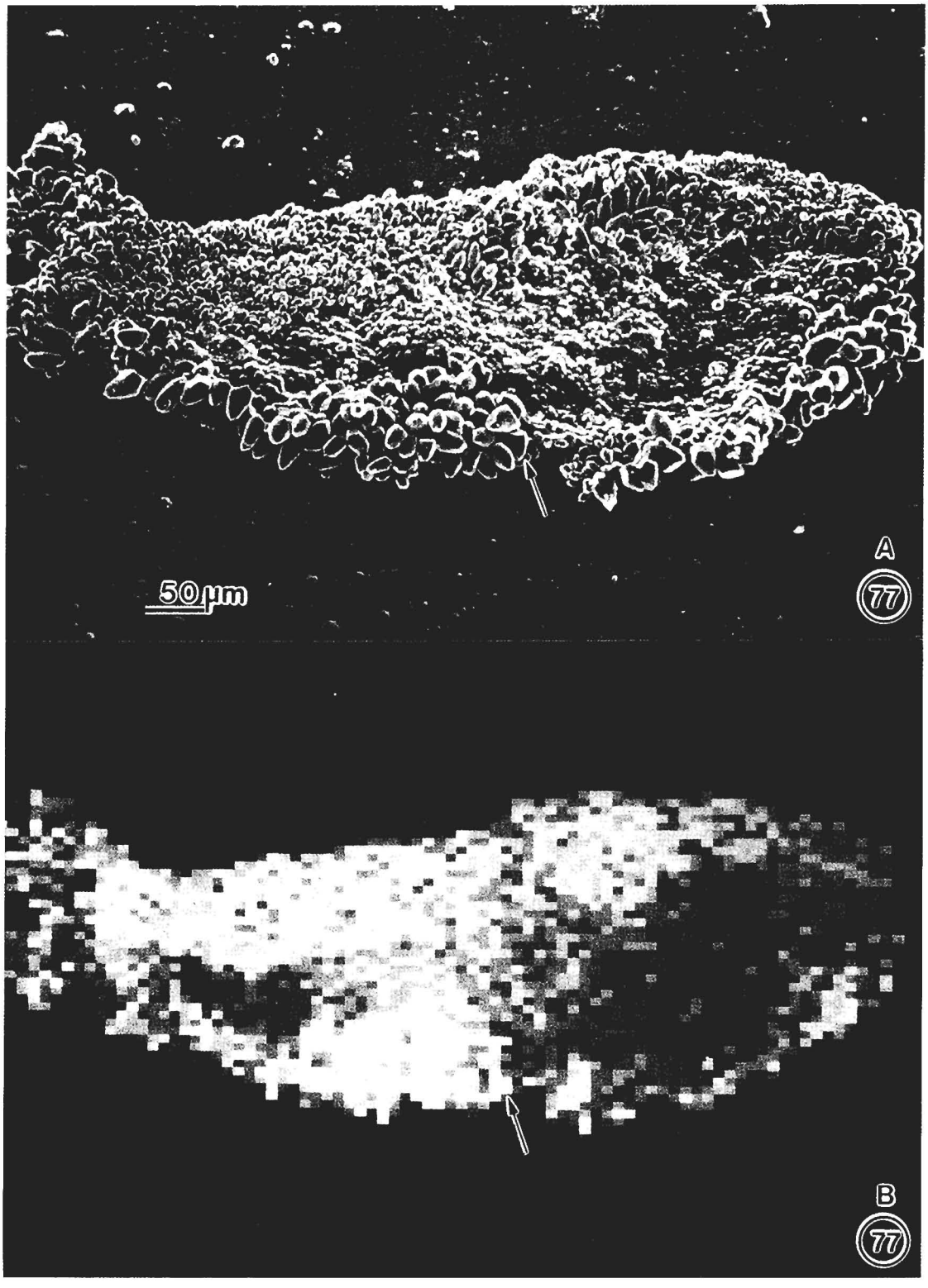


Fig. 77. A. Saccular otoconial membrane isolated intact and used for X-ray microanalysis to estimate calcium content in different regions of the membrane. Note larger crystals on the periphery of the membrane (arrow) also shown in hamster (Figs. 63, 64). **B.** Digitized (pixelized) image of the isolated saccule above to estimate electron probe X-ray microanalysis of the membrane. Lighter pixels represent high concentration of calcium on the crystals. This image demonstrates that larger otoconia such as these seen in abnormal specimens do not necessarily contain more calcium than smaller otoconia. Please see Figures 48, 49 of otoconia from Lamprey for comparison. The Lamprey otoconia are large but hallow, a condition that is easily created by introxication with various drugs (Marmo, 1967; Purichia and Erway, 1972; Harada and Sugimoto, 1977; Marmo and Balsamo, 1977; Harada et al., 1978; Wright and Hubbard, 1981; Wright et al., 1982; Anniko et al., 1984; Lim, 1984; Fermin and Igarashi, 1986; Igarashi et al., 1993a).

glycan synthesis and carbonic anhydrase inhibitors in the inner ear (Lim, 1973; Erway et al., 1986; Campos et al., 1990a), of which glycosaminoglycans were shown to be intimate components of the avian otoliths (Fermin et al., 1990, 1995b; Fermin, 1993). The implicit genetic-enviromental relationships of the above findings raise many interesting, but as yet unanswered, questions in animal models and in humans.

Several authors have described variations in the elemental composition of otoconia with aging. For instance, Anniko et al. (1984) pointed out that otoconia in elderly humans contain increased levels of phosphorus, and suggested this finding as a sign of age-related degeneration in which calcium-calcite is replaced with calcium-phosphate. This change probably preceded the morphological changes described in the aged utricule and saccule. In the degenerated otoconia with roughened surface pits, parallel slits and grooves seen in aged Wistar rats, calcium levels were significantly lower (sign of demineralization) than in normal otoconia from the same maculae. Although demineralization was similar in peripheral zones of the utricle and saccule, the process was apparently different in central zones of both maculae (Campos et al., 1990a). Recent work by (Takumida and Zhang, 1997) has shown that calcium concentration is lower in aged guinea pig otoconia. These authors found calcium to correlate linearly in young animals with the P, S, Cl and K^+ also present in otoconia. However, the Ca^{++}/P, Ca^{++}/K^+ and Ca^{++}/S ratios are generally not maintained in aged animals, suggesting that aging influences the metabolism of these elements in the vestibular organ, resulting in alterations of the otoconia mineral composition with potential consequences for the turnover rate of elements in otoconia if it exist at all.

Aminoglycoside-induced toxicity can also alter otoconia elemental composition (Cañizares et al., 1990) showed that the earliest effect of gentamicin in adult mice was to decrease calcium levels in otoconia smaller than 4 μm in diameter, but not in larger mature otoconia. Mechigian et al. (1979) found that the rate of ^{45}Ca incorporation decreased by 30-40% in the otolithic membrane of both the saccule and utricule of adult guinea pigs treated with ototoxic doses of gentamicin or streptomycin. Further studies with this method have shown that streptomycin specifically interferes with calcium uptake in otoconia (Zhang et al., 1996a). Oral administration of tetracycline to guinea pigs induced the deposition of the antibiotic (as viewed with the fluorescent microscope) on the outer surface of otoconia. A finding that was recently duplicated by (Kido, 1997) in chicken corroborating previous hypothesis put forward by one of us that mineralization of the organic matrix occurs gradually from outside toward inside the crystals (Fermin and Igarashi, 1985a,b, 1986; Fermin et al., 1987), following a pattern of formation that conforms to a sequence of events proven in other systems such as bone (Fermin et al., 1995b). Like otoconia, bone requires mineralization after deposition of an organic template. Thus far the strongest evidence presented for both mammals and birds is that otoconia templates are first secreted in a similar manner as other gelatinous membranes are secreted in the endolymphatic space and are later mineralized. Furthermore, the coexistence of calcified and uncalcified membranes such as that of the horizontal canal cupula and the utricular otoliths requires microenvironmental controls so finely tuned that only a differential distribution of macromolecules able to attract different ions to each membrane seems reasonable (Fermin et al., 1990, 1995b; Fermin, 1993). While other mechanisms of otoconia formation and mineralization are certainly possible the data presented thus far does not favor alternate hypotheses such as extrusion of otoconial bodies from the supporting cells. Not a single paper has yet presented credible evidence at the transmission, scanning and light microscope levels to prove alternate hypothesis. If indeed, preotoconial bodies form prior to the development of the otoconial membranes in the utricle and saccule in mammals and the maccula lagena of birds (Jorgensen, 1970; Lavigne-Rebillard et al., 1985), transmission electron microscopy of such bodies should be forthcoming, but none has yet been shown (Harada, 1978, 1982, 1983; Nakahara and Bevalander, 1979). There are numerous proven histochemical reactions that precipitate the likely candidates for molecules making up the otoconial membranes, and such histochemical stains with the transmission electron microscope should yield macromolecules making up the otoconial membrane during early development and regeneration (if it occurs) after damage.

In quantitative EPMA studies of adult mice a direct linear association between calcium and potassium in saccular and utricular otoconia was not maintained after exposure to gentamicin, which suggests that this aminoglycoside antibiotic interferes with the calcium-potassium equilibrium in the otoconia. A dose of 200 mg/kg gentamicin twice a day for 5 days did not affect calcium in the mineral phase of otoconia, but did increase potassium in both saccular and utricular otoconia. The increase in potassium may reflect a modification in the composition of the endolymph resulting from cellular damage at the plasma membrane (Campos et al., 1994). Streptomycin ototoxicity can give rise to giant otoconia. An increase in solute concentration, a decrease in levels of calcite growth-inhibiting agents such as PO_4^{3-} and Mg^{2+}, and an increase in the production of organic matrix molecules, which in turn leads to higher numbers of seeding-nucleating sites. The net result is an increased rate of crystal growth (Ballarino and Howland, 1986; Campos et al., 1990b).

Elemental composition of the gelatinous membranes

The gelatinous membrane that supports the otoconia consists of a randomly cross-linked filamentous network forming strands that uniformly distribute the forces of inertia during motion (Kachar et al., 1990). It has become increasingly clear that detailed knowledge of the

biochemical environment of the otoconia, including the gelatinous membrane, is essential to understand the complex interactions between organically controlled and purely physical processes that result in biomineralization of portions of the membranes (Ross and Burkel, 1973; Ross and Pote, 1984) and not others. Immunohistochemical studies reported proteoglycans, including keratan sulfate (Fermin et al., 1990, 1995b; Munyer and Schulte, 1991; Fermin, 1993) and chondroitin sulfate (Munyer and Schulte, 1991), in the gelatinous membranes of the endolymphatic duct. Glycoconjugates were located in the gelatinous membrane and otoconial layers (Fermin et al., 1990, 1995b) have been found to differ in carbohydrate composition (Suzuki et al., 1992). Nine major proteins (Khan and Drescher, 1990) were identified in the saccular otolithic membrane by SDS-polyacrylamide gel electrophoresis, and numerous biochemical analyses of gelatinous membranes support a complex composition and probably complicate homeostatic mechanisms both in the vestibular endolymph and the auditory endolymph (Thalmann et al., 1987, 1990, 1993). Nevertheless, the biochemical composition as well as the microstructure of the gelatinous membrane in vivo are not known (Fermin et al., 1990; Takumida et al., 1992). Although EPMA has been used to study inner ear tissues, these studies have focussed mainly on the elemental composition of the otoconia (Anniko et al., 1984; López-Escámez et al., 1992a) instead of the gelatinous membrane layer because of difficulties in the preparation of inner ear samples due to a probable redistribution of ions during preparatory conditions (Anniko and Wroblewski, 1981). In addition, there was for a long time a lack of suitable standards for quantitative analysis of inner ear membranes (López-Escámez et al., 1993b) until recently when quantitative electron probe X-ray microanalyses were successfully done using salts dissolved in dextran solutions as appropriate standards (Crespo et al., 1993; López-Escámez et al., 1993c). In mice, phosphorous and potassium concentrations were inversely correlated, whereas phosphorous and sulfur concentrations were directly correlated (Crespo et al., 1993). Gentamicin ototoxicity in mice selectively decreased the concentration of P and S in the gelatinous membrane of the saccule, but left the utricle unaffected. The concentration of potassium increased in the utricular gelatinous membrane (López-Escámez et al., 1994). Although the mechanism of ototoxicity in the gelatinous membrane is unknown, the ability of aminoglycosides to block calcium channels may disrupt the ionic balance the endolymphatic fluid and thus affect the biochemical composition of the otoconial membrane. In general, the above differential effect of aminoglycosides on different ions of the membranes suggests that different mechanisms act concomitantly to maintain the exquisite homeostasis of the endolymph that permits proper transduction of mechanical into neural signals by hair cells (Zenner et al., 1994).

The macular organs and their contribution to vestibular evoked potentials

Linear vestibular evoked potentials (VsEPs) are neural responses produced by brief translational motion of the head. These responses to linear cranial motion can be recorded noninvasively from the surface of the skull in birds and provide a useful means of monitoring vestibular function in developing and mature animals. The avian VsEP represents compound action potentials of the vestibular nerve and corresponding central relay projections (Jones et al., 1997, 1998). It is clear that the responses depend strictly upon an intact vestibular labyrinth and are independent of the cochlea (Jones and Pedersen, 1989; Jones, 1992; Jones et al., 1997). However, the extent to which the macular organs contribute to responses remains to be shown. *A priori*, one expects the macular organs to be the primary source of sensory activity underlying the translational VsEP since it is well known that these organs respond to linear acceleration of the head and linear acceleration pulses are used to elicit the VsEP. Two recent lines of inquiry in a Jones' laboratory provide evidence that the maculae of the saccule and utricle are involved in the generation of the VsEP. First, VsEPs elicited by stimuli in the horizontal plane exhibited a strong preference for medially directed motion, a response feature anticipated for macular but not ampullar receptors. Second, abnormal VsEPs were found in animals with congenital abnormalities restricted to the sacculus thus suggesting a critical contribution by receptor in that organ.

Under the influence of linear cranial acceleration or a changing gravitational vector, otolith structures of the vestibular system are thought to undergo shearing motion relative to the underlying sensory epithelium. The shearing action is believed to provide an adequate stimulus to stereocilia of hair cells and, in turn, it results in the activation of primary vestibular afferents (De Vries, 1950; Fernández and Goldberg, 1976; Breuer, 1981). In addition, each hair cell is endowed with a specific directional sensitivity, which is determined by the orientation of stereocilia and kinocilium (Wersall, 1956; Lowenstein and Wersall, 1959; Flock et al., 1962; Flock, 1964). Collectively, hair cells and their respective polarization vectors are arranged systematically over the macular epithelium (Wersall, 1956; Flock, 1964; Wersall et al., 1965; Lindeman, 1969; Rosenhall et al., 1977).

Evidence from selective stimulation of vestibular organs

The pattern of polarization vectors in the utricular macula suggests that: 1) primary afferents should also exhibit preferred activation vectors; and 2) most horizontally directed forces should evoke a unique pattern of afferent activation corresponding to its direction. Many physiological investigations have critically tested and refined these predictions (Fernández et al., 1972; Loe et al., 1973; Fernández and Goldberg,

1976; Tomko et al., 1981; Dickman et al., 1991). Notably, for the overall population of afferents measured, investigators commonly described a majority of neurons responding best to an 'ipsilateral tilt'. In these cases, a majority of fibers innervating the utricular macula evidenced polarization vectors responding best to a laterally directed (ipsilateral) otolithic shearing motion.

In all previous VsEP studies in the bird, stimuli were delivered in the naso-occipital axis and no attempt was made to identify hypothetical polarization vectors for the response. Our work in this area has evaluated responses to linear translation in the lateral axis (interaural, ±y axis). Responses to cranial translation in opposite directions in the interaural axis (left and right translation) were compared for evidence of a "preferred population polarization vector". The response activity of each ear was isolated and compared for each stimulus. In the context of the utricle, epithelia on each side of the cranium present mirror images of polarization vectors due to the bilateral symmetry of the organs (Fermin, 1995; Fermin et al., 1995c). We recently tested the hypothesis that VsEPs elicited by lateral cranial translation reflects a preferred stimulus direction and this preference or not should be of opposite sign in the two ears. Based on published findings from utricular afferents, the preferred direction should correspond to an 'ipsilateral tilt'.

Fig. 78 schematically illustrates the lateral stimulus axis and skull coupling arrangements. A dental acrylic pad, anchored to the skull with self-tapping screws, was bolted to the steel arm of a stimulus shaker. Rigid coupling to the skull resulted allowing cranial translation stimuli to be delivered in the ±y axes. During translation in the +y direction the left ear received a '*Normal*' stimulus whereas the right ear receives an '*Inverted*' stimulus. During translation in the opposite direction (-y) this relationship was reversed. Responses from each ear were recorded during each of these stimulus directions and response amplitudes compared. Typical VsEPs recorded in the bird are illustrated in Fig. 79. Also shown are the stimulus and peak scoring strategies used in the present study. Stimuli were acceleration ramps having a duration of 2ms and jerk amplitude of 1g/ms as shown. In the present study, only the earliest components of the response were considered (P1 and N1) because these components are believed to reflect the activity of vestibular primary afferents (Nazareth and Jones, 1997).

Representative vestibular responses to linear translation in the interaural axis are shown in Fig. 80. Recordings from the right side (vertex to right mastoid) are shown. Note that before application of tetrodotoxin (TTX), the onset peaks P1 and N1 were of maximum amplitude only during movement in the -y direction. This direction is defined here as a '*Normal*' stimulus polarity for the right ear. During the '*Inverted*' stimulus (+y direction) P1-N1 is significantly reduced if not eliminated. These results clearly reveal a preference for a stimulus of *Normal* polarity. Indeed this was seen in both ears of all animals (n=15) studied. To rule out the

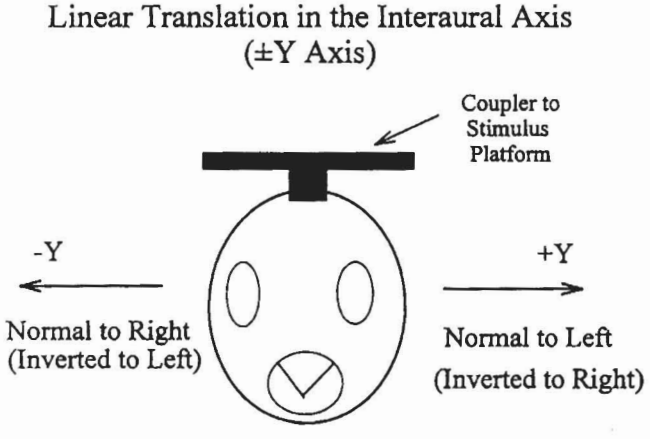


Fig. 78. Diagram of lateral stimulus axis and skull coupling for measurements of linear translation and estimation of macular contribution to the vestibular evoked potentials obtained. The computer controlled set up delivered a stimulis that is not perceived by an observer naked eye, and barely felt on touch.

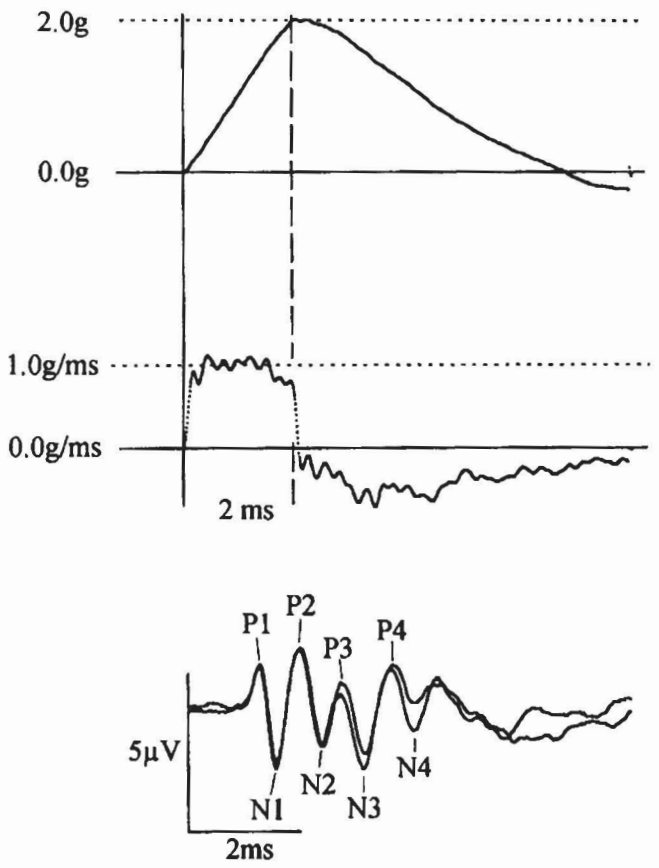


Fig. 79. Examples of typical VsEP recorded from birds showing also the stimuli, amplitude and duration with emphasis on the early components of the response namely P1 and N1. The strength, duration and polarity of the stimulus are shown on the top, and the positive (P) and negative (N) components of the response as well.

possiblity that the recording leads were picking up activity from the contralateral ear during these recordings, we applied TTX to the ear ipsilateral to the recording electrodes (TTX to Right Ear). After TTX application to the right ear, the onset peak P1-N1 virtually disappears for both *Normal* and *Inverted* stimulus polarities. This demonstrates that the ipsilateral ear produced the large P1-N1 component. The intact left ear and its neural relays through the brainstem produced all activity remaining after unilateral TTX application. This was confirmed when a second TTX application was made to the contralateral side (left ear) eliminating all residual response activity (bilateral TTX).

The results clearly show that VsEPs are sensitive to the direction of stimulation and that responses from each ear exhibit a preferred stimulus direction in the interaural axis. Moreover, this preference is of opposite sign in the two ears. These findings are consistent with the hypothesis that VsEPs are elicited by linear acceleration pulses and are generated primarily by the maculae. Since one expects cranial translation in the interaural axis to activate primarily utricular maculae and minimally affect saccular maculae, these findings further support the hypothesis that the utricular macula contribute significantly to the VsEP.

The specific direction of the preferred vector found in the present study does not match that predicted by head tilts and single unit recordings. Our '*Normal*' stimulus was the preferred polarity here. These stimuli are presumed to cause an adequate shearing motion which is directed medially (towards the opposite ear) and which would correspond to static tilts with the recorded ear up. This is the reverse of preferences observed in single unit studies. These findings are interesting in that they may provide additional evidence that responses to acceleration transients reflect activity in a selected set of vestibular neurons, most likely those described as being of "irregular" type and perhaps those sensitive to jerk components of stimuli. Whether this would explain differences in a general directional preference remains to be tested. Another plausible explanation that remains to be explored is that the two methods preferentially recruit neurons from different regions of the utricular epithelium.

Evidence from congenital saccular deficits

Belgian Waterslager canaries (BWC) are a strain of canary bred for their distinct low frequency song. Their unique song is due presumably to the fact that these birds have abnormal cochlear sensory epithelium and high frequency hearing loss (Oka et al., 1986; Weisleder and Rubel, 1992, 1993; Gleich et al., 1994a,b). In addition to cochlear abnormalities, BWC display hair cell abnormalities in one vestibular organ, the sacculus. Recall that the sacculus is a macullar vestibular organ that is responsive to linear acceleration. Abnormalities described for the saccule include fewer hair cells per given area, and shorter hair cell stereocilia whose bundles display no orderly orientation or staircase pattern. These morphological abnormalities affect sensitivity and transduction capabilities of the hair cells and consequently, neural activation. If the sacculus contributes in a substantial way to the generation of VsEPs to linear acceleration, then it can be hypothesized that disruption of the saccular epithelium would result in changes to the BWC VsEP waveform compared to normal controls.

Jone's laboratory recorded VsEPs to linear acceleration from BWC and a normal strain of canary, Yellow Choppers (YCC). Comparisons between the two species were made for VsEP response peak onset latencies, peak amplitudes, and thresholds. Onset latencies reflect the relative amount of time required for receptor transduction, transmission across the first synapse, and activation of primary afferent neurons of the vestibular nerve. Response peak amplitudes reflect the recruitment of neurons and the synchronization of firing patterns among the afferent neurons responding to the vestibular stimulus. VsEP thresholds provide estimates of the physiological sensitivity of the peripheral vestibular sensory system. Together, these measures provide sensitive, objective and noninvasive means for assessing peripheral vestibular function with strong otolithic contribution.

Fig. 81 shows VsEP response waveforms from one YCC and one BWC. On average, YCC show robust VsEPs similar to those already reported for birds (see Fig. 2, Jones, 1992; Jones and Jones, 1995; Jones et al.,

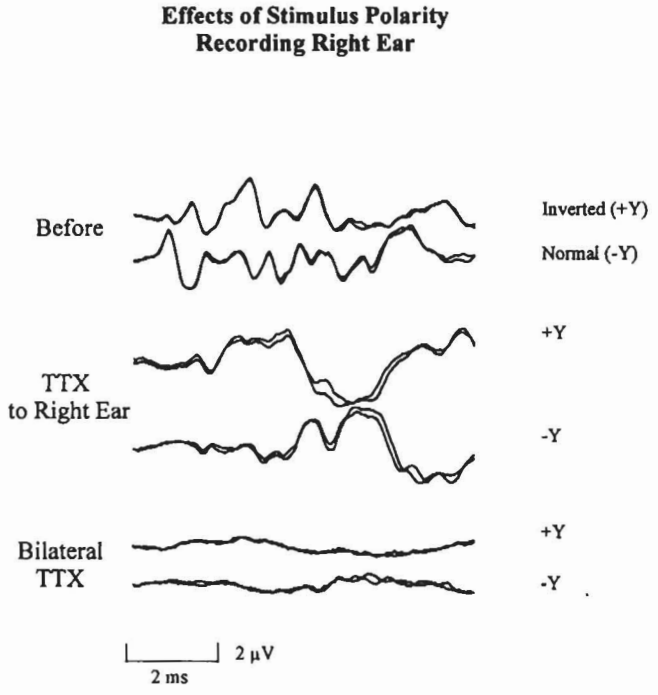


Fig. 80. Effect of stimulus polarity when recording from the right ear of the bird before and after application of tetrodotoxin (TTX) to the vestibular nerve. Bilateral application of TTX eliminates both P1 and N1 components suggesting that these components of the response are contributed by the nerve.

1998a,b) while BWC demonstrate VsEPs that are significantly reduced in amplitude and show slightly elevated thresholds. Mean VsEP thresholds for BWC were -22.5±0dBre: 1.0g/ms (N=5) compared to -24.75± 1.5dBre: 1.0g/ms (N=4) for normal YCC. These results suggest that the sacculus contributes substantially to the generation of VsEPs to linear acceleration. The precise generators for the remaining low amplitude BWC VsEP remain unknown, but the candidates would have to be capable of producing the precise timing of neural activation that is a primary requirement for the VsEP (Jones et al., 1998b). Normal hair cells of the BWC utricle, and semicircular canals, would be capable of producing this critical activation. Indeed, BWC VsEP response peak latencies appear to be normal suggesting that normal activation timing of the contributing neurons is present. The above studies of birds illustrate that damage restricted to the sacculus (hence, sparing both the utricle and canal organs) significantly alters the VsEP. Moreover, the VsEP shows evidence of a population polarization vector which would be an expected attribute of macular responses. Together these results provide compelling evidence that the macular systems of both the utricle and saccule contribute significantly to the linear VsEP. There is no positive evidence that canals participate in the generation of the VsEP. However, we can not rule out entirely any contribution a canal may have until studies permit testing non-invasively canals function.

Concluding remarks

The above data from different aspects of vertebrates otoliths illustrate the complexity and importance of macular organs for the survival of each species. In each case, from older primitive forms to newer forms (Figs. 46-62), modifications of the vestibular apparatus, and thus the inner ear, have favored anatomical modifications that enhanced differential perception of motion through the physical displacement of the otoliths due to linear and/or angular acceleration. The exquisite arrangement of the macular motion detectors (utricle and saccule of mammals and macula lagena of birds) in the vestibular system of newer species suggests that the acquisition of more refined and complex functions arose from a carefully orchestrated evolutionary programs that retained in the newer forms the most energy saving mechanisms of the older forms. The best example of this condition is the retention of a fine meshwork of organic fibrils from Lamprey's adult otoliths into the embryonic mammlian and avian otoliths. Even more fascinating is the retention of such meshwork as stabilizing and elastic spring-like-loaded membrane that permits energy dissipation while at the same time allowing the necessary deformation that culminates in receptor stimulation. And of course the potential piozoelectric properties that a spring-like-loaded flexible membrane may impart to the otolithic organ (Ross and Pote, 1984; Ross and Donovan, 1986) deserves a completely separate discussion, but so far direct evidence from experimental manipulations of the otoliths has not been forthcoming. If the piozoelectric properties of the otolithic membrane can be demonstrated to be intrinsic part of them, the concept has potential implication for receptor stimulation, energy dissipation and electrical current and/or field generations in the endolymphatic space under altered gravity such as that provided by space flights and the upcoming international space station.

On evolutionary trends

During evolution, the transition and modifications of the sensory receptors actuated by the otolith also favored the differential displacement of sensory receptors to detect acceleration in all possible directions. Thus, not only did the actuator or test-otolitic-masses (Ross, 1981; Ross and Donovan, 1986) changed with the demands of the emerging niches of the new environments, but the hair cells adapted new modifications that may or not may be related to transitional changes. For instance, older species have type II hair cells or modifications thereoff in the macular organs (Highstein and Baker, 1985; Highstein et al., 1987; Guth et al., 1994), whereas newer species have type II and also true type I hair cells to which special adaptation of afferent neurons were also developed. The emergence of newer and more refined species (Fig. 82) may have adopted otolith with mineralization patterns that favored each species emerging environment (Fig. 83) such that each crystalline form may have taken advantage of existing

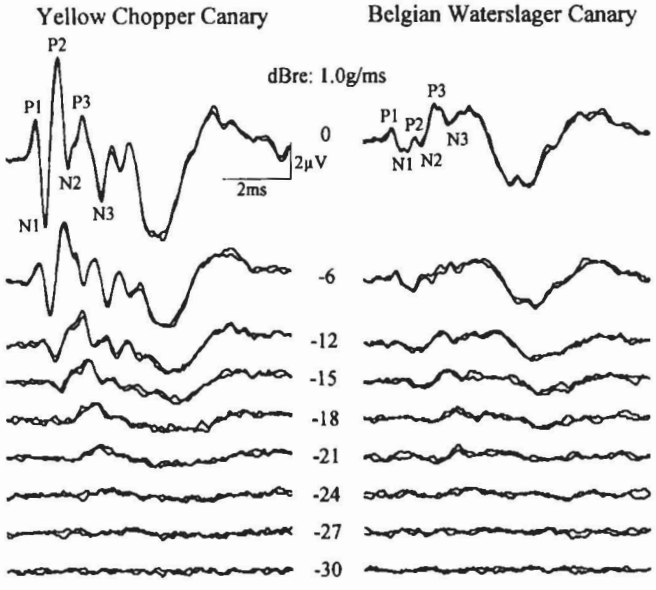


Fig. 81. VsEP from Yellow Chopper and Belgian Waterslager Canaries showing a diminished amplitude response recorded from the Waterslager canary on the right. These canaries have saccular defects sparing the utriculus and the canals. The shallow response suggest that the saccule contributes greatly to the VsEP recorded and imply a contribution of the otoliths toward these potentials. Note that response to stimulus differ between these species attesting to the sensitivity of the method. Morphologically, macular damage vary in each species.

macromolecules (Carlstrom et al., 1953; Carlstrom and Engstrom, 1955; Carlstrom, 1963) in each corresponding environment. The arrangement of hair cells in the striolar and non-striolar regions of each maculae from older to newer species was elegantly presented by (Lewis et al., 1985), and that work remains to date an accurate source of information to evaluate diversity of innervation of the maculae in the diverging vertebrate forms. Such modifications of the receptor innervation contributes today in newer species to sophisticated mechanisms of acceleration detection that were carefully examined by pioneers (Smith, 1956) of inner ear anatomy (Wersall and Lundquist, 1966; Engström, 1968) and contemporary workers such as (Goldberg, 1991), (Ross et al., 1991) and others (Flock, 1964; Jorgensen and Andersen, 1973; Kessel and Kardon, 1979; Baird and Lewis, 1986; Fernández et al., 1990; Chimento et al., 1994). More recently, biochemical and molecular properties of the afferents neurons are appearing giving even a more sophisticated but complex picture of the organization of afferent receptors from invertebrates to mammals (Cazin and Lannou, 1976; Honrubia et al., 1985; Kevetter and Perachio, 1985; Favre et al., 1986; Chover, 1989; Ehrenberger and Felix, 1991; Soto et al., 1991; Usami et al., 1991; López et al., 1992; Meza et al., 1992b; Budelmann and Williamson, 1994) and birds (Rubel, 1978; Wold, 1981; Peusner et al., 1988; Fermin, 1995; Fermin and Martin, 1995; Fermin et al., 1995c, 1997).

As far as the transduction of mechanical to neural energy in the endolymphatic duct is concerned, the relevant point of discussion and contention as it relates to otoliths properties; has been and will continued to be the contribution that a large and concentrated accumulation of macromolecules known to be part of the otoliths play in the transduction process. In newer species the endolymphatic space (lumen), where the transduction takes place, is a closed-conduit with a pressure-relief valve namely the endolymphatic duct and sac. We know from diseased temporal bones that the Reissner's membrane may provide a relief valve function as well, by distenting with the unfortunate eventual development of hydrops (Meyer zum Gottesberge-Orsulakova and Kaufmann, 1986; Horner and Rydmarker, 1991; Yoon et al., 1991). Thus, a future avenue for research in this field is the uncontaminated analysis of the otolithic membranes and endolymphatic content under various disease conditions that involve alteration of the macromolecular content of the endolymphatic space.

The endolymphatic duct is an intriguing environment where self assembly of organic crystals may take place. The building materials (macromolecules) available in the endolymphatic space (Santi and Anderson, 1986; Ross et al., 1987a; Thalmann et al., 1987; Lim and Rueda, 1990; Prieto et al., 1990; Santi et al., 1990; Thalmann et al., 1993; Fermin et al., 1995a,b) and the structures that are assembled in that space point to energy saving mechanisms from evolutionary efficient adaptions (Lima-de-Faria, 1995a) that maximize energy utilization and self assembly. Periodic structures such as the macromolecular arrays found in the gelatinous membranes of the endolymphahtic duct favor self assembly mechanisms and may, under appropriate

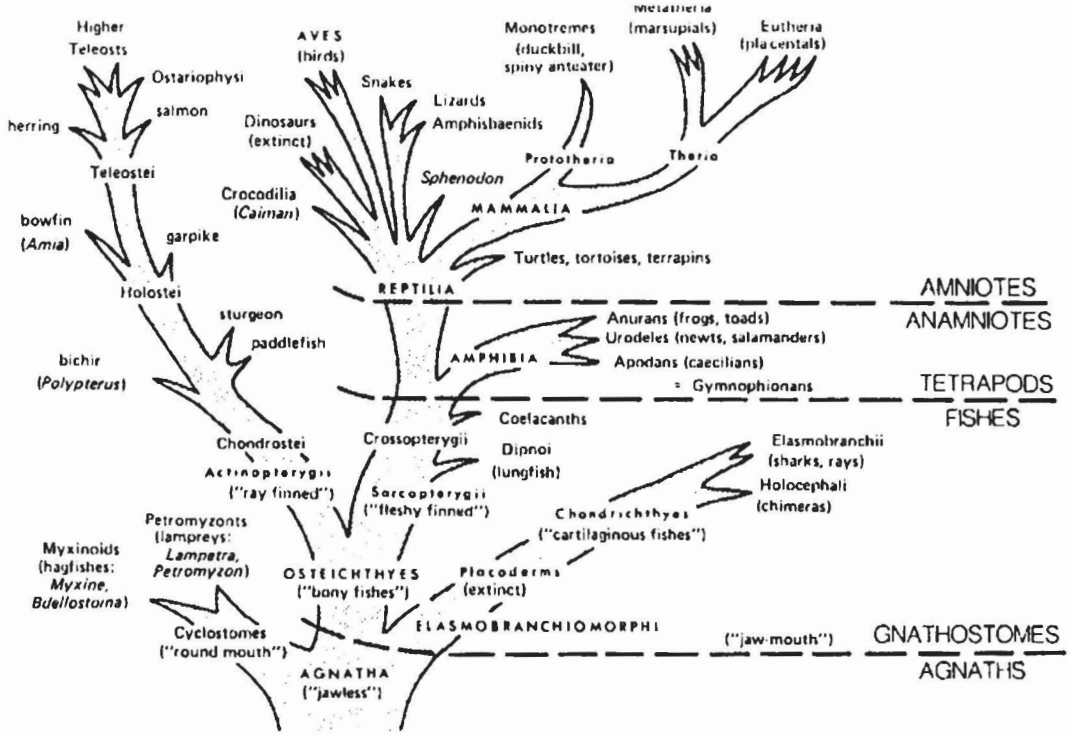


Fig. 82. Simplified evolutionary tree of vertebrates with extincts forms not included. Used with permission from the publisher (Lewis et al., 1985). Please compare to Figure 58. While birds and mammals diverged early, both have common ancestors.

conditions (such as nucleation), follow the principle of "self-assembly-critically" (Bak and Chen, 1991) that plays an intergral role in the biological periodicity and the retention of energy saving mechanisms by organisms during evolution. Assembly of periodic structures such as the crystals found in the endolymphatic duct probably obey the laws of biological periodicity which (Lima-de-Faria, 1995a) describes as being "characterized by an equally unexpected recurrence of groups of properties that emerge at relatively regular intervals, as the biological complexity increases throughout the evolutionary scale." On his provocative work he goes on to say that "...the similarity of properties rests on the electronic configuration of single atoms or of key atom groups" a case that is corroborated by the essential role that atomic elements play on the key molecules of living organisms such a chlorophyll and hemoglobin. In them magnesium and iron atoms respectively are more important than the remainder complex portions of these molecules.

On otoconia genesis and mineralization

The endolymphatic environment has retained the elements that best promote, with energy efficient mechanisms, the assembly of structures that emerged with the acquisition of specific functions such as transduction of mechanical into neural energy. From the opened neuromast of lateral line organs (Colmers et al., 1984) to the semi-closed sand grains filled otocysts (Fig. 53) to the sophisticated avain and mammalian hexagonal otoconia (Figs. 63-66) contained in a closed loop environment, the manifestations of biological periodicity are evident. First, final patterns can be produced by different molecules such as different proteins folding by the same aminoacids or crystals by organic fibrils interacting with different ions (Lima-de-Faria, 1995b). Of the seven distinct crystallographic patterns known, similar minerals forms could arise from totally different mineral classes. The triclinic, monoclinic, orthorhombic, tetragonal, trigonal, hexagonal and cubic crystallographic forms have different elements that impart each form its characteristic crystallographic property. Otoconia in different species may have acquired the conditions that best fit their position in time and retained them to this date. In newer species with organs of differing phylogenetic ancestry, such as the saccule and the utricle (Figs. 46, 47, 58A), different forms of crystals may be found in a single species (Marmo et al., 1983; Pote and Ross, 1991, 1993; Pote et al., 1993b). Interestingly, different forms of crystals appear when the homeostasis of the endolymphatic space or lumen (EL) is altered (Lyon, 1955b; Campantico, 1967; Balsamo et al., 1968, 1969; Erway et al., 1970; Lim, 1971; Lim and Erway, 1974; Wright et al., 1979a, 1982; Johnsson et al., 1980; Marmo et al., 1981b; Wright and Hubbard, 1981; Erway et al., 1986; Fermin and Igarashi, 1986; Igarashi et al., 1993a) suggesting that homeostasis of the fluids and macromolecules in the EL must be maintained within very narrow ranges.

In line with the energy saving strategies preserved during evolution of biological systems, it is worth mentioning that self-assembly processes outnumber cumbersome processes that require too much energy to complete. Energy is the crucial factor of self-assembly, since it determines the transformations that stabilize biological structures during evolution (Lima-de-Faria,

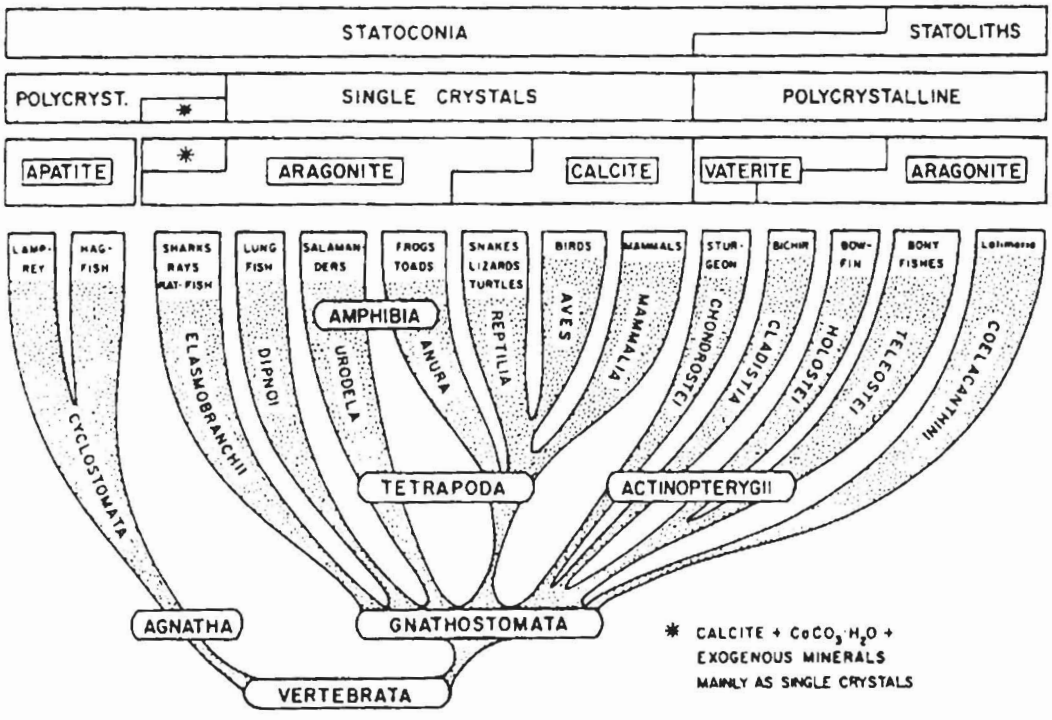


Fig. 83. Crystals of different otolithic forms according to an evolutionary tree. Used with permission from the publisher (Carlstrom, 1963). Please compare to Figure 58, and note crystalline forms that were incorporated during evolution into the otoliths. Nonetheless, the acquisition of the minerals that determine the crystalline forms on each group appeared more recently during evolution, probably influenced by their respective environments.

1995d). It is not surprising that otoconia of newer emerged species ended up with the hexagonal crystallographic forms that allow for more than 2,000 combinations or patterns. Crystals reveal the internal order that atoms are obliged to follow and in the case of calcite otoconia shapes are governed by the internal organization of its main calcium carbonate components. The preservation of internal atomic orders permits the precise assembly of crystals without the direct intervention of genomic functions. Otoconia, shells, bones and spicules all contain calcium with final forms that are dictated by its association with organic building blocks that in the endolymphatic space are provided by glycoproteins and polylactosaminoglycans (Fermin et al., 1995b), a proposition that was earlier suggested by (Belanger, 1960). Thus, atoms allow changes of crystallographic forms but within predetermined thresholds. For instance, boracite can only become cubic, as quartz does, but can never become hexagonal like otoconia calcite does (Lima-de-Faria, 1995c). This brings us to the mineralization of otoconia in mammals and aves which under normal conditions crystalizes in the hexagonal pattern. This pattern is one of the most stable, and the most stable structures known are based on the hexagonal repeats. The hexagonal motif has been termed universal, and found from the smallest to the largest structures that have stable infrastructures. Viruses, geodesic disks, cell cytoskeleton, buckyballs, pollen grains, ions such as potassium and muticellular organisms such as a volvox retained the hexagonal organizational pattern which (Ingber, 1993) and (Lima-de-Faria, 1995a) considered dominant forces shaping evolutionary processes due to their energy conservation nature.

It is not surprising then, that the development of otoconia was described in terms that accommodate the above principles of energy preservation. One of us showed transmission electron microscopy data, with and without histochemical decoration of macromolecules, that the initial stage of formation of the avian otoliths consisted of the deposition of a gelatinous organic matrix over the putative sensory areas of the presumptive maculae (Fermin and Igarashi, 1985a,b). Similar morphological events were demonstrated in mice by (Anniko, 1980), who hinted to the existence of a very short critical period of development when mineralization of otoconia occur. He also pointed out the "re-organization of material secreted into the extracellular space that we later showed in chicks to contribute to otoconia templates (Fermin et al., 1987). This last work showed transmission electron microscopy data indicating that the accumulation of the primitive membrane followed the same pattern of deposition as the non-calcifying tectorial membrane of the auditory organ (Cohen and Fermin, 1985). Similar secretory cisterns filled with material were shown by (Kawamata and Igarashi, 1993), whose works suggested a gradual deposition of organic substance as well. Similar ideas were then echoed also by (Kido, 1985) who showed with scanning electron microscopy a meshwork of material over the nascent epithelia before recognizable otoconia were emerged in either the striolar or extrastriolar regions. A gradation in the deposition of organic material and appearance of well formed otoconia away from microvilli of the supporting cells was also shown by (Veenhof, 1969b) in the mouse. The deposition of the organic matrix (Marco et al., 1971) was altered by substances known to interfere with enzymatic processes that maintain homeostasis in the endolymphatic space (Marmo, 1965, 1966; Fermin and Igarashi, 1986). Further evidence from our group also showed that segmentation of the nascent membrane occurred first away from the secreting epithelial front (Figs. 40, 41) and that older otoconia first appeared away from the supporting cells microvilli (Figs. 8-10) in this review and in previous publications above. A gradual mineralization, as opposed to an abrupt mineralization that previous workers have conceptualized but not demonstrated, is supported by the gradual decoration of macromolecules with histochemical stains (Figs. 18-42). In fishes, the involvement of the oganic matrix as important constituent of the otoliths was presented by (Saitoh and Yamada, 1989) who highlighted the presence of spherules during the early genesis of the otoliths.

Many theories of ocotonial formation were formulated in the past, departing from the our original hypothesis of data-supported scheme, but transmission electron microscopy data for the alternate hypotheses have not been published. Developmental data from both mammalian (Lim, 1973; Lim and Erway, 1974; Salamat et al., 1980) and avian (our data) otoconia supporting the deposition of an organic matrix and gradual mineralization of such matrix (Fermin, 1993; Fermin et al., 1995b) has not yet been proven wrong with substantiating alternative data. Thus, until new convincing evidence from transmission electron microscope is presented for the mammalian otoconia formation that deviates from the process documented in this review for aves we assume that in mammals the identical process takes places. In other words, if there is an intermediate stage between what (Salamat et al., 1980) showed in the developing rat and what so many others have shown to be similarly shaped hexagonal crystals in adults birds and mammals, a well controlled transmission electron microscopy study should illustrate it. Particularly because the make up melliu of the endolymph and the gelatinous membrane has been well documented for decades in both mammals and birds.

The old debate between alternate views of otoconia formation best illustrated by a series of papers in the 80's remains an interesting one (Harada, 1978; Nakahara and Bevalander, 1979, 1980; Peacor et al., 1980; Nakahara et al., 1981). Nonetheless, data presented on those contrasting views and newer data corroborate pioneering work on otoconia development (Lyon, 1955a,b; Belanger, 1956, 1960; Campantico, 1967; Veenhof, 1969b) that otoconia begin by secretion of an organic meshwork of disorganized fibrils. A silver methamine staining method was used by Igarashi et al. (1993b) to

suggest that different macromolecules were present in the organic matrix of the otoliths, a concept that was previously demonstrated in chicks by one of us (Fermin, 1993; Fermin et al., 1995b) and others. Perhaps the low resolution images used for older illustrations and conclusions in the original works, as opposed to later higher resolution transmission electron microscopy analysis, conceptualized the idea of an abrupt appearance of the otoconia in the endolymphatic space. For instance (Vilstrup, 1951) showed vacuole-like compartments filled with material, but the nature of the vacuoles was not demonstrated leaving unanswered questions about the role that such inclusions may play in otoconia formation. Unfortunately, even work in lower species fails to support the concept that otoconia suddenly appear in the endolymphatic space (Ballarino et al., 1985; Wiederhold et al., 1986; Pedrozo and Wiederhold, 1994; Hallworth et al., 1995). One of these work (Wiederhold et al., 1995) showed that at stage 33 development of the Japanese Newt a small number of formed otoconia appear, but no transition between this appearance and the origin of precursors macromolecules was demonstrated. The above group also question why in the Newt some otoconia only originate after stage 51 (Steyger et al., 1995), but they did not showed histochemical evidence of gradual appearance of precursor molecules that may appear. Thus, in lower species too, there is the need to clarify the emergence of otoconia at critical periods of development that are probably missed by studies that do not examine developmental critical stages within narrow ranges.

It appears that in the chicken there is a weight-dependent regulation of otolith grow (Howland and Ballarino, 1981), with an exponential increase of calcium carbonate incorporation after stage 36 (E10) according to Ballarino and Howland (1984). A direct relationship between body weight and macular size was previously demonstrated in primate as well (Igarashi et al., 1984), a concept that goes hand in hand with the idea of adaption to emerging environments discussed below.

On otoconia composition

Different studies have estimated different values for calcium and other molecules in adult otoconia (Carlstrom et al., 1953; Carlstrom and Engstrom, 1955; Wislocki and Ladman, 1955; Belanger, 1960; Carlstrom, 1963; Degens et al., 1969; Ross, 1979; Marmo et al., 1981b; Vinnikov et al., 1981b; Campos et al., 1985; Erway et al., 1986; Drescher et al., 1989; Fermin et al., 1990, 1995b; Kawamata, 1990b; Pote and Ross, 1991; Tachibana and Morioka, 1992; Fermin, 1993; López-Escámez et al., 1993b,c; Pote et al., 1993a,b; Lychakov and Lavrova, 1994a; Hallworth et al., 1995; Takumida and Zhang, 1997). According to Anniko and Wroblewski (1983), calcium levels in mice are higher in the central zone of the otoconia than at the periphery. Such differential distribution may be related to otoconial growth patterns over the maculae, but this supposition needs corroboration. Crespo et al. (1985) analyzed the distribution of elements in the central and peripheral zones of mouse otoconia, and reported that calcium and P levels were homogenous throughout, whereas Mg, S and potassium levels varies between these zones. Quantitative EPMA has shown that the WP (weight percent) of calcium in adult mice is 38.97±1.27 in the saccule and 39.61±0.89 in the utricle; these data are close to the 40.04 WP of pure crystalline calcite (Fig. 77A,B). A significant amount of potassium is present in 90% of the otoconia (López-Escámez et al., 1992a) in the saccule (1.32±0.16) and 95% of those in the utricle (1.01±0.14). Calcium and potassium ions may be used to balance the charges of the sulfated anionic groups in carbohydrate-protein complexes and in glycosaminoglycans as well as in acidic amino acids and phosphate groups in matrix proteins, as described for dentine mineralization (Sánchez-Quevedo et al., 1989; Höhling et al., 1991) and suggested that they may also occur in the otoliths (Fermin, 1993; Fermin et al., 1995b). In this connection, calcium and potassium concentrations were found to correlate linearly in otoconia of the saccule and utricle in adult mice (López-Escámez et al., 1992b; Campos et al., 1994), not surprising when one estimates that otoconial membranes from the utricle, saccule and lagena macula all seem to have similar composition (Fermin et al., 1995b). Similar composition of gelatinous membranes (Fig. 33) would be expected if indeed those membranes were all influenced by similar mechanisms inside a close loop system that only has one pressure relief valve namely the endolymphatic sac and duct. The contribution that the sac and duct have on the formation (SavinVasquez, 1955; Yamane et al., 1984; Yoshihara et al., 1992) were considered and it was subsequently shown that the sac may be an important player in the process of otolith formation and homeostatic control (Campantico, 1967; Marmo et al., 1981a; Imoto et al., 1983; Kawamata, 1990a).

On physical and chemical alterations of the otoliths

Potential turnover rate of molecules in the endolymphatic duct and embryonic, young immature or adult otoconia was reported (Ross and Peacor, 1975; Harada, 1982; Imoto et al., 1983; Yamane et al., 1984; Kawamata, 1987), but the exact nature of such turnover was not conclusively demonstrated (deVincentiis and Marmo, 1966; Ross, 1979; Kawamata and Igarashi, 1995). A differential incorporation of tetracycline into rat otoconia was noted by (Kawamata and Igarashi, 1995) suggesting that a differential growth between central and peripheral portions may take place leading to a central core.

Incorporation of radiactive calcium into already formed otoliths (Ross, 1979) suggest that calcium is exchanged (turnover) but conclusive evidence is still lacking. On the other hand, recent experiments that examined the incorporation of tetracycline into developing bone and otoconia suggest calcium is added gradually to embryonic and developed otoconia (Canalis and Lechago, 1982; Kawamata and Igarashi, 1995;

Zhang et al., 1996a,b; Kido, 1997). Of those studies (Kido, 1997) findings support our hypothesis of otoconia formation by showing with fluorescent labeling of tetracycline that: 1) Calcium uptake rate was higher away from the epithelia precisely where we demonstrated with histochemical methods to be most concentrated (Fermin and Igarashi, 1985b, 1986) and now illustrated in this work (Figs. 10-20), and 2) that the incorporation rate of new calcium added occurred at the periphery of each crystal first and then advanced toward the central core. These concepts we enumerated before in our work (Fermin et al., 1987, 1990; Fermin, 1993; Fermin and Martin, 1994; Fermin et al., 1995b) and corroborate now in this work (Figs. 4, 7, 8, 10, 13-15, 17, 19, 20) each with a clear central core even in the TEM and STEM modes of whole clorax etched otoconia.

Calcium needed to first mineralize otoconia and then to maintain any turnover rate that may exist in the otoliths may be sequestered to the areas needed through selective secretion and possibly in granular form which is found in histochemical decoration of calcium near the secretory epithelia of an otherwise clean endolymphatic duct (Fig. 18). We showed these data previously (Fermin and Igarashi, 1985b) and concluded that such granules may serve for delivering calcium to the sites they are needed, because they were readily precipitated by calcium precipitating agents. The granules were also extracted before and after precipitation with chelating agents such as EDTA (Figs. 12-16). Very high resolution of otoconia matrices (Salamat et al., 1980; Mann, 1986, 1988; Fermin, 1993) with TEM showed that fragmentation of otoconia crystals always yielded fracture surfaces where the minimal calcite units could fit such that seeding nuclei on the prelayed organic matrix (Figs. 22-24). Unfortunately, in every study conducted to date, the ultrastructural arrangement of the organic matrix lattice (Figs. 29-31) does not match the perfect crystallographic lattice of calcite. The seemingly paradoxical mismatch between the organic matrix lattice and crystal lattice of calcite could be explained by considering the irregular fracture faces of calcite demonstrated by (Mann, 1986, 1988), and in fact stereo pair preparations of whole otoconia (Figs. 27, 28, 36, 37) showed that there is a stratificaiton of layers which corresponds not only to the irregular fracture faces shown by Mann above, but also would fit granular calcium to start seeding. Thus, the paradox is not really as big as originally thought even by our own group, because the calcite crystals will grow in different directions as opposed to crystals of quartz which generally grow and fracture in concentric layers (Lima-de-Faria, 1995d). Finally, otoconia of embryonic chicks histochemically reacted with polycationic stains that precipitate macromolecules of the forming otoconia and found in the endolymphatic duct, show a gradual accumulation of such molecules rather than their abrupt appearance in newly hatched otoconia (Figs. 35, 42, 43). In each case granular aggregates are observed at the periphery of the crystal from where can be removed by sonication or by chemical reactions.

It is then reasonable to assume that physical or chemical forces able to perturb the normal homeostasis of the endolymphatic duct would alter the delicate balance that must be maintained in order for the gelatinous membranes to remain healthy. Drugs that remain in the duct, which has a slower than systemic turnover rate, alter homeostasis in the duct and damage otoconia (Purichia and Erway, 1972; Ross and Williams, 1979; Harada, 1982; Wright et al., 1982; Lim, 1984; Campos et al., 1994; Gauldie and Xhie, 1995). Incorporation and retention of essential macromolecules into the otoconial matrices may also be influenced by physical forces that at 1G on earth shaped the formation of crystals such as calcite with net effect on the ultimate fate of otoconial membranes and/or otoliths. Hypergravity (+2G force) continously applied to chick embryos from 0-18 days affected the size and shape (Figs. 66-70) of the developing avian (Hara et al., 1995) otoconia and produced severe behavioral deficits at hatching. Hamsters exposed to hypergravity and then allow to stand at 1G (Sondag et al., 1995a,b, 1996a,b) showed some changes in the size of mid-large (Figs. 63, 64) otoconia but not of small (Fig. 65) otoconia. Interestingly, the behavior of the hamster was affected even after 8 months after expossure to hypergravity and returned to 1G (Sondag et al., 1995a), as it was the behavior of tadpoles developed in hypogravity (Briegleb, 1968). The behavioral deficit observed could result from afferent nerve misfiring (Ross, 1983) because temporary exposure of developing animals to hypogravity was shown to cause persistent threshold shifts (Fermin et al., 1996). These results are in line with our previous observations that rearrangenments of the vestibular systems by many variables including alteration of gravity persist for long time after the variable is removed (Fermin et al., 1996). Chicks exposed to 2G under different conditions (Ballarino and Howland, 1984) of rotation as those exposed in Hara's experiment showed no effect on the size and shape of otoconia. Thus, the jury is still out on this issue and should prove a good source of experiments in the future. New studies should address the issue of critical periods of development when otoconia genesis is most likely to affected reversible or irreversibly. None of the studies above reconciled such issues. In addition, the exposure-non-exposure protocols should be designed to coincide with the known and predicted changes of epithelial changes in the maculae of the animals as to best estimate the contribution of the tissues before, during and after innervation is accomplished. There is however not doubt that systemic administration of aminoglycosides (Campos et al., 1994) affect the maculae and the otoconial membranes of rats (Figs. 71-76), but when, how, and what exactly happens is not fully understood.

The elements in the crystals are governed by the internal forces of their atoms (see discussion above). Ultrastructurally one would expect that the elements in otoconia would be viewed in the matrix of the crystals and to that effect, we showed previously that granular

calcific components abound in the endolymphatic space (Fermin and Igarashi, 1985a,b) from where they are probably sequestered to the matrix during mineralization. Similar granules were reported by Anniko (1980) suggested that calcium probably emanated from the surface of the epithelia, and in fact, potassium pyroantimonate precipitation of the endolymphatic space components showed higher accumulation of precipitate near the planum semilunatum (Fermin and Igarashi, 1986), a very active secretory area of the otolithic organs (Fig. 18). Subsequent reports from Newt otoconia examined with atomic forced microscope (Hallworth et al., 1995) showed 20-50 nm diameter granules (subunits) as being part of the crystals, dimensions that fits those previously reported by us (Fermin, 1993) and unfortunately not correlated by these authors or discussed for potential significance. In addition, there was no mention how the atomic force microscope (AFM) tip influences the end results. TEM yields direct two projections of the otoconial subunits, whereas AFM provides a relief picture similar to that obtained by rastering across a stone road as shown in teoleost otoliths (Gauldie and Xhie, 1995). In this study, the internal structure of the fish otolith was shown to consist of rows of filaments with granular structures in between. In either case, damage caused by microscopic radiation and other external forces can induce defects (Pradere and Thomas, 1990) in the crystal lattice images (Mann, 1986, 1988; Addadi and Weiner, 1992). These last authors emphasized: 1) The fracture surfaces of calcite faces do not resemble the smooth and straight planes of inorganic calcite, 2) Like in tooth, the complex ultrastructural alignment of many nucleation site contributes to distortion of crystal lattice projections, 3) Crystal morphology (Malkin et al., 1996) is modified by impurities from fluids, 4) Influences of organic macromolecules are important in the regulation of mineral growth, 5) It appear that fragments from otoconia range between 50-100 nm and occur as irregular curved units (Fig. 23).

On the functional contributions of otoliths

The technique we used provided a direct electrophysiological measurement of neural activity within the peripheral vestibular nerve (Jones, 1992; Jones and Jones, 1995; Jones et al., 1997) connecting the maculae to the brain stem, and the data demonstrates a relevant contribution of the otoliths toward vestibular function. Vestibular responses were elicited using linear cranial acceleration transients (Jones and Pedersen, 1989; Jones and Schiltz, 1989) and were recorded noninvasively from the surface of the skull as described in detail elsewhere. Early components of the compound vestibular response (e.g. positive and negative peaks of component P1 & N1, Fig. 79) have been shown to reflect activity within the vestibular nerve itself whereas later components likely represent activity of second or higher order postsynaptic activity. The physiological sensitivity of the vestibular apparatus was estimated by measuring vestibular response thresholds (Jones and Pedersen, 1989; Jones and Schiltz, 1989; Jones, 1992; Jones and Jones, 1993; Jones et al., 1993), expressed in dBre:1.0g, where 1.0g=9.81 m/sec^2. Response activation latency was measured and defined as the onset latency of the compound action potential (component 1). Response amplitudes were also measured at stimulus levels of 1.0g (0dB). The strategies utilized by our group should prove useful for demonstrating contribution of the otoliths toward vestibular function, in different species and under different experimental conditions in ground at 1G or hypergravity and in microgravity of space orbiting structures.

Technical notes

Animals

a) Chicks (*Gallus domesticus*) were used for otoconia genesis study. All chicks were sacrificed by cervical dislocation after ip. Nembutal anesthesia, then processed as described below. Staged embryos were used as in previous studies (Fermin et al., 1990) to see if keratan sulfate changed during development. b) For the evolutionary significance of the otoliths fifteen species of Black Sea Fish were used and prepared for observation as previously described (Lychakov, 1988c, 1991, 1993). Briefly, specimens were collected, sacrificed and fixed in aldehydes. The specimens were then dehydrated, critical point dried, coated and photographed with a scanning electron microscope. Similar procedure was used to photographed the 2.0G exposed chicks and rodents. c) For measuring vestibular evoked potentials birds were anesthetized with Equithesin as previously described (Jones and Pedersen, 1989; Jones, 1992; Jones et al., 1997).

Gel Electrophoresis

The temporal bones were removed and placed in phosphate buffered solution (PBS) while dissection of individual end organs was carried out. Specific details for preparing materials and gels have been previously reported (Dunbar, 1987). Briefly, OM were dissected from unfixed tissue and solubilized in electrophoretic buffer. Samples of otoconia from over the maculae were carefully dissected out under an Olympus BHZ stereo microscope with fine forceps. Otoconial samples were then separated and aspirated into a micropipet that contained 1x Leammli's buffer and placed in a Eppendorf tube containing the same solution. Samples for SDS-PAGE were boiled for 2 minutes by placing the tubes in a boiling water bath, then kept at -20 °C until used. Samples for isoelectric focusing (IEF) were snap frozen in liquid nitrogen and stored at -70 °C until used. SDS-PAGE of OM was first done with either utricle, saccule or macula lagena separately, but since no difference was observed, otoconia from all maculae of 5 different embryos were combined for subsequent electrophoretic separations. Blood and tissues were always processed concomitantly as controls with the OM.

Controls for 1-D gels

The whitish and denser consistency of the OM facilitated the separation of the OM from the surrounding inner ear tissue when using either regular transmitted or dark field illumination. The utricle, saccule and macula lagena (Jorgensen, 1970; Lavigne-Rebillard et al., 1985) were physically separated from the rest of the membranous labyrinth before extraction of the OM was performed. The OM used for 1-D gels was placed immediately in the denaturing buffer and boiled for two minutes before storing it. An average of two minutes from sacrifice of the chick to extracting the OM was maintained during dissection. Tissue and a blood sample were always included in each run to account for tissue and blood contamination that is unavoidable with the procedure utilized.

Histochemical reaction with phosphotungstic acid (PTA)

To demonstrate fibril packing in the OM and otoconia, chicks were processed as previously described (Fermin, 1993). Chicks were perfused and fixed through the left ventricle with 2% paraformaldehyde-2% glutaraldehyde, tissues were then dissected, washed with PBS, and dehydrated in ethanol 50%, 70%, 80%, 90% and 95% for 15 min each. Then the tissues were incubated overnight at 60 °C in 100% ethanol containing 1% PTA with 3 drops of 95% ethanol per 10 mls of PTA solution. The next day the tissues were rinsed with cold 100% ethanol, infiltrated in 1:2 mixture of 100% ethanol/LR White for 6-7 hrs, then infiltrated in 100% LR White overnight and polymerized in the oven at 60 °C overnight. Thin sections were cut and viewed unstained. For stereo pairs images, tissues sections were photographed at plus and minus 15 degrees, developed and the photographs aligned at equal magnifications.

Aminoglycoside induced vestibular disturbances

Streptomycin sulfate (STP) was from PISA Laboratories (Mexico) and sterile physiological saline solution (0.85%, SPS) was prepared with NaCl (Sigma). Twenty-day-old male Long Evans rats were used in this study. Ten animals were injected daily intramuscularly in the thigh in alternated hindlegs for 48-57 days with 400 mg/kg body weight of STP dissolved in SPS. Five rats served as controls and received SPS injections for the same time interval and conditions as their experimental comates. Seven of the 57 day treated rats and three of the SPS-injected animals, were used to follow recovery for 8-12 weeks and did not receive any STP or SPS beyond the 57th day. After completion of each experimental manipulation animals were deeply anesthetized with 475 mg/kg body weight chloral hydrate (Sigma) and transcardially perfused with 300 ml of SPS followed by 350-400 ml of 4% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4 at 4 °C, over a period of 15 min. Animals were decapitated, the cerebral mass removed and auditory bullae were extracted in the same cold fixative for postfixation after drilling a hole in the bone to ensure proper entrance of solution. Bullae were further postfixed in buffered 1% osmium tetroxide, dehydrated and embedded in Araldite. After polymerization, the plastic outside of the bony labyrinth was removed with a razor blade, the bone was chipped off with a sharpened steel stick and the membranous labyrinth was divided into the five vestibular organs and half turns of cochlear duct. After evaluation by phase-contrast microscopy as whole mounts, selected portions of otolithic organs were mounted in vise chucks and sectioned with an ultramicrotome at one-micron thickness, either perpendicular or parallel to the endolymphatic surface. The sections were stained with methylene blue and azure II and examined by bright-field microscopy.

Hypergravity effect on otoconia

Rodents: To determine the effect of hypergravity on the morphology of the otoconia, patches of the utricles and saccules were fixed in 2.5% gluteraldehyde + 0.5% paraformaldehyde in phosphate buffer solution (0.1M, pH 7.4). After rinsing in distilled water and air-drying, the specimens were prepared for calcium content analysis and scanning electron microscopy. To determine the calcium contents of the otoconia, the specimen were sputter-coated with carbon and subjected to energy dispersive X-ray (EDAX, DX4) element analysis. For electron microscopical scanning (ISI SS40), the specimens were mounted on aluminium stubs and coated with gold. Photos were made to determine the effect of hypergravity on size and shape of the otoconia and on the distribution of utricular areas with small, medium-sized or larger otoconia. We calculated the area of each zone in relation to the total surface of the otoconial layer (the relative area of each otoconial size type).

Chicks: We used developing chick embryos, incubated from 0-18 days. Fresh eggs were placed in an incubator and were exposed to 2G on a centrifuge by the original apparatus. Effective 2G loads were obtained by rotating the apparatus at a constant velocity of 327.5 degree/s with the mounted incubator located at 55cm from the center of rotation. The vestibules of the chick embryos were excised and dissected, and fixed in 2.5% glutaraldehyde. The utricles were removed in phosphate buffer (0.1M, pH 7.4), postfixed with 2% osmic acid and dehydrated in graded ethanol. The tissues were dried with critical point dryer, coated with a layer of platinum and examined under a scanning electron microscope.

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