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REVIEW OF STATOCONIA FORMATION IN BIRDS AND ORIGINAL RESEARCH IN CHICKS (Gallus domesticus)

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Abstract

This paper reviews published materials on statoconia formation in birds, and emphasizes works dealing with the embryonic chick (Gallus domesticus) saccule and utricule. Histological, biochemical and histochemical aspects of forming statoconial membranes and statoconial crystals of mammals are included. Results from our work with chick embryos permitted us to conclude that statoconia probably do not form by seeding of a subunit around central core. Instead, immature statoconia may emerge already formed, from a segmenting mass of organic material that seems to be secreted by the supporting cells of the saccular and utricular maculae. Crystallization of each statoconium may involve seeding of multiple subunits around many nucleation centers in the organic matrix. Following these processes, calcium (sometimes granular) attaches to immature statoconia and become subsequently incorporated between the fibrils of the organic matrix starting at the peripheral zone and advancing toward the central core. Our transmission electron microscopy findings, histochemistry and X-ray microanalysis complements of other investigators, who used chicks with light microscopic studies. These results agree with the notion that the secretion of an organic matrix constitutes the first step toward the formation of the statoconial membrane and statoconia. We show ultrastructurally how statoconia may be assembled from the organic matrix before they acquire their characteristic geometric shapes.

KEY WORDS: Statoconial membrane, statoconia genesis, calcification, chick embryo, saccule, utricle,

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Introduction

Statoconia are microscopic structures, usually of geometric shape, that are contained in sac-like structures within the membranous labyrinth of vertebrates. In addition to the utricular and saccular maculae found in mammals, birds have a lagenar macula which also contains statoconia. This paper will deal primarily with the utricular and saccular maculae and their statoconia in chick embryos.

When the head moves in response to linear acceleration, statoconia in the maculae tend to stand still, while the cilia of the hair cells move and produce a complex but ordered set of neurological events that culminate with detection of gravity and linear acceleration (Oosterveld, 1982). Statoconia provide indispensable cues for sensory perception of the environment. Although abnormality of the vestibular nerve and nuclei contribute to postural imbalance and ataxia, the vestibular end organs and statoconia provide the primary input for detecting gravity. Without the utricular and saccular maculae and/or statoconia in these structures, vertebrates have great difficulty keeping body balance. Several species with genetically defective otoliths show erratic behavior and are sometimes unable to walk upright (Lyon, 1955a,b; Erway et al., 1970; Purichia and Erway, 1972; Rouse et al., 1984; Lim, 1984). Thus, the study of statoconia formation and mineralization constitutes an important step toward the understanding of balancing mechanisms.

Two main hypotheses underline ongoing investigations in our laboratory in regard to statoconia formation in the chick: 1. The segregation of cell type, the cytodifferentiation of the vestibular sensory epithelia, and the synaptogenesis of afferent and efferent fibers onto hair cells follow well defined gradients, and the events are coordinated in time and space (stage and location). 2. Statoconia may detach from a primitive otolithic membrane by segmentation, and at least part of the

process of calcification occurs by the incorporation of calcic granules into the organic matrix.

In this paper, previous works on adult and developing statoconia of birds are reviewed. In addition, original research that corroborates the second hypothesis described above is presented.

Literature Review

General Comments

The earliest embryological sign of a vestibular apparatus is a thickening of the ectoderm on both sides of the head at the rostral caudal level, approximately at the middle of the hindbrain. Progressive invagination of the ectodermal layer leads to the formation of a concave cavity that is called the otic pit. By closing, the pit becomes a vesicle and evagination of its walls leads to the formation of three primary ducts, namely the endolymphatic duct, the utricles, and the saccules. These mechanisms seem to be the same in those species examined. Probably the best description about changes of the otic placode was provided by Bast and Anson (1949) in the human embryo. The progressive changes of invagination of the ectodermal layer were later described by O'Rahilly (1963), also in human embryos.

Similar changes in the ectodermal layer of the otic placode were later described and illustrated by Meier (1978). He showed that in the chick embryo, invagination of the otic walls occurred at about 40-45 hours of incubation. He hypothesized that pseudostratification of the placode occurred even earlier, and that it was the result of crowding of cells from continued division within set boundaries of the epithelia. For a more complete and recent review of inner ear early development, see Structure and Function in the Vertebrate Auditory System by Rubel (1978). Inductive interactions involved in the early stages of otocyst development have been extensively studied in many vertebrates. For extensive treatment of this subject, the reader is referred to a chapter on "Developmental Mechanism of Mammalian Inner Ear Formation" by Van de Water (1984).

Bird's Statoconial Membrane and Statoconia

De Vincentiis and Marmo (1963) studied acid phosphatase activity in chick embryos and examined their activity in the utricular and saccular maculae, the cupula and the tectorial membrane of the cochlea. He found an intense alkaline phosphatase activity from the 7th day of incubation (stage 31) until about the 12th day of incubation (stage 38). Three years later, de Vincentiis and Marmo (1966a) studied amino-acid incorporation into the epithelia of the inner ear with particular attention to the otolith organs. For this purpose they used tritiated leucine and proline, and determined that there was a higher incorporation of these amino acids in the cupula of the semicircular canal cristae than in the otoliths. De Vincentiis and Marmo (1966b) could not find any calcium incorporation on the 4th day of incubation (stage 25). The first indication of radioactive calcium incorporation (see Preston et al., 1975) was found in the endolymphatic sac at about the 5th day of incubation. This observation indicates that the endolymphatic sac could actively participate in statoconial formation (Savin-Vasquez, 1955) and removal (Imoto et al., 1983; Yamane et al., 1984). Experiments that further illustrate this possibility are described below.

De Vincentiis and Marmo (1966b) also showed that on the 5th day, an intense PAS activity was found in the same locations where calcium was incorporated. The PAS activity was later located in the saccule, and by the 6th day it was present in the utricle. Between the 7th and 12th days of incubation, radioactivity decreased. From the progressive changes observed between the time of the appearance of the first hints of otolithic membrane, and the first calcium incorporation, de Vincentiis and Marmo (1966b) hypothesized that the otoliths were formed in two steps that involved: (1) secretion of a membrane, probably organic, and (2) the calcification of this membrane. These two steps were found to be vulnerable to the effect of carbonic anhydrase inhibitors (de Vincentiis and Marmo, 1968; Erulkar and Maren, 1961).

Differences between acid phosphatase and alkaline phosphatase were studied later by Marmo (1965). He showed that acid phosphatase activity was present in the saccule and the gelatinous substance of the otoliths at about stage 37 (11 days of incubation). Similarly, a high activity of acid phosphatase was observed in the endolymphatic sac as early as 3 days of incubation (stage 19). Later during development, phosphatase activity was found distributed throughout the epithelia in a non-preferential manner, but it seemed that acid phosphatase was compartmentalized intracellularly while alkaline phosphatase was found outside the cells.

The activity of the respiratory enzymes, cytochrome oxidase and succinic dehydrogenase have also been examined in the developing chick ear. Marmo and Castaldo (1972) found these enzymes to be distributed throughout the epithelia nonpreferentially. The enzymes' activity was higher during periods of active cell division and growth. However, at later stages, when the epithelia had finished division, the enzymes were only present in areas undergoing drastic and rapid transformations, where large amounts

of ATP would be needed. They indicated that in the saccule, high metabolic activity is required for CO_3^{--} formation from CO_2 in order to precipitate $CaCO_3$ in the presence of Ca^{++}, all necessary steps for otolith calcification. These processes require high amounts of ATP for energy.

Studies on the composition of the otolithic membrane of chick embryos indicated that it was formed by complex muscins in which various carboxylic and sulphuric acid radicals were present (Balsamo et al., 1968). These investigators used radioactive sulphate, Alcian blue, as well as PAS and Toluidine blue stains at various pHs and determined that indeed, proteins with different charges reacted with these stains differently.

Tetracycline, which is known to be incorporated into forming bone, was found to be distributed equally in the otolith and adjacent bones of the same animal as judged by the auto-fluorescence of this antibiotic (Balsamo et al, 1968). However, they determined that during the early stages of development, there was a much higher tetracycline activity in the saccule of the young chicks than in the utricle. This is an important observation because many lines of evidence indicate that the saccule may have a directing role during otolith formation. Except for the endolymphatic sac, the saccule remains the most active structure during early development. Balsamo et al., 1969a, hypothesized that statoconia were formed first in the saccule, then in the utricle, and later in the macular lagena of the chick cochlea by a process that involved the following reaction: $H_2O + CO_2 = H_2CO_3$. Subsequently, Balsamo et al. (1969b) determined that carbonic anhydrase was necessary for the reaction described above during maximum formation of $CaCO_3$, which is necessary for the calcification of statoconia. The carbonic anhydrase enzyme was found to be necessary during the highest turnover rate of $CaCO_3$ and this was corroborated by the use of radioactive calcium. Later, Marmo and Castaldo (1973) found that radioactive calcium was not present before the 8th day of incubation. They also showed many small granules of radioactive calcium over and around statoconia of 13-day-old embryos. However, no mention was made of the possible association of the granules and the organic matrix, despite the fact that this group of investigators had previously proposed that the organic matrix might be required during the formation of the otolith. The radioactive granules shown by Marmo and Castaldo (1973) in their study could correspond to the electron-dense granules observed by us with the TEM (Fermin and Igarashi, 1985a, 1985b). The granules were enhanced with osmic potassium pyro-antimonate which is known to selectively outline calcic deposits within and between cells (Schechter, 1976). Marmo and Balsamo, (1977-1978), using scanning electron microscopy, showed that polymorphic otoliths might be precursors of adult types. They determined that a slow decalcification of the statoconia resulted in subunits which, under the scanning microscope, resemble crystal grown in vitro instead of the skeleton of the organic matrix. For similar findings in mammals, see Ross and collaborators (Ross and Peacor, 1975; Ross et al., 1976; Ross, 1979, 1983; Ross and Pote, 1984). An interesting observation from these studies is, when the statoconia were decalcified, they seemed to regress to younger shapes, i.e., the characteristic dumb-bell shape of young statoconia was more pronounced. This condition is now believed to result from the loss of support that is normally provided by calcic components crystallized between the organic matrix fibrils. As a consequence, the harsh conditions of the critical point drying procedure tend to collapse the center of the statoconia, which is known to remain relatively empty in relation to the peripheral zone. Collapsed centers of young statoconia during scanning electron microscopic preparation have also been shown by Ballarino and Howland (1982, 1984) and Howland and Ballarino (1981) in young chicks.

Epithelia of the Utricle and Saccule

Understanding the development of the epithelia that supports the statoconia is very important for a proper evaluation and comparison to be drawn between the two structures. Although the PI and colleagues have described a developmental gradient of the vertebrate auditory labyrinth (Cohen and Fermin, 1978, 1985; Fermin and Cohen, 1984a, 1984b), similar studies remain to be done in the vestibular labyrinth.

A number of investigators have studied different aspects of inner ear formation in the chick. Knowlton (1967) described the development of the chick labyrinth, nerve and brain centers. However, because of the breadth of her study, and because electron microscopy was not used, details of synaptogenesis were not addressed in her study, mainly because electron microscopy was not included. Ginzberg and Gilula (1979, 1980) offered a general overview of differentiation of hair cells and supporting cells as well as mechanisms of cell death in the innervation of hair cells, formation of basal laminae, and more extensively, modifications of intercellular junction as important factors for the reshaping of the epithelia of the inner ear. They also showed that well developed chemical synapses, consisting of presynaptic ribbons with vesicles and postsynaptic densities, were common in the labyrinth of the 7 day-old chick embryo. Their transmission

electron microscopy analysis also determined that most synapses formed between the 4th and 8th day of incubation. Unfortunately, in these studies the developmental sequence that the vestibular labyrinth follows, i.e., what is the progression of change in relation to the embryo age, was not addressed. Several studies have partially examined this issue in mammals (Campos et al., 1984; Ciges et al., 1983; Mbiene et al., 1984; Sanchez-Fernandez and Rivera-Pomar, 1983, 1984; Sans and Chat, 1982). Furthermore, the Ginzerg and Gilula (1980) study does not allow one to determine in which macula the synapses first appeared. This last question in particular is very important for interpreting the maturation of the vestibular apparatus and the functional significance of the otoliths, since the various vestibular end organs (maculae and cristae) are of different ontogenetic origins, and each carries out a different function.

A few reports have dealt with the relationship between the development of peripheral and central vestibular nuclei of the chick. Levi-Montalcini (1949), in a very extensive study of the peripheral and central nervous system of the chick embryo, determined that synaptic connections play a major role in the completion of differentiation and maintenance of nerve cells in the inner ear and brain centers. Particular interest has been directed by others toward an analysis of the spoon endings on the nucleus vestibularis tangentialis (Hinojosa and Robertson, 1967; Peusner, 1980, 1984) and modifications of the peripheral epithelia (Fink and Morest, 1977), the role of neurotransmitters (Meza et al., 1982) and the effect of afferent deprivation on peripheral and central nuclei (Decker, 1970; Fritzsch, 1981).

From the aforementioned brief review, it is clear that much work needs to be done to completely understand the development of the vertebrate labyrinth, in particular the development of the statoconial membrane, statoconia and epithelia that support the otoliths. The chick embryo is considered an excellent model because it is representative of other higher vertebrates including mammals (Hamilton, 1952; Heaton, 1975; Rubel, 1978; Parks and Cohen, 1982; Beck et al., 1983); also the chick inner ear is more easily accessible anatomically than the inner ear of many higher vertebrates (Fermin and Igarashi, 1983).

The elegant work of Dr. Lim's (Lim, 1974) and Dr. Ross's (Ross and Pote, 1984) groups in the United States, as well as Dr. Nakahara's (Nakahara and Bevelander, 1979) and Dr. Harada's group (Harada and Tagashira, 1981) in Japan have provided answers to many questions about adult statoconia and the maturation of developing statoconia of mammals. Wright's group (Wright and Hubbard, 1982) has also contributed important data on human statoconial development with the SEM. However, the initial stages of statoconia appearance have not been documented ultrastructurally. In this paper, we illustrate the initial stages of statoconia formation, propose a mechanism for statoconia genesis in birds, and show data indicating that statoconia may detach from a primitive otolithic membrane by segmentation, and at least part of the process of calcification involves calcic granules.

Materials and Methods

General Comments

Osmium tetroxide-potassium pyroantimonate (OKP) was utilized in conjunction with the carbonic anhydrase inhibitor DIAMOX in order to determine possible relationships between calcium (granules) and the formation and segmentation of the otolithic membrane. Carbonic anhydrase inhibitors have been shown to hinder statoconia formation in the chick embryo (de Vincentiis and Marmo, 1968), and to alter the behavior of the offspring from normal mammalian parents (Erway et al., 1970, 1971). Acetazolamide is a carbonic anhydrase inhibitor that is often used clinically in the treatment of glaucoma and also as a diuretic (Windholz, 1976). It is available under the generic name of DIAMOX and when administered to chick embryos in doses high enough to hinder statoconia formation, it increases the mortality rate of the embryos. However, the surviving embryos develop without gross anatomical abnormalities and have normal looking maculae and sensory epithelia (de Vincentiis and Marmo, 1968). Despite its effects, DIAMOX does not seem to interfere with most of the cytodifferentiating processes, since hair cells, supporting cells, and nerve fibers develop normally as far as shape, size, and cytoplasmic appearance are concerned. However, it is not known whether DIAMOX alters the entire process of otolith formation, or whether the organic matrix will form, but in the absence of calcium (which could be a determining factor for fibril assembly and/or maturation), the matrix formed may simply disintegrate and the fibrils become reabsorbed.

Embryos

White leghorn fertile eggs (Gallus domesticus) were used in this study. The eggs were kept in a humidity and temperature controlled incubator (Petersime model 1) that automatically turned the eggs at regular intervals in order to improve the survival rate of the embryos. Embryos were staged according to Hamburger and Hamilton (1951). Specimens were dissected out under

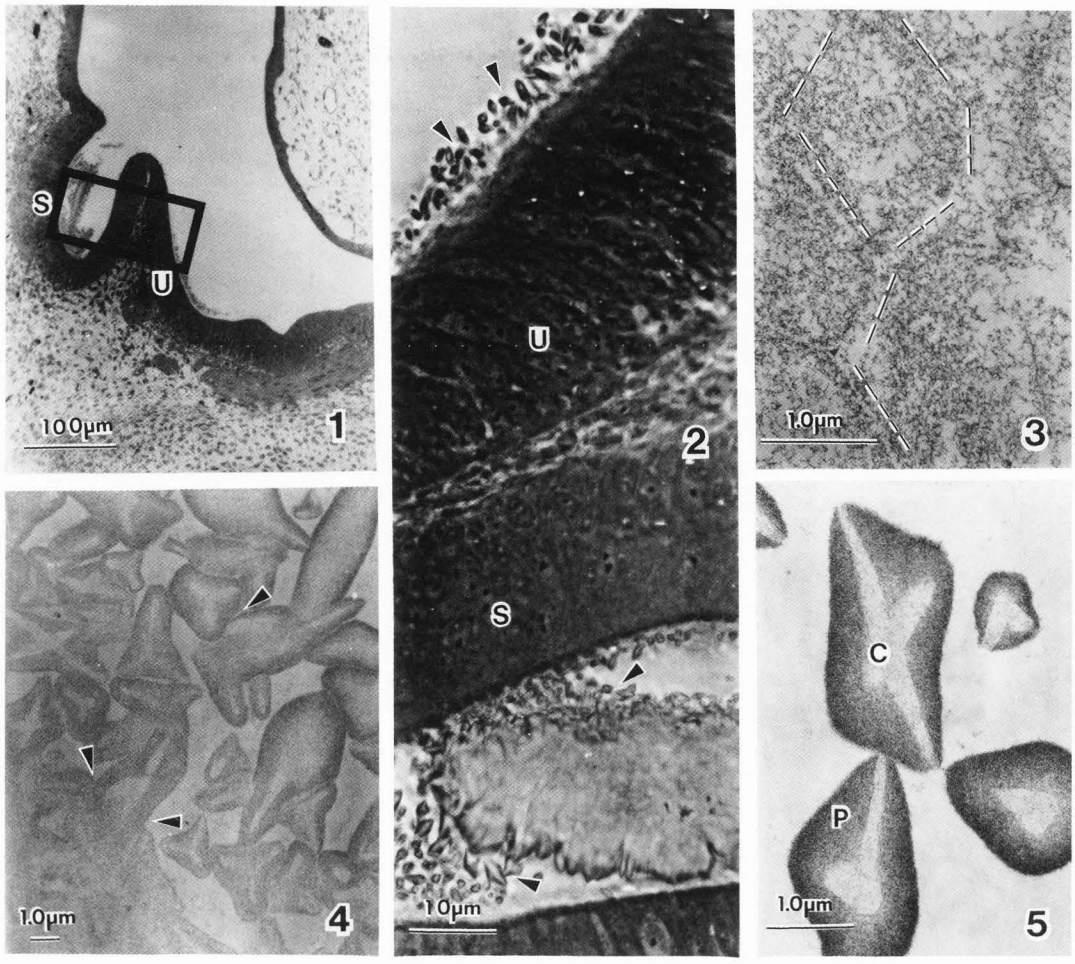


Figure 1. The organic matrix over the maculae seems to aggregate and form an immature otolithic membrane through a process that resembles the secretion of the tectorial membrane in the cochlea. Segmentation of the immature, uncalcified, otolithic membrane in the saccule (S) gives origin to statoconia. Embryo #899, stage 30. Utricle (U).

Figure 2. Higher magnification of area boxed in figure 1. Fibrous material secreted by the supporting cells of the saccule (S) thickens at its outer edge (arrows) and individual statoconia begin to form. This process continues and primitive statoconia are produced by segmentation of the membrane. The fibrous substance remains in contact with the microvilli of the supporting cells. The process of segmentation was seen in the saccular macula between stages 26-30. The saccular macula might serve as a locus for statoconia initiation and might also provide the necessary material for the assembly of utricular statoconia. In the utricle (U), statoconia were not seen attached to the statoconial mass. Embryo #899, stage 30.

Figure 3. TEM micrograph showing part of the segmentation process (dotted lines). Separation lines appear in the lower part of the membrane and statoconia appear. Embryo #251, stage 29.

Figure 4. The upper portion of the otolithic membrane contains individual statoconia, some seen here still attached to the segmenting part of the membrane (arrows). Embryo #896, stage 29.5.

Figure 5. By the time statoconia separate from the statoconial mass, their periphery (P) has thickened, but the central core (C) is almost empty. Embryo # 896, stage 29.5.

the microscope (Otto et al., 1984), and fixed with aldehydes.

The embryos intended for TEM were fixed in 3% pure glutaraldehyde in Ø.2 M cacodylate buffer. The osmolarity (385-41Ø MosM) of the solutions was monitored with a vapor pressure osmometer (Wescor, 51ØØC). Embryos were post-fixed in 1% osmium tetroxide and embedded in Araldite 5Ø2.

Specimens were sectioned entirely (edge to edge) as follows: 1) five 5-µm thick sections were cut and saved, unstained, 2) then two 2-µm thick sections were cut, mounted on glass slides and stained with 1% paraphenylenediamine and 1% Toluidine blue (1:1, v/v), dried and cover slipped, and 3) every 1ØØ µm, ten 7Ø-9Ø nm thick sections were collected on copper grids, and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). These specimens were then photographed with a JEOL 1ØØCX TEM. Embryos intended for light microscopy observations only were fixed in 3% biological-grade glutaraldehyde in Ø.1 M phosphate buffer and embedded in methacrylate plastic (JB-4, Polysciences # Ø226). The inner ear of embryos from this latter group were cut serially at 5 µm, stained with a mixture of Ø.2% Toluidine blue and Ø.1% basic Fuchsin in 25% alcohol, and mounted on glass slides as usual.

Carbonic Anhydrase Inhibitor

Acetazolamide (carbonic anhydrase inhibitor) was used in order to prevent otolith formation (Erulkar and Maren, 1961; de Vincentiis and Marmo,1968). Six to 1Ø mg/egg (average egg weight was 55 grams) of acetazolamide sodium (DIAMOX from Lederle # NDC Ø2Ø5-4466-96) was injected into the yolk sac of fertile eggs by visualizing the embryos using an egg candler. Surviving embryos without signs of gross malformation were harvested and processed as described above.

Calcium Stain

For the demonstration of calcium in selective portions of the inner ear, the tissue was fixed in potassium phosphate buffer-3% glutaraldehyde, and immersed in the same fixative in the refrigerator at least overnight. Sodium phosphate or sodium cacodylate buffer were not used because these agents tend to yield non-calcic precipitates in tissue that has been incubated in OKP. Next, the inner ear was dissected out under the microscope, and the tissue rinsed in the same buffer. The membranous labyrinth was post-fixed in 1% osmium tetroxide-2% potassium pyroantimonate (Schechter, 1976; Jansen et al., 1982), embedded in plastic, and processed as described above.

X-ray microanalysis

Semi-thin sections (9Ø-15Ønm) were collected on copper, beryllium and nylon grids, scanned with a JEOL 1ØØCX TEM, and microanalyzed with a Kevex detector in a Tracor Northern NS-88Ø. The sections chosen for this group were adjacent to those used for regular TEM examination. Elemental analysis was restricted to electron dense granules in the matrix of statoconia. Photographs (STEM mode) from the areas where the X-ray microanalysis is conducted, were obtained in order to determine the location of the calcic components within individual statoconia.

Ethylene Diamine Tetraacetic acid (EDTA)

EDTA is a disodium salt usually obtained as dihydrate crystals. In solution, it has a pH of 5.3. As a weak acid, it tends to display CO_2 from carbonates. It is used as a sequestering agent, a chelating agent and an anticoagulant (Windholz, 1976). Thin sections were incubated in 1ØmM solution of EDTA at 5Ø°C for one hour in order to extract calcic components (decalcification). Sections chelated with EDTA and/or incubated in the same vehicle in which the EDTA solution was made, were used as a control to determine the specificity of EDTA chelating properties.

Results and Discussion

Data presented here were obtained from a total of 32 embryos. Of this total, 21 were embedded in Araldite 5Ø2 and examined with the electron microscope, and 11 embryos were embedded in JB-4 plastic and examined only with light microscopy. Thirteen embryos were normal and 19 belonged to experimental groups. Data obtained from these specimens have corroborated previous observation from this laboratory, indicating that in the chick (birds) each statoconium detaches as a single organic unit from a segmenting immature organic membrane.

Between stages 26-30, a statoconial mass begins segmentation in the saccule of chick embryos and statoconia are formed (Figs. 1-4). The abundance of material available for statoconial formation in the saccule over the utricle at first could indicate that the saccule might serve as a locus and source of material for statoconia formation, both in the utricle and saccule itself. The lower portion of the otolithic membrane (in contact with supporting cell microvilli) acquires stress-like lines (Fig. 3) and statoconia progressively obtain various geometric shapes before separating from the upper portion of the otolithic membrane proper (Fig. 4). Young statoconia have open canaliculi-like channels connecting the central core with the endolymphatic space. The periphery remains relatively denser than the center after segmentation takes place (Fig. 5). Progressively the periphery of each statoconium becomes denser (Fig. 6), but the central core remains relatively empty, and

EDTA does not alter it (Fig. 7). As statoconia mature, the fibrils of the organic matrix are packed more tightly than the fibrils of the organic substance holding statoconia together (Fig. 8). Similar ultrastructural characteristics were seen in holding statoconia together (Fig. 8). Similar ultrastructural characteristics were seen in statoconial masses of embryos injected with the carbonic anhydrase inhibitor DIAMOX, but the organic matrix of these masses did not aggregate to form typical statoconial units (Fig. 9).

Histochemically, calcium was detected ultrastructurally in association with statoconia around stage 36 (10 days of incubation) when most statoconia are partially calcified. Granules ranging from 20 to 150 nm in diameter were seen in the supporting cells' cytoplasm in the endolymphatic space, in the organic matrix of the otolithic membrane and on the statoconia (Fig. 10). The granules were primarily calcium because when chelated (Fig. 11), they no longer showed calcium peak with X-ray microanalysis. Other elements (e.g. osmium) did show up in the analysis.

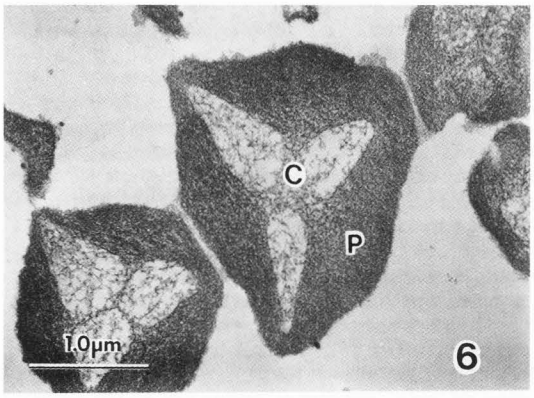
We have taken advantage of DIAMOX's ability to produce partial inhibition of statoconial formation, to examine the fate of the calcium that remains in the inner ear following interference of the metabolic pathway for the enzyme carbonic anhydrase. In the saccule of 11-day old embryos, statoconia were absent, and in their place were giant statoconial masses surrounded by a ring of organic matrix substance (Figs. 12-14, also Fig. 9). While the epithelia of the macula (Fig. 13) seemed relatively normal, the statoconia were not. Available calcium seemed to have aggregated, but did not associate itself with the organic matrix since the two parts of the masses (ring and central mass) remained completely separated from each other. Furthermore, the central mass did not react with osmium, and did not stain with either toluidine blue or paraphenylenediamine, probably because elemental calcium crystallized before associating itself with the matrix. Normally, statoconia stain very intensively with this mixture.

In the macular epithelia, cytoplasmic organelles seemed normal. Cisterns filled with material similar to the fibrils of the organic matrix were seen in the supporting cells (Fig. 15). Granules that reacted positively with OKP were detected in the apical cytoplasm and were also seen facing the endolymphatic space (Fig. 16). The OKP positive granules were associated with available organic matrix fibrils (Fig. 17) and seemed to get progressively denser as they exited the cytoplasm (Fig. 18). The presence of these OKP positive granules indicated to us that calcium was available in these embryos in the same form that it was available in normal embryos (Fig. 10), but perhaps it was not present in sufficient quantities, or some factor required for the proper utilization of free calcium was altered by DIAMOX, These materials may allow us to further evaluate the origin and fate of calcic granules since the OKP reaction facilitates visualization of the granules as they emerge and are utilized for calcifying statoconia.

Our results appear contradictory to the previous notion of statoconia formation in other species, in which statoconia have been thought to form by seeding a single unit around a nucleus (James et al., 1969). For instance, fish statoliths form by gradual growth of periodical layers around a nucleus (Pannela, 1971). In the chick, however, statoconia do not seem to form by this process. Instead, statoconia separate from an amorphous mass of fibrillar material which is probably secreted progressively by the supporting cells of the saccular and utricular macula. The secreted material remain intimately attached to the microvilli of the supporting cells (Fermin and Igarashi, 1985a). Secretion of the substance making up the otolithic membrane resembles the process by which supporting cells secrete the tectorial membrane in the basilar papilla. However, details of this process on a stage by stage basis, particularly during critical periods of development, as it has been shown for the cochlea (Cohen and Fermin, 1985; Fermin and Cohen, 1984a,b), need to be analyzed in the vestibule, because knowing when the main bulk of the membrane is secreted in normal animals will help to understand how the process of secretion is altered by teratogens injected into the embryos.

Results from Mammals Relevant to Our Results

Our results are in partial agreement with those obtained by other investigators working with mammalian species. Lyon (1955a,b) showed that in the mouse, the otoliths were formed in three parts: (1) gelatinous, (2) granular calcium, and (3) organic (fibrillar) matrix. She also showed that distribution of glycogen and many other substances within the developing ear, differed among the utricle and saccule. Of interest is the finding that phosphatase activity decreased first in the saccule as the embryo matured. She hypothesized that maybe the saccule served as the histogenetic activity center. However, there was no clear indication as to what part of the epithelia or which epithelia of the macula secreted the otolith. In a different work using Pallid mice, Lyon (1955b) showed that during development, a PAS positive material was always present in the saccule, and later in the endolymphatic sac, in spite of the fact that calcium was not being deposited in the macula of these genetically deficient mice. In addition, it has been found by us in the chick (Fermin and Igarashi, 1985a,b)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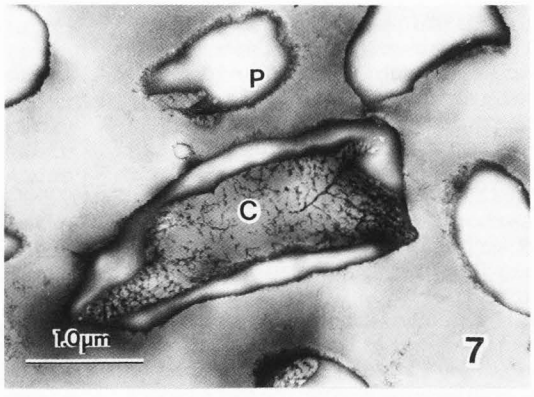
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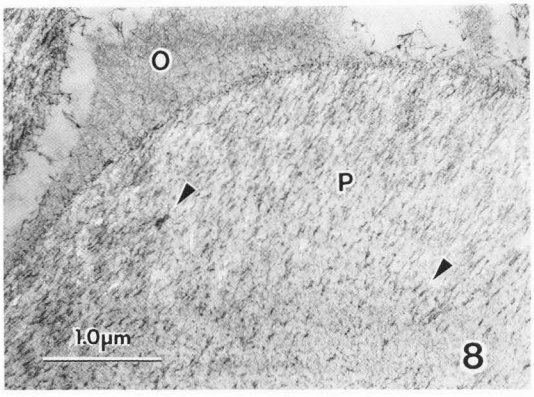
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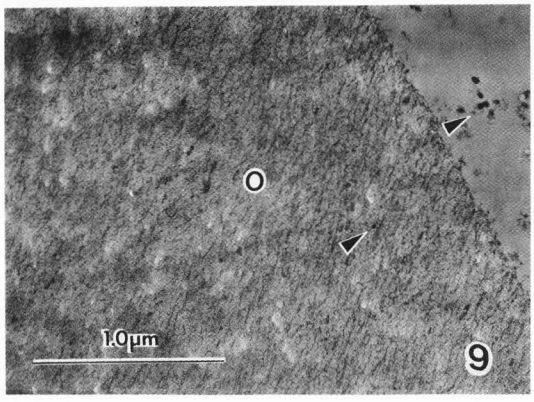
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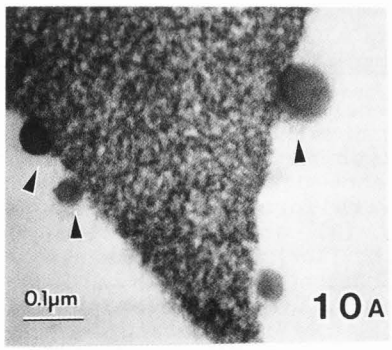
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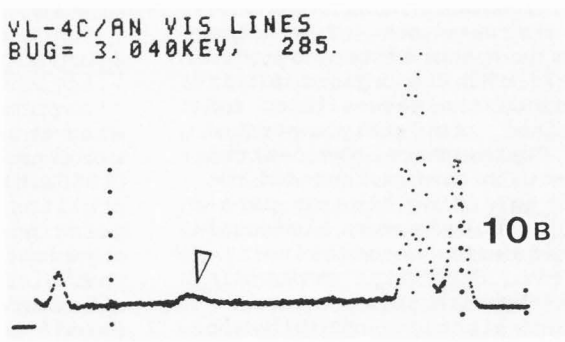
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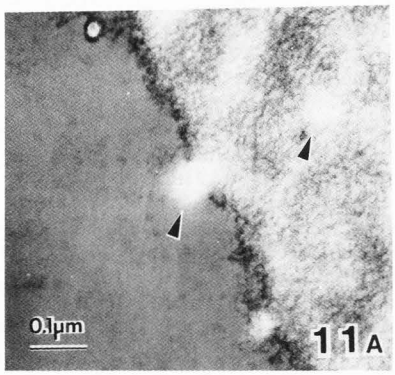
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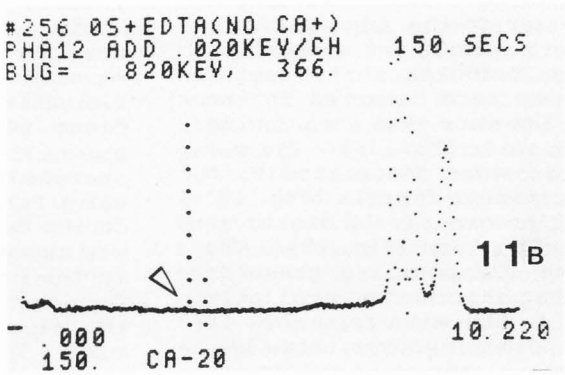
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Figure 6. Canaliculi connecting the endolymph with the central core (C) of the statoconia are later closed as the peripheral (P) area of each statoconium becomes denser. Embryo # 558, stage 36.5.

Figure 7: EDTA does not affect the central core (C) of statoconia, indicating that little or no calcium is present in the core. As statoconia mature, the central core becomes progressively smaller and calcium is extracted from the periphery (P). Embryo # 558, stage 36.5.

Figure 8. By Stage 41 (15 days of incubation), no granules were observed in the statoconia of normal embryos, and the positive antimonate reaction was more intense in the periphery of the statoconia (P) than in the organic matrix holding statoconia together (O). A positive reaction is seen (arrows) within the fibrils of the organic matrix, possibly because calcium is deposited between the fibrils of the organic matrix as part of the calcification process. Embryo # 600, stage 41.

Figure 9: In embryos injected with DIAMOX, the organic matrix (O) formed, but it did not lead to assembly of individual statoconia. Shown here is part of the outer ring of a giant statoconium, which was also seen in embryo # 699 (Figures 12-14). Electron dense granules (arrows) are seen within the fibrils and outside. Embryo # 700, stage 36.

Figure 10. Dense granules (20-150 nm in diameter) were intimately attached to the periphery (arrows) of statoconia (A). Although low, a small concentration of calcium was obtained. Part of calcium in the granules was probably extracted by the preparation methods (B, arrow). Embryo # 256, stage 37.

Figure 11. The granules were readily extracted (arrows) with EDTA (A). After chelation with EDTA, the granules showed no calcium peak (B, arrow).

as in this mutant mouse, that there was granular material deposited over the macula before the appearance of the organic matrix. Combined observations from these two studies in normal and Pallid mutant mice allowed Lyon to suggest that an organic matrix was involved in the formation of the otolith during development of the vestibule.

Belanger (1960) determined that in the rat, statoconia were heterogeneous crystaline bodies and probably grew by coalescence and fusion of small particles. He further noticed that statoconia contained calcium and phosphorus and the material was distributed in two main regions - a peripheral area and a central area that he designated as a soft core. He found no keratin present in either the cupula or the otolithic membrane. Radioactive sulfate was present in higher proportion in the cupula than in the otolithic membrane. Similarly, PAS stained the cupula more intensively than the otolith. From his observations, he hypothesized that there probably existed a neutral protein fraction around the statoconia for stimulating the precipitation of calcium, which was later incorporated into statoconia.

Carlström et al. (1953) examined statoconia in humans, guinea pigs, and rabbits and found that they were composed primarily of calcite. They determined the specific weight of human statoconia as well as a specific gravity and found it to be about 2.7 - 2.9. Later, Carlstrom and Engstrom (1955) speculated that human statoconia may be made up of small, well aligned units that allow statoconium to behave as a single crystal.

Veenhof (1969) found a strong parallelism between bone and statoconia formation. Through use of X-rays, he found conclusive evidence for two different crystalline structures in statoconia; calcite and an organic matrix which was of a low X-ray density. He thought that the organic substance may have been made up of medium molecular weight units. Some of these observations were later amplified by Lim (1973) who suspected that the organic matrix was a prerequisite for statoconia formation. This study allowed Dr. Lim also to hypothesize that dark cells in the vestibule participated in the removal of statoconia during trauma and disease.

Working with Erway, Lim (Lim and Erway, 1974; Lim et al., 1978) determined that melanization in the inner ear appeared to be improved by manganese supplementation. This study corroborated earlier observations by Erway et al. (1970) and Erway et al. (1971) in which these investigators showed that zinc and manganese were essential for the proper development of the otolith (Purichia and Erway, 1972).

Harada (1978) showed morphological evidence that the supporting cells of the macula produce calcium because, with X-ray microanalysis, granules observed inside the supporting cells produce a high, strong peak of calcium activity. In subsequent work, Harada (1982, 1983), Tagashira and Harada (1982), and Takumida and Harada (1984) repeatedly found calcium in the supporting cells of the utricular macula of guinea pigs. Anniko (1980) showed that elemental calcium increased in the endolymph during calcification.

A new concept of statoconia formation was put forward by Nakahara and Bevelander (1979) who showed that statoconia already existed as small immature statoconia in very early stages of formation. They called these bodies "pre-otolith" but did not show

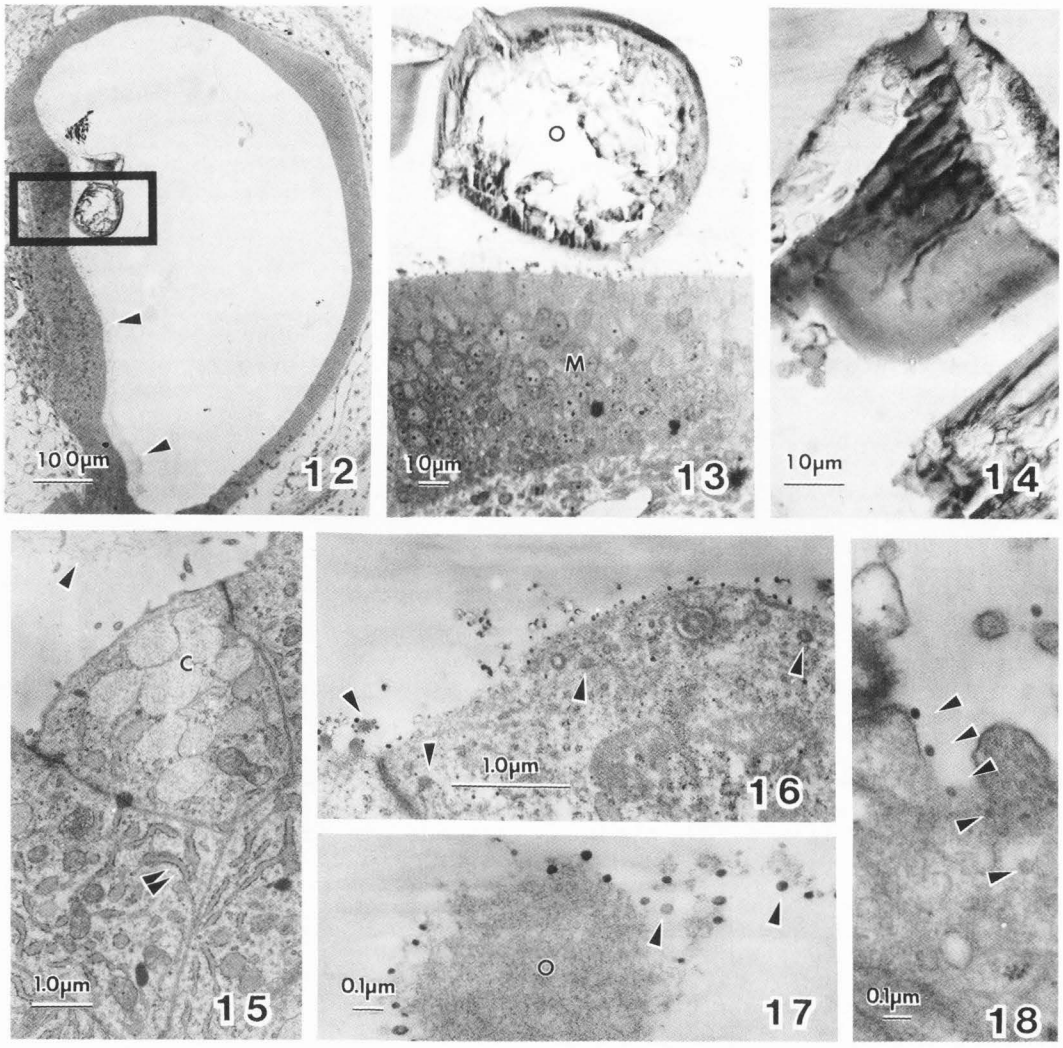

Figure 12. Saccule from a 10 day-old embryo injected on the 3rd day of incubation with the carbonic anhydrase inhibitor DIAMOX. Normal development of statoconia has been modified, and the organic matrix is scarce (arrows). Available calcium and the organic matrix produced may have fused and formed two giant statoconia in this ear. No statoconia were seen in the utricle of this embryo. LM.

Figure 13. Higher magnification of area outlined in Figure 12. The giant statoconia (O) over the macula (M) have numerous small glasslike bodies enclosed by a ring of amorphous material. LM.

Figure 14. The giant statoconium formed in this ear lacks the organization and geometric shape that characterizes individual statoconia in normal ears. LM.

Figure 15. Cisterna (C) filled with material similar to the organic matrix (arrows) is seen in cells adjacent to the saccular and utricular maculae. Dense, non-fibrillar material is also seen in the rER cisterna (double arrow). TEM.

Figure 16. OKP positive granules are seen in the endolymphatic space associated with dense granules (arrows) which resembles material illustrated in Figure 15. TEM.

Figure 17. Fibrillar material of the organic matrix (O) is closely associated with OKP positive granules (arrows). TEM.

Figure 18. An increased density of granules was seen in the apical end of some cells (arrows). The granules could react with substances in the endolymph and become denser. TEM.

where these bodies came from. At 15.5 days of gestation, the mouse otolith showed aggregation of the organic matrix, and at one day after birth, the matrix formed a tube-like structure in which needle-shaped crystallites were initiated. These changes were observed one day later in the utricle (Nakahara and Bevelander, 1980; Nakahara et al., 1981).

Ross and her group (Salamat et al., 1980; Peacor et al., 1980) have utilized rats' adult and developing statoconia and have shown that statoconial proteins consist of a mixture of amino acids generally comparable to those found in biomineralized materials that contain polymorph calcium carbonate. From the excellent work coming out of Dr. Ross' laboratory, one of the most fascinating concepts, is the idea that statoconia may have piezoelectric properties, i.e., that they may have the property or capacity of being electrically charged.

Scanning electron microscopy of human infants (Wright et al., 1979a,b; Wright and Hubbard, 1978, 1981, 1982) has shown that the macula sacculi and the macula utriculi continue to grow after birth. This group also showed that abnormal infants with no statoconia in the utricle and saccule usually did have a gelatinous layer that seemed normal. Although not discussed by the investigators, this finding implies to us that the production of a gelatinous layer (organic matrix) and the formation and calcification of statoconia are probably two separate processes, providing more evidence on the originally proposed hypothesis of two steps during statoconia formation. In other words, in the first step, an organic matrix is secreted, and in the second step, the statoconia are assembled and are progressively calcified.

Consequently, our results fill in the gap left behind by previous work in birds which did not show the ultrastructural appearance of primitive statoconia before they acquire their characteristic geometric shape. We show that, at least in the chick, each statoconium forms from the organic matrix and not by seeding of a single unit around a nucleus. Until similar mechanisms of statoconia formation are also shown for mammals, i.e., during early stages of statoconia genesis, one cannot say for certain that statoconia formation in both species is patterned after identical mechanisms. Nonetheless, results obtained to date in both species indicate that statoconia formation may share some common mechanisms in different species.

In the enclosed micrographs we illustrate some of the results obtained to date. Explanations are offered in the legends. These results allow us to conclude that: a) The otolithic membrane is probably secreted by the supporting cells of the saccular and utricular macula (stage 23-26). The fibrils of the membrane are attached to the microvilli of the supporting cells. b) The immature organic matrix segments, and gives origin to immature statoconia (stage 26-28), which continue to accumulate on top of an existing fibrillar membrane. c) Individual statoconia become progressively denser from the periphery to the center (stage 29-34). d) Calcic granules (20-150 nm in diameter) attach to young statoconia and are later incorporated into the organic matrix (stage 30-38). e) The central cores of statoconia remain less electron-dense than the periphery. More calcic material is chelated from the periphery than from the central core. f) Calcic components become sandwiched into the fibrils of the organic matrix arranged similarly to what has been shown in rat statoconia (Salamat et al., 1980). We are continuing these studies in order to establish developmental gradients of the maculae, and to further corroborate these observations.

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Discussion with Reviewers

J. Ballarino: Newly-formed otoconia are extremely sensitive to decalcification, particularly during the early stages of development. Fixation with glutaraldehyde has been shown to result in the decalcification of newly-formed otoconia in fetal rats (Salamat et al., 1980, Ann. Otol. Rhinol. Laryngol. 84: 22-36) and embryo chicks (Ballarino et al., 1985, Amer. J. Anat. 174: 131-144). Since fully calcified otoconia have been reported (Ballarino et al., 1985) for the same stages in chick embryos in which you are seeing "segmentation membranes", one is forced to conclude that these "segmentation membranes" are actually decalcified otoconia. Is there any evidence for the existence of segmentation membranes in material which has been prepared using cryomethods or other techniques which do not expose the otoconia to fixatives?

In answering this question, the authors are requested to keep in mind the following:

1. The techniques of using the tips of forceps for monitoring calcification is not a reliable method for measuring the calcification of otoconia which are 2-10 μm thick (chick embryo stages 27-31). I suggest the use of polarized light microscopy to observe freshly dissected otoconial membranes which are spread under mineral oil. This technique takes five

minutes to prepare and has the added benefit of localizing calcite deposition in unfixed specimens. With a powerful light source, much information can be obtained at 1000X magnification.

2. The fact that segmentation membranes have been "identified now in 4 different embryos", does not dismiss the fact that this material is decalcified. Until the authors document the presence of their segmentation membrane in frozen, unfixed specimens under TEM, their results will be called into question.

3. The reason the authors do not see a segmenting membrane in their older embryos is that otoconia from older embryos are less sensitive to decalcification (Ballarino et al., 1985). When freshly dissected embryonic otoconia are viewed under polarized light microscopy, then subsequently exposed to glutaraldehyde, and reexamined under polarized light microscopy, significant decalcification can be observed in stage 27-32 chick embryos, but not in older embryos. Apparently, the otoconia of older embryos are more resistant to decalcification.

4. The article by Ballarino and Howland (1980), to which the authors refer, also has decalcification artifacts. This paper was unfortunately published before we realized the error in our methods. I regret that we did not discover this mistake before it was published and that our reviewers did not catch the mistake. This paper can not be used as evidence for segmentation membranes. The authors are requested to refer instead to the accompanying paper by Ballarino et al. (1985) for a more reliable documentation of otoconial development.

Authors: The work you refer to (Ballarino, et al. 1985) does indeed show calcified otoconia, but it does not show quantitatively that all otoconia at those stages (29-32) are calcified. The hypothesis about segmenting membranes only requires that not all otoconia are calcified at once, but gradually. Your own paper points out that "chick otoconia pass through the growth sequence at different rates". Furthermore, your comment would imply to me that the cross linkage of molecules in the organic matrix following glutaraldehyde fixation is a weak one, and that somehow, the otoconia fuse again after they have separated. While this is possible, prior work with rat otoconia (Salamat et al., 1980), found no difference between the matrix of calcified and decalcified otoconia. This would argue against your notion of fusion after decalcification. This issue requires further studies, now underway in this laboratory. As to the invalidation of the data presented, due to absence of using cryomethods, we showed secondary evidence to support the data (see Dr. Roomans' comments). We will pursue these issues further in other studies to corroborate these observations. In response to the use of forceps, we did not base the conclusion presented on this paper or that observation, instead, we pointed out that if "all" otoconia are calcified at stage 29, as you advocate, we would have encountered in 6-day-old embryo otoconia the same resistance we saw after final calcification (newly hatched). In other words, at 6 days, a great portion of the otoconia mass was very soft, elastic and sticky rather than hard, rigid and crystalized. Furthermore, count per minute (CPM) was greater at 11 days (X=1267) than at 8 days (X=776), significant at p=0.05 and with Nann Whitney rank order test. Thus, in view of the fact that your paper does not show quantification, demonstrating that all otoconia are calcified at 6 days, some of them could very well exist normally as we have illustrated.

G. M. Roomans: (1) Loss and redistribution of Ca during fixation and other preparation steps (sectioning on water) has been demonstrated repeatedly (reviewed by Morgan 1979). During fixation, Ca can be removed from its physiological location and precipitate elsewhere (Barnard 1981). The only safe methods are the cryomethods which, however, are notoriously difficult to apply to this particular tissue, even though successful attempts have been made (Meyer zum Gottesberge-Orsulakova and Kaufmann 1985).

(2) Although the pyroantimonate method may have been accepted ten years ago, it is at the moment not considered to be reliable. That it is still often used reflects, rather, a reluctance to use the much more demanding cryomethods. Apart from the calcium redistribution during the concurrent aqueous fixation, the method has other problems: it doesn't detect Ca concentrations below about 10 mM and it doesn't detect firmly bound calcium: the Ca has to be solubilized to react with the capturing agent. According to Morgan (1979) it is even questionable whether pyroantimonate is particularly effective in retaining calcium.

(3) With regard to fig 10B, it is not certain that the peak actually represents Ca; at best it is a convoluted Ca-Sb peak. Antimony's L lines overlap the Ca lines and without careful deconvolution the presence of Ca may not be taken for granted. I agree, however, that the absence of the signal after EDTA is an argument in favor of Ca.

(4) The risk for artifacts evidently is largest when the otoconia are not fully mineralized. The techniques used in this paper probably miss the very onset of calcification. Some redistribution, in

particular at the subcellular level, may occur.

Authors: Your comments are well taken and will be considered in future studies of this sort. It is interesting though that the pyroantimonate reaction was not diffused through the scala media, an acellular fluid environment in which calcium could diffuse freely if unbounded. Curiously enough, the precipitate was located only on or near the otoliths (Fermin and Igarashi, 1985). In relation to the possible artifactual origin of the granules, the close association of these granules favors our hypothesis or implies that the cross linkage glutaraldehyde creates with the organic matrix is so weak that, while still in glutaraldehyde, the organic matrix opens up to let these granules in and then reassociates again. While this is a attractive idea, the data obtained thus far does not allow this conclusion.

J. Saunders: What is the importance of the statoconia being electrically charged?

Authors: The issue of statoconia electricity has been touched upon by Dr. Ross in her elegant work. If indeed statoconia are piezo electric, then the notion of displacement by weight should be modified. This point, however, is not stated in her paper and we have no intention to do so. Dr. Saunders is referred to that work for further information.

J. Saunders: The authors note that the precipitation of $CaCO_3$ is a necessary process in otolith calcification. Moreover, these processes require "high amounts of ATP for energy". Where does this energy (ATP) come from in the extracellular environment of otolith formation?

Authors: ATP utilization by living cells is well established. Involvement of ATP in statoconia formation was brought up by the investigators cited therein. ATP utilization in the process leading to otolith calcification cannot be ruled out. In the case of the organic matrix and the otolithic membrane in general, the precursor substances are probably produced inside the cell but are assembled outside. ATP, in any living system, is produced intracellularly (see any freshman biology textbook) and comes from, among other sources, glycolysis.

J. Saunders: If the saccule serves as the "locus and source" of material for statoconia formation in both the saccule and utricle, where do statoconia form from in the lagena?

Authors: We have no answer for this question. It is only to be assumed that otoconia originate in the lagena macula in the same way that they do in the utricle and the saccule. Where they come from is a different issue which requires further investigation. Again, the precursor substances are probably produced locally in the macula themselves. We do not advocate that statoconia in the utricle come from the saccule, but we believe that the saccular statoconia (especially large ones) could serve as a possible source of stored material for the utricular statoconia, saccular statoconia, and probably lagenar statoconia. This needs to be investigated, and our research will try to accomplish this. We have recently prepared several 6 day-old embryos (stage 29) injected with ^{45}Ca and found that there was almost no label in the utricle when compared to the almost 1Ø fold label seen in the saccule (Fermin, unpublished observation).

Y. Harada: You said statoconia are produced by segmentation of statoconial mass in the saccule. How is the formation process of statoconia in the utricle?

Authors: The process of segmentation in the utricle is identical to that in the saccule. The process of statoconia formation follows the sequence that has been reported for development of the inner ear in the chick embryo by earlier investigators.

Y. Harada: How does DIAMOX affect the formation of statoconia? Does DIAMOX affect the segmentation process of statoconial mass?

Authors: DIAMOX probably affects some limiting factor (most likely a calcium-binding protein). Results obtained thus far indicate that this might be the case, but other studies now in progress will give a better answer to this question.

Y. Harada: In our experiment, giant statoconia are also found in the mouse injected with DIAMOX. We have speculated that once formed typical statoconia are changed to giant statoconia by DIAMOX. Do you have any comments on our speculation?

Authors: Dr. Harada's speculation is a possible one, but other, more attractive to us, is that DIAMOX interferes with limiting factor which probably belongs to the same class as carbonic anhydrase. A possible candidate will be a calcium-binding polypeptide of relatively low molecular weight. Biochemical assays of primitive otolithic membrane will probably answer this question. This is because one can selectively dissect the membrane prior to when most of the statoconia are calcified. Consequently,

one is dealing with organic matrix primarily. We have, so far, 3 gels from three different groups of chicks (n=10 each group) and found that there are at least 5 major proteins in the membrane.

Y. Harada: Where do you think the calcic granules are produced?

Authors: Similar to Dr. Harada, we think the granules are produced primarily, but not exclusively, by the supporting cells of the maculae.

H. Nakahara: The authors stated that carbonic anhydrase inhibitor produced the partial inhibition of statoconial formation, how can you rule out the involvement of the general weakening of metabolic activity due to the inhibitor's effect on other carbonic anhydrase-containing cells such as erythrocyte?

Authors: Although not stated in the review, the involvement of a general weakening of the metabolic activity of the body due to the effect of DIAMOX in other cells containing carbonic anhydrase enzymes has not been overlooked. However, because carbonic anhydrase has been shown to be preferentially accumulated in the ear fluids and tissues (Drescher, 1977), it is generally accepted that DIAMOX does indeed have a preferential affinity for inner ear fluids and tissues. Moreover, those embryos showing lack of statoconia and / or deformation were anatomically normal, and display normal behavior. One cannot disregard the systemic effect of DIAMOX and secondary effects related to the results discussed. If, however, the systemic effect of DIAMOX was secondary rather than primary, one will also see the same phenomena in adult-injected specimens. The fact that both components (organic matrix and calcium) are present but not associated with each other might indicate that a limiting factor is the culprit.

Additional References

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