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(March, 1993)

STUDIES ON THE ANATOMY AND MICROSTRUCTURE OF THE INNER
EAR OF TILAPIA (*OREOCHROMIS NILOTICUS*), WITH SPECIAL
REFERENCE TO THE OTOLITH GROWTH
IN THE OTOLITHIC ORGAN*1*2

Setsuo SAITOH*3

Anatomical and fine structural investigations of the inner ear of *Oreochromis niloticus* were conducted to establish the morphological basis of otolith (sagitta) growth with reference to the daily increment formation. The saccular (otolith-holding) wall was composed of a single layer of cells classified into three different types of epithelia, the sensory epithelium, the transitional epithelium and the squamous epithelium. A patch of mitochondria-rich cells was located in the squamous epithelium. The otolithic membrane and the otolith were located over the sensory epithelium of the macula. The otolithic membrane consisted of two distinct layers, the upper gelatinous layer and the lower subcupular meshwork. The subcupular meshwork extended over the entire undersurface of the otolith, whereas the gelatinous layer was limited to the macula region which corresponded to the center area of the otolith. The cytoplasmic extrusions and microvilli-originated vesicles were observed on the sensory and transitional epithelial cells, respectively. The sensory epithelium of the crista ampullaris (non-otolith-holding) was morphologically the same as that of the saccular macula, and similar secretory extrusions were existed on the cell surface, but there were no transitional epithelium cells bearing microvilli. The quantitative distribution and calcium content of the secretory spherules on the transitional epithelium showed a diurnal change. Materials in the microvilli-originated vesicles, or spherules on the transitional epithelium, were suggested to play the most important role in the otolith growth.

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*1 ティラピアの内耳、特に耳石器官と耳石の成長に関する解剖学的、微細構造学的研究

*2 The present work was submitted as a partial fulfilment of the requirements for Doctor's degree in Fisheries Science at Hokkaido University in 1992
(本論文は、平成4年1月北海道大学へ提出した学位審査論文である。)

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1. INTRODUCTION

In the analysis of fish resources, the age determination of individual fish is very important and the otolith is one of the most excellent age characters. Calcified tissues such as scales and bones have been used for age determination of fishes since more than 200 years ago (Dean et al., 1983). Later, some studies have demonstrated the presence of annuli in otoliths of temperate fishes as are induced by the results of slow and fast growth periods. The reliability of these annuli of otoliths for age determination has been validated through work with fishes of known histories and of tagging and recapturing experiments.

Since the finding of the presence of daily growth increments in fish otoliths (Pannella, 1971), many investigations have been carried out to clarify the microstructure and growth layers of the otolith (Pannella, 1974; Brothers et al., 1976; Taubert and Coble, 1977) and to utilize the increments as an indicator of daily age of fish (See Beamish & McFarlane, 1987). Also the otolith microstructure has been studied to substantiate age determination on a daily basis (Morales-Nin, 1987). It is now considered that one can estimate the age of a fish using the otolith microstructure even if it lacks annuli as in the case of some tropical species or the fish is in very young growth stages.

The validity of daily increment formation in the fish otoliths should provide fishery scientists with a very powerful tool. If the temporal history of a fish is recorded in the otolith as a permanent physiological calendar, it would make possible to analyze seasonal and short-term events in the daily increment record. Thus the use of otoliths for estimating ages, growth patterns and life histories, particularly in previously unaged fishes, would promote population studies for resource managements. Tanaka et al. (1981) and Mugiya et al. (1981) suggested that the daily nature of increments are based on the formation of calcium-rich and organic matrix-rich layers in accordance with the photoperiod as an external time cue for the internal biological clock. However, the increments are not always formed daily under a slow-growing condition (Geffen, 1982). On the other hand, it sometimes makes difficult to assess the validity of daily ages by appearance of subdaily rings, false rings, and very narrow indistinct rings (Campana and Neilson, 1985).

A growth increment is a bipartite structure, usually formed over 24 h, consisting of accretion and discontinuous zones (Watabe et al., 1982). When viewed with a light microscope, the accretion zone appears as a broad, translucent band, while discontinuous zone is of relatively narrow and opaque. When an otolith plane having a series of daily growth increments is etched with a weak acid and viewed with a scanning electron microscope (SEM), accretion zones appear as relatively wide, light-etched bands while discontinuous zones are of narrow and deeply-etched. There have been made very few investigations about the mechanism of formation of these bands, especially from cellular aspects of otolith daily growth, and many problems remain to be solved for more accurate use of these structures as an information source of the life history of fish.

The inner ear in fishes lacks bony labyrinth and is located in each side of the posterior part of the cranial cavity. The typical membranous labyrinth of the inner ear in cartilaginous and bony fishes consists of three semicircular canals and three otolith organs. All are filled with a lymphatic fluid called the endolymph, or otolith fluid in otolithic organs. The semicircular canals and the utricle, one of the otolith organs, are responsible for the control of equilibrium and locomotion, while the other two otolithic organs, the saccule and lagena, function as receptors for both audition and gravitation (Lowenstein, 1971). The otolith organs are membranous sacs each containing a single, dense calcareous otolith or many calcareous otoconia on the sensory epithelium, the macula, which extends on the medial wall of each sac. The otolith or otoconia are separated from the sensory epithelium by the otolithic membrane which is a structure composed of glycosaminoglycans (Mugiya, 1968) and helps keep the otolith in position relative to the sensory epithelium.

In bony fishes, there are three kinds of solid calcareous otoliths; lapillus in the utricle, sagitta in the saccule and asteriscus in the lagena. These three kinds of otoliths differ from one another in size and shape. The sagitta is usually the largest and is most often used for age determination. In ostariophysid fishes, the asteriscus is the largest in the calcareous otolith. Each otolith has a species-specific shape that is used for analyzing systematic relationships in taxonomy of both extant and fossil fishes. These otoliths are composed of calcium carbonate in the form of aragonite in teleosts (Morales-Nin, 1987).

The otoliths of teleosts contain an organic matrix, composed of glycosaminoglycans, lipids (Mugiya, 1968) and proteins known as otolin (Degens et al., 1969). The proteins consist of a large proportion of acidic amino acids such as aspartic acid and glutamic acid. This matrix is seen in cross-sections of the otolith as fibrous materials in both alternating thin organic (discontinuous zone) and inorganic (accretion zone) layers surrounding the central core (Watabe et al., 1982). The core is a crystallized central region which consists of several calcareous spherules called the primordia, and is surrounded by a thick calcified layer. The organic substance is found also in calcified incremental layers as fine fibers between single crystals (inter-crystalline) and even within calcium crystals (intra-crystalline) (Dunkelberger et al., 1980). Vice versa, the organic layers have some calcium crystals. A similar intracrystalline matrix is found in molluscan shells (Mutvei, 1970) and spicules of coelenterates (Dunkelberger and Watabe, 1974). However, the sources of these organic materials and calcium and the way of incorporation into the otolith have not been positively elucidated.

The otolith is surrounded by otolith fluid (endolymph). The electrolyte composition of saccular endolymph is characterized by a high potassium and a low sodium concentration, and therefore the endolymph has a nature of intracellular fluid (Ormerod, 1960). Moreover, a considerably high bicarbonate ion concentration as well as a high value of pH, and a low calcium (diffusible) concentration compared with those of serum (Enger, 1964; Mugiya and Takahashi, 1985) are to be noted. According to Shichiri (1985),

ionic components of saccular endolymph are highly supersaturated to the calcium carbonate of otolith. In the sacculus, the medial wall including the macula region was postulated to be the site of calcium secretion (Mugiya, 1974).

Mugiya (1968) suggested that the organic matrix of otolith is produced at the macula region and secreted into the otolith fluid (endolymph). Dunkelberger et al.

(1980) postulated that the subcupular meshwork, the lower layer of the otolithic membrane which covers both the macular sensory and the primacular non-sensory regions, is incorporated into the otolith as an organic matrix constituent.

The inner ear of higher vertebrates than teleosts has been studied mostly from the medical point of view concerning the constituents of endolymph and the structural characteristics. Most studies have been carried out with reference to the sensory region, and a few investigations have treated the otolith (otoconia) formation. Salamat et al. (1980) suggested that some cells in the macula region secrete precursors of otoconial constituents into aqueous endolymph and then the precursors are calcified into a crystalline structure. Harada (1983) studied the otoconial membrane (a structure which corresponds to the gelatinous layer) and the sensory epithelium of the guinea pig inner ear, and proposed that calcium to be incorporated into otoconia is secreted from supporting cells of the sensory epithelium.

Dale (1976) observed the otolithic membrane and the otolith surface in cod, and found that some uniform spherules were embedded into the otolith. He concluded that the spherules were the 'building units' of the otolith. Sokolowskii (1986) and Yamada and Shimizu (1988) observed fibrillar strands extending from the macular cells to primordial spherules of the otolith in embryonic toadfish and medaka, respectively. However, there are no descriptions on the ultrastructural characteristics of the membranous labyrinth in teleosts in relation to the otolith growth.

This study was performed to contribute to the knowledge of the comparative anatomy of the inner ear and to promote understanding of the mechanism of otolith growth in fish. Using tilapia as materials, the research aimed at: 1) Characterizing ultrastructure of the otolithic membrane, otolith and other structures of the membranous labyrinth, 2) Identifying cells which are responsible for secretion of otolith materials and 3) Examining the presence of a diurnal rhythm of the secretion. In addition, a comparative study on the distribution of mitochondria-rich cells in the labyrinths of tilapia (non-ostariophysid fish) and goldfish (ostariophysid fish) was conducted to define the role of mitochondria-rich cells.

II. MATERIALS AND METHODS

Hundreds of juvenile and adult tilapia, *Oreochromis niloticus*, which were about 2 cm and 10 cm in total length, respectively, were used as materials. The fish were kept in 60 liter aerated freshwater aquaria at temperature maintained at $27 \pm 1^\circ\text{C}$. They were

fed *ad libitum* with a sufficient supply of commercial pellets for carp.

Juvenile fish were used for light microscope (LM) and transmission electron microscope (TEM) observations of the membranous labyrinth. Adult fish were used for gross anatomy of the inner ear, and for studies by scanning electron microscopy (SEM) and electron-probe X-ray microanalysis (EPMA) of secretory structures of the otolithic organs.

For a comparative study with tilapia, juveniles of goldfish, *Carassius auratus*, approximately 2 cm in total length were used as materials for examination of the distribution of mitochondria-rich cells in the membranous labyrinth by LM and TEM. Rearing conditions were almost the same as that of tilapia.

For LM and TEM studies, animals were decapitated, and the heads were immersed in a primary fixative of Karnovsky (1965). The primary fixative was a mixture of 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1M cacodylate buffer (pH 7.4). After 30min the heads were cut along the median line (tilapia) or trimmed (goldfish), and then fixed with the same fixative for another 5 h at room temperature. The head pieces were rinsed overnight in a cold buffer solution, and then decalcified. After rinsed in the same buffer solution, they were postfixed in a secondary fixative which was 1 % OsO₄ in the same buffer solution, and then dehydrated in ethanol. The specimens were embedded in epoxy resin by the routine procedure of Luft (1961). After sectioned with an ultramicrotome Porter-Blum MT-1, ultrathin or thick sections were subjected to an electron staining with saturated aqueous uranyl acetate (Sjöstrand, 1967) for 15min at room temperature for electron microscopy or stained with toluidine blue for light microscopy. All examinations of ultrathin sections were performed with a Hitachi H-300 transmission electron microscope operated at 75kV.

For studies of gross anatomy, SEM and EPMA, the heads of adult fish were cut sagittally and fixed with the primary fixative. Some specimens were fixed with the same fixative containing 4 % cetylpyridinium chloride (CPC) added to stabilize glycosaminoglycans (Watabe et al., 1982). The head pieces were washed with cold buffer solution and dehydrated through a graded series of ethanol. For gross anatomy, surrounding bones and cartilages were removed carefully in 70% ethanol until the entire labyrinth was loosened from its bony cradle. For SEM and EPMA observations, the membranous saccular and ampullar capsules were opened with a razor blade and the otolith was extricated gently from the sensory macula. After dehydration, the capsules were dried with the critical-point drying method, the insides coated with carbon or gold under vacuum and examined with a JEOL JSM 25 SEM and EPMA.

Materials and procedures specific in each observations or experiments will be described in each chapter.

Statistical analysis : Differences between pairs of means of morphometric and EPMA measurements were tested by the Student's t-test. Significance was accepted at a $p\text{-value} < 0.05$.

III. MORPHOLOGY OF MEMBRANOUS LABYRINTH

The morphology of the inner ear of teleosts has been studied by many investigators (Flöck, 1964 ; Hama, 1969 ; Lewis and Nemanic, 1972 ; Saito, 1973 ; Dale, 1976 ; Popper, 1976, 1977 ; Jenkins, 1977, 1979 ; Coombs and Popper, 1982 ; Platt, 1983 ; Popper and Platt, 1983 ; Saidel and Popper, 1983). However, most of these previous work have been carried out from a neuro-physiological points of view. Cellular contributions to the formation of otoliths or otoconia in vertebrates have not been studied extensively.

Enger (1964) reported that endolymph in the teleost inner ear showed high potassium and low sodium concentrations compared with the perilymph in the cranial cavity. However, no morphological basis on the mechanism of ionic regulation of endolymph has been presented.

A morphological study was conducted to characterize the ultrastructure of the tilapia labyrinth, with emphasis on a comparative aspect of the sacculus which contains otolith to the ampulla which is a non-otolith holding sac. Also, the localization and ultrastructure of mitochondria-rich cells in the teleostean labyrinth were examined in both goldfish (ostariophysid fish) and tilapia (non-ostariophysid fish).

1. Materials and Methods

The head of adult tilapia was cut into two halves along the median line and immersed immediately in the primary fixative at least 2 weeks. The head piece containing the inner ear was then washed overnight with the same cold buffer solution, and dehydrated in a series of graded ethanol. In 70% aqueous ethanol, the head was further dissected until the whole labyrinth was loosened from its bony cradle. The specimens were observed unstained dipping in 70% ethanol under a binocular dissecting microscope.

For light microscope (LM) observations, juvenile tilapia (*Oreochromis niloticus*) and goldfish (*Carassius auratus*), both approximately 2 cm in total length, were used as materials. The juvenile fish were decapitated and the heads were immersed immediately in the primary fixative. After 30min they were cut along the median line (tilapia) or trimmed (goldfish), and then fixed for further five hours at room temperature with the same fixative. After rinsed overnight in the same bufer solution, the head piece containing the ear was decalcified in 10% EDTA, pH 7.4, for 3 days at 4 °C. After rinsed overnight in the cold buffer solution, the head piece was postfixed with the cold secondary fixative for 1 h. Plastic sections of 1 μ m thick were stained with 1 % toluidine blue in 1 % aqueous borax on a hot plate (Mercer and Birbeck, 1972).

Fish used for transmission electron microscope (TEM) observations were the same as described for light microscope observations. Their heads were processed in the same way described above up to embedding. Ultrathin sections were subjected to an electron staining and examined by TEM.

For scanning electron microscopy (SEM), adult *Oreochromis niloticus*, approxi-

mately 10cm in total length, were used. The adult fish were decapitated after bleeding by caudal section to avoid blood contamination. The cranium was opened dorsally, and the brain was removed carefully to expose the inner ears. Then the head was immediately immersed in the primary fixative, and trimmed down to two small blocks each containing the labyrinth. After this procedure, a block was placed in a vial containing the primary fixative and kept for at least 48 h. Some specimens were fixed with the same fixative but with 4 % cetylpyridinium chloride (CPC). Then the block was washed overnight with a cold buffer solution. It was then postfixed with the secondary fixative for 1 h and dehydrated through a series of graded ethanol. The specimens were further dissected in 70% aqueous ethanol to an appropriate size for observations by SEM. The ears were then dissected from the tissue block. The membranous otolithic capsules were opened and the otoliths were gently pulled away from the sensory maculae. One of the membranous labyrinth ampullae was opened and the crista ampullaris was exposed in the same way. After dehydration the capsules and otoliths were dried by the CO₂-critical point method using amyl acetate as an intermediary fluid, placed on an aluminium stub, coated with gold in vacuum, and observed by SEM.

2. Observations

1) Gross anatomy

The gross structure of the membranous labyrinth of *O. niloticus* is shown in Fig. 1 a. The membranous labyrinth lacking the bony labyrinth is suspended directly in the cranial cavity. It is composed of two major membranous compartments; the sacculus to which the lagena is attached at the posterior end, and the utriculus from which the three semicircular canals are extended. The ampullae are bulbous swellings of the anterior parts of the anterior and lateral semicircular canals and of the posterior part of the posterior semicircular canal. The two vertical canals, the anterior and the posterior, are connected at the apex of the sinus superior which opens at the middle of the utriculus.

The maculae, namely the areas of the sensory epithelium of the utriculus, sacculus and lagena are covered, respectively, by a compact calcareous stone, the otolith. The utricular macula is horizontally elongated whereas the saccular macula and the lagenar macula are vertically oriented.

The utriculus, sacculus, and lagena each contains a compact calcareous otolith, the lapillus, sagitta, and asteriscus, respectively. The size of the otolith is related to the volume of each endolymphatic sac (Fig. 1 a). The sagitta is the largest and occupies almost the entire space of the sacculus, while the asteriscus is the smallest. The terminals of the three semicircular canals are bulged to form the ampullae in each of which is also the sensory area without loading an otolith on it.

2) Sacculus

Histological characteristics of the sacculus: A transverse section of the middle part

of the sacculus of *O. niloticus* is shown in Fig. 1 b. The sensory epithelium of the macula region is the thickest in the saccular wall. The connective tissue layer lining the saccular epithelium is highly vascularized at the macula region to form a capillary bed. From the connective tissue, nerve fibers innervate the sensory cells of the macula through the basement membrane.

The periphery of the sensory epithelium is transformed into a thinner transitional epithelium, which in turn gradually becomes thinner to form the extremely thin squamous epithelium at the lateral and opposite sides of the macula. The squamous epithelium is composed of a single layer of thin epithelial cells and a patch of mitochondria-rich cells. The loose connective tissue beneath the squamous epithelium is vascularized with a few capillaries near the epithelial lining.

The large space of the saccular cavity is filled with a clear fluid, or endolymph. The otolith lies over the sensory and transitional epithelium region interrupted by the otolithic membrane.

Sensory epithelium : The sensory epithelium is composed of a single layer of hair cells and supporting cells. In the survey view (Fig. 2 a), the hair cells were observed to extend half-way down the epithelium interposed alternately by supporting cells. Tight junctions are connected these cells at the apical surface. The hair cells are embraced by supporting cells which generally extended from the surface of the epithelium to the basement membrane. The apical end of the hair cell is provided with a bundle of sensory hairs. The ovoid nucleus is located in the middle of the cell and surrounded by a more or less dense population of mitochondria. Mitochondria are also found congregated at the apex of the cell immediately beneath the free surface, which bears microvilli or hairs. The supporting cells are more electron dense than the hair cells. The nuclei of the supporting cells are found at various levels below the middle of the epithelium. This caused the sensory epithelium to give an appearance of a pseudostratified epithelium. The nuclei of the supporting cells also stained more heavily than those of the hair cells. Toward the basement membrane wide intercellular spaces are often formed between the supporting cells.

Figure 3 a illustrates the positional relationship between the sensory epithelium, the otolithic membrane and the otolith (sagitta). Overlying the sensory epithelium is the otolithic membrane which is in contact with the inner surface of the otolith (sagitta). The otolithic membrane is composed of two distinct layers ; the upper layer or the gelatinous layer is a mass of a gelatinous substance, which forms the cupula for sensory hairs of the macula, and the lower layer or the subcupular meshwork is a meshwork of granular or finely fibrillar substances occupying the subcupular zone. The gelatinous layer is in close contact with the otolith. The electron density of the gelatinous layer is lower than that of the subcupular meshwork. Many vesicular structures are found in the subcupular zone beneath the gelatinous layer. Abundant cytoplasmic extrusions are bulging from the apical surface of the sensory epithelium into the saccular lumen. Vesicular dilations, about $0.5\mu\text{m}$ in width, are found at the distal end

of microvilli of the supporting cells (Fig. 3 b).

The cytoplasmic extrusions showed the same electron density as the subjacent apical cytoplasm (Figs. 3 b, 4 a). They often contained some vesicles of $0.3\text{--}0.8\text{ }\mu\text{m}$ in diameter (Fig. 4 b) and numerous ribosomes (Figs. 3 b, 4 a). These apocrine-type extrusions often occurred close to the apical junctional complex (Fig. 4 a). Sometimes they were seen to collapse in the subcupular zone (Fig. 4 c). These extrusions were also confirmed in scanning electron micrographs as spherules of $1.5\text{--}3\text{ }\mu\text{m}$ in diameter (Fig. 5 a). They were located between the hair bundles, showing a round (Fig. 5 b) or an irregular (Fig. 5 c) shape. In some cases, a few holes were observed on the surface (Fig. 5 d).

Transitional epithelium : Cells of the transitional epithelium are cylindrical in shape gradually changing to a low cuboidal form more distal from the sensory area (Fig. 2 b). Relatively wide intercellular spaces developed sometimes with large distensions (Fig. 6). Desmosomes are present between neighboring cells. The rough endoplasmic reticulum and mitochondria are prominent from the basal to the apical cytoplasm. Presence of numerous microvilli on the cell surface is a characteristic feature of the transitional epithelium (Figs. 2 b, 6). Many Golgi apparatuses indicating a highly active phase are observed in the supranuclear region (Fig. 7 a). Flattened saccules and electron-lucent microvesicles of $0.1\text{--}0.2\text{ }\mu\text{m}$ in size are predominant Golgi elements. The saccules are dilated at the distal end in association with the rough endoplasmic reticulum (Fig. 7 b), which is well developed from the base to the top of the cell. Mitochondria are scattered throughout the cytoplasm often in association with cisternae of the rough endoplasmic reticulum (Fig. 7 c). At the basal portion of the cell (Fig. 8 a), the rough endoplasmic reticulum and free ribosomes are densely packed. Distorted mitochondria formed by invagination of cytoplasm were often seen (Fig. 8 b).

Vesicular dilations at the top of the microvilli were frequently observed on the surface of the transitional epithelium (Fig. 9 a). Some of these microvesicles seemed to be liberated free in the lumen, showing inside a few membranous structures (Fig. 9 a, b) or many minute vesicles (Fig. 9 c). Sometimes they seemed to be fusing together (Fig. 9 d).

The microvesicles were observed also by SEM as spherules of $0.5\text{--}0.6\text{ }\mu\text{m}$ in diameter on the transitional epithelium of the perimacular region where numerous microvilli were seen (Fig. 10). Some of the secretory spherules on the middle portion of the perimacular region (Fig. 10a, b) seemed to be formed by fusion of several small spherules. They showed a rough-surfaced appearance probably caused by dehydration procedure. On the anterior and posterior parts of the perimacular region where microvilli are not so abundant, the secretory spherules are almost smooth-surfaced (Fig. 10c, d). Some membranous structures covering the cell surfaces were observed when specimens were fixed with the primary fixative containing cetylpyridinium chloride (CPC) (Fig. 10d).

The vesicles and microvilli on the transitional epithelium gradually reduced in

number as distant from the macula region (Fig. 11).

Squamous epithelium : The squamous epithelium is composed of very flattened cells, about $1.5\ \mu\text{m}$ in height, exhibiting very few microvilli on the apical surface facing the lumen (Fig. 2c). Light and dark cells are distinguished lying alternately or at random. Both the cells have a few mitochondria, the poorly developed rough endoplasmic reticulum and sometimes inconspicuous Golgi apparatuses close to the nucleus. The basement membrane is thin and the connective tissue is of an areolar type.

The plasma membrane between adjacent cells is indented involutedly that a canalicular network sometimes occupies almost the entire cytoplasmic area (Fig. 12a).

On the surface of the squamous cells, secretory blebs which looked like a mass of amorphous material, or of many vesicles, are frequently observed (Fig. 12b). Some of the blebs contain a few electron-lucent vesicles or slightly electron-dense material (Fig. 12c). The blebs are also identified by SEM as variously sized spherules of about $1-10\ \mu\text{m}$ in diameter. They are in various shapes such as of oval (Fig. 13a), round (Fig. 13b), or amorphous (Fig. 13c), and sometimes in a linked form (Fig. 13d).

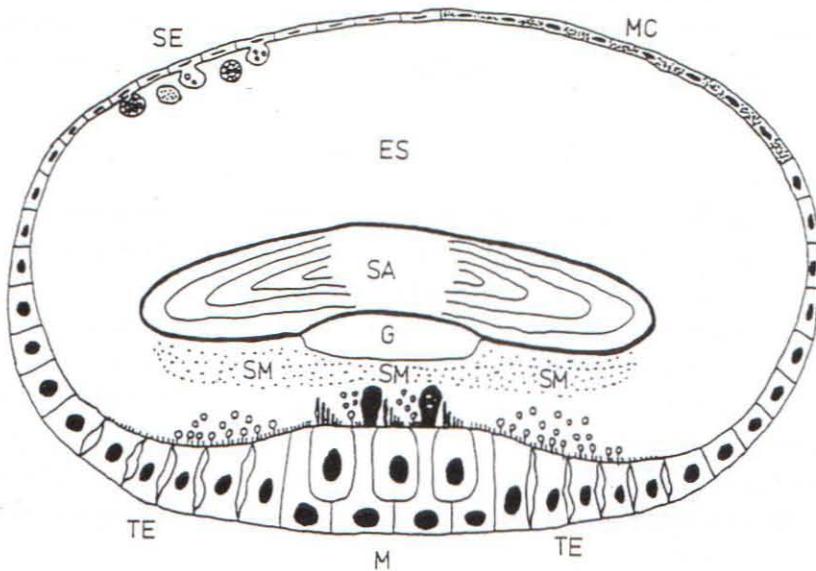
In a survey view by SEM (Fig. 14a), there are distinguished two different regions as to the presence of the blebs. In the region where the blebs are present (Fig. 14b), the terminal bars of individual cells are not obvious, but in the region where the blebs are absent (Fig. 14c), the cell boundaries are generally delineated by microvillar patterns.

As described already, the squamous cells are generally very flattened, but sometimes have well developed rough endoplasmic reticulum (Fig. 15a), or numerous vesicles and electron dense bodies associated with Golgi apparatus (Fig. 15b).

Mitochondria-rich cells : The mitochondria-rich cells varied in size and shape. They are generally squamous or cuboidal and 3 to $5\ \mu\text{m}$ in height. The most obvious cytological characteristic of the cells is the presence of abundant mitochondria with dilated cisternae of the smooth endoplasmic reticulum. The nucleus is oval and located in the central part of the cell (Fig. 2d). The cytoplasm contained numerous oval- or rod-shaped mitochondria and extensive endoplasmic reticulum. The apical surface of the cell is convex with a few microvilli, and the basal area is lined with the comparatively smooth basement membrane. Many capillaries are distributed in the connective tissue below the mitochondria-rich cells.

Otolithic membrane : The otolithic membrane extending over the saccular macula consists of two different layers, the upper gelatinous layer and the lower subcupular meshwork (Fig. 3a). The gelatinous layer is composed of a less electron-dense, smooth material than that of subcupular meshwork. Between the subcupular meshwork and the epithelial surface is the subcupular zone into which hair bundles of sensory cells protruded. The gelatinous layer is limited to the area above the sensory macula corresponding to the sulcus acusticus of the otolith, but the subcupular meshwork extends over the transitional epithelium beneath the entire otolith. Both layers have finely textured amorphous materials.

SEM observations of the gelatinous layer (Fig. 16a, b) revealed a honeycomb



Text-fig. 1. Schematic illustration of a transverse section through the middle part of the sacculus of *Oreochromis niloticus*. The saccular wall consists of sensory epithelium, macula (M), transitional epithelium (TE), squamous epithelium (SE), and mitochondria-rich cells (MC). ES, endolymphatic space; SM, subcupular meshwork; G, gelatinous layer; SA, otolith (sagitta).

structure with numerous holes, about $5\ \mu\text{m}$ in diameter. Sensory hairs protruded into the holes of the gelatinous layer (Fig. 16c, d). The outer surface facing the otolith is smooth.

The results of observations of the saccular epithelium and the otolithic membrane in tilapia, *O. niloticus*, is summarized as follows (Text-fig. 1) : The saccular wall is composed of three types of epithelia ; sensory epithelium, transitional epithelium, and squamous epithelium with a patch of mitochondria-rich cells. The sensory epithelium is composed of hair cells and supporting cells. Apocrine-type secretory materials extrude from the apical surface of the cells of the sensory epithelium into the saccular lumen. Another type of secretion is performed by the supporting cells as vesicular dilations of the distal ends of their microvilli. Contiguous to the sensory epithelium, the transitional epithelium cells gradually change from a cylindrical to a cuboidal form more distant from the sensory area. Vesicular dilations at the tips of the microvilli are more frequent on the transitional epithelium ; some detach from the microvilli free into the subcupular space as vesicles. Secretory blebs of various shapes with various electron densities occur on the squamous epithelium cells. A cluster of mitochondria-rich cells containing dilated cisternae of the smooth endoplasmic reticulum are aggregated at a portion of the squamous epithelium. The otolithic membrane consists of two distinct layers, the upper gelatinous layer and the lower subcupular meshwork. The subcupular

meshwork extends over the area of the sensory and transitional epithelia beyond the entire undersurface of the gelatinous layer which is limited only over the maculae region. The gelatinous layer corresponds in position to the sulcus acousticus at the central area of the otolith.

3) Ampulla

Sensory epithelium : The sensory epithelium of the crista ampullaris is morphologically the same as that of the saccular macula showing components of two types of cells, hair cells and supporting cells (Fig. 17b, c).

The cytoplasmic extrusions containing some vesicles, $0.2-0.5\ \mu\text{m}$ in diameter, were found close to the apical junctional complex of the sensory epithelium (Fig. 17d), showing the same feature observed in the sacculus. By SEM configurations, many extrusions appear as round bodies of about $3-5\ \mu\text{m}$ in diameter, arising among bundles of sensory hairs (Fig. 18b). No extrusions are noticed at the peripheral surface of the crista (Fig. 18c) where sensory hairs grow thick and squamous epithelium is adjacent directly to the sensory area.

Squamous epithelium : Epithelium contiguous to the sensory epithelium is a squamous type. There observed no transitional epithelium in the ampulla. The cells adjacent to the sensory epithelium are morphologically the same as those of the squamous epithelium of the sacculus. They are flat about $2\ \mu\text{m}$ in height having few microvilli (Fig. 19a), and the cytoplasm is very complexed with infoldings and indentations between neighboring cells (Fig. 19b, c). No vesicular dilations of the distal end of microvilli were observed in TEM profiles.

SEM observations of the surface of the squamous cells around the crista ampullaris revealed a few microvilli but no secretory spherules.

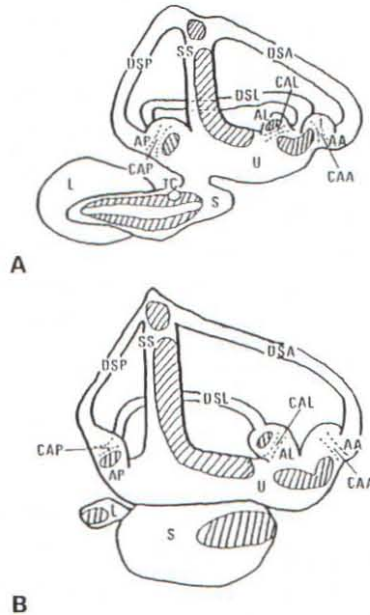
Cupula : The cupula above the crista is composed of numerous vesicular structures, and among which are electron dense cytoplasmic extrusions (Fig. 17b, c, d). SEM observations of the cupula revealed solid bodies or a pile of many solid blocks (Figs. 20a, 35a), into which sensory hairs from the crista are intruded. No subcupular meshwork was observed below the cupula (Fig. 20a, b).

2 - 4. Mitochondria-rich cells

Gross anatomy of the membranous labyrinth : The three semicircular canals, the sinus superior and the utriculus in goldfish (Text-fig. 2A) and tilapia (Text-fig. 2B) are almost similar in gross structure. In goldfish, the utriculus, sacculus and lagena are connected with each other through broad openings, and the right and left sacculi are joined by a duct called the transverse canal. The sacculus takes a slender and elongated shape and the lagena is relatively large. In tilapia, on the other hand, the sacculus is a simple ovoid sac completely separated from the utriculus, and the small lagena is attached to the posterior end of the sacculus.

Localization of mitochondria-rich cells : In both goldfish and tilapia, mitochondria-rich cells are distributed in patches in the three semicircular canals, the sinus superior and the utricle in a similar pattern (Text-fig. 2 A, B). Mitochondria-rich cells in the ampullae of all the semicircular canals are always found in the epithelium adjacent to the crista ampullaris (Fig. 21a). In the anterior ampulla, the cells are located almost completely surrounding the opening to the utricle. In the lateral ampulla, they are located on both the utricular and the canal sides of the crista in two groups ; one extending to the utricular opening and the other forming an oval patch in the canal base. In the posterior ampulla, the cells are located only on the utricular side. In the middle portion of the utricle (Fig. 21b), The mitochondria-rich cells are found along the lateral wall, independently of those in the anterior portion. The lateral wall of the sinus superior is occupied by a large elongated patch of mitochondria-rich cells extending from the middle portion of the sinus superior. Separated from this large patch, a small patch is found on the top of the lateral wall of the sinus superior.

The distribution of the mitochondria-rich cells in the sacculus and the lagena in goldfish is different from that in tilapia. In goldfish, the cells can be seen in the transitional epithelium around the macula of the sacculus (Fig. 21c) and on the basal wall of the transverse canal which is continuous with the transitional epithelium of the



Text-fig. 2. Schematic representations of a median view of the left membranous labyrinth of goldfish (*Carassius auratus*) (A) and tilapia (*Oreochromis niloticus*) (B). The hatched areas indicate those occupied by mitochondria-rich cells. AA, ampulla anterior ; AL, ampulla lateralis ; AP, ampulla posterior ; CAA, crista ampullaris anterior ; CAL, crista ampullaris lateralis ; CAP, crista ampullaris posterior ; DSA, ductus semicircularis anterior ; DSL, ductus semicircularis lateralis ; DSP, ductus semicircularis posterior ; L, lagena ; S, sacculus ; SS, sinus superior ; TC, transverse canal ; U, utricle.

sacculus (Fig. 21d). The cells cannot be seen in the lagena. In tilapia, on the other hand, the mitochondria-rich cells are observed in the lateral wall of the sacculus. They are also observed in the lateral wall of the posterior portion of the lagena in a pattern similar to that in the sacculus.

Ultrastructure of mitochondria-rich cells: The most conspicuous cytological characteristics of the mitochondria-rich cells are the presence of abundant mitochondria and dilated cisternae of the smooth endoplasmic reticulum. The cells in both goldfish and tilapia are basically similar in ultrastructural features.

In the ampullae they are columnar, about $10\text{ }\mu\text{m}$ in height, and have an oval nucleus (Fig. 22a). Some vacuoles are found in the cytoplasm. Medullated fibers run through the connective tissue beneath the cells. Epithelial cells often seen between the mitochondria-rich cells have electron-dense cytoplasm with poorly developed cell organelles.

Mitochondria-rich cells in the utricle and the sinus superior often show their nuclei located in the apical portion of the cells. The cell surface is relatively smooth, but occasionally it is equipped with poorly developed microvilli, and the cell boundaries are generally indistinct (Fig. 22b). Some of the cells, however, reveal indented cell boundaries connected with a few desmosomes in the apical and the basal regions (Fig. 22c). Dilated cisternae of the smooth endoplasmic reticulum are remarkable in the cytoplasm. Numerous glycogen granules are sometimes detected in clusters or scattering among mitochondria (Fig. 23a). Capillaries with the fenestrated endothelia are noticed in the connective tissue beneath the mitochondria-rich cells (Fig. 23b).

In the sacculus and the lagena of tilapia, the mitochondria-rich cells are basically similar in ultrastructural features to those in the utricle and the sinus superior. They are generally squamous or cuboidal, 3 to $5\text{ }\mu\text{m}$ in height, as are other epithelial cells.

The mitochondria-rich cells in the sacculus (Fig. 23c) and the transverse canal (Fig. 23d) of goldfish are similar to those in the ampullae in size and shape, but their apical surfaces are provided with many microvilli, and indentations of the cell boundaries between them are less complicated.

3. Discussion

Most previous work on the morphology of the teleostean inner ear has been done from a neurophysiological perspective (Hama, 1969; Lewis and Nemanic, 1972; Saito, 1973; Popper, 1976, 1977; Jenkins, 1979; Coombs and Popper, 1982; Platt, 1983; Popper and Platt, 1983; Saidel and Popper, 1983). Ultrastructural characteristics of the membranous labyrinth or the sacculus relative to formation and growth of the otolithic membrane and the otolith are poorly understood.

The otolithic membrane, lying between the otolith and the sensory cells, has been considered to be an apparatus for mechanoreception of gravitational forces. Generally, a layer called the "tectorial reticulum" is found between the surface of the sensory

epithelium and the cupular layer in the labyrinthine mechanoreceptor organs in vertebrates from fishes to mammals (Wever, 1971). The otolithic membrane of the sacculus in *Oreochromis niloticus* was composed of two clearly separable layers of different electron densities, the upper gelatinous layer and the lower subcupular meshwork. This feature is consistent with observations of the saccular otolithic membrane in *Gadus morhua* (Dale, 1976) and *Fundulus heteroclitus* (Dunkelberger et al., 1980). Dale (1976) suggested that the gelatinous layer is a structure precipitated from endolymph but the subcupular meshwork is a secretory product of the sensory epithelium. Dunkelberger et al. (1980) reported that the gelatinous layer is composed of tightly arranged fibrous materials that overlie only the sensory macula, but the subcupular meshwork consists of fibers in a loose network extending beyond the sensory macula. The present study revealed that the subcupular meshwork of *O. niloticus* is also extending over the entire undersurface of the otolith, whereas the gelatinous layer is limited to the macula region which corresponds to the central area of the otolith.

Vesicular dilations of the distal ends of microvilli of the supporting cells and the transitional epithelium cells appear to be liberated free in the lumen. The resultant vesicles are probably identical with the "veils" observed in the otolith organs of pigeons (Dohlman, 1971) and chinchillas (Lindeman, 1973). According to Dohlman (1971), the "veils" secreted from microvilli of the supporting cells are gradually packed together into the gelatinous layer of the otolithic membrane. If this is also the case for tilapia, the gelatinous layer might be derived from the vesicular dilations of microvilli of the supporting cells. However, the sensory epithelium has many cytoplasmic extrusions, which appeared to collapse and scatter the contents in the subcupular zone under the gelatinous layer. The fact that the subcupular meshwork abuts the microvilli-bearing cells of the sensory and transitional epithelia suggests the possibility that the vesicles from the microvillous secretion are incorporated into the subcupular meshwork, rather than into the gelatinous layer. The cytoplasmic extrusions of the sensory epithelial cells may be responsible for the formation of the gelatinous layer.

Cytoplasmic extrusions were also observed on the sensory epithelium of the ampulla, on which only the cupula, a structure corresponding to the gelatinous layer of the sacculus, was located. Therefore, cytoplasmic extrusions of the sensory epithelium cells seem to be related to the formation of the cupula in ampulla as same as the formation of the gelatinous layer in the sacculus.

The subcupular meshwork was observed on the saccular macula, but not on the ampullar crista, where otolith did not exist. Vesicular dilations of the distal end of microvilli were found on the sensory and transitional epithelium cells of the sacculus but not on those of the ampulla. It is suggested that vesicles derived from microvilli may play an important role in the formation of subcupular meshwork which may be an essential structure for the otolith formation.

Teleostean otoliths are composed of polycrystalline calcium carbonate, mainly in the form of aragonite (Morales-Nin, 1987 ; Mugiya, 1974). The needle-like crystals are

reported to grow by fusion of calcareous spherules (Nishio, 1926). Also in mammalian otoconia, calcite crystals may originate from multiple calcareous subunits seeded on the organic matrix (Ross and Peacor, 1975 ; Salamat et al., 1980). However, cellular contribution to the otoconial formation has not been elucidated. Secretory vesicles, attached to or extruded from the sensory epithelium cells into the vestibular lumen at the site of developing otoconia, were observed in frog (Harada, 1972), chicks (Balsano et al., 1969), and mammals (Marco et al., 1971 ; Nakahara and Bevelander, 1979 ; Salamat et al., 1980 ; Harada, 1983). These vesicles are assumed to contain some precursor materials of the organic matrix of otoconia.

In SEM profiles of the sacculus in *O. niloticus*, numerous spherules are found to occur by microvillous secretion of the transitional epithelium cells. Although their chemical nature remains unknown, a membranous structure in association with the secretory spherules became visible on the transitional epithelium when fixed the tissue with a fixative containing CPC. Because CPC stabilizes and preserves glycosaminoglycans (Watabe et al., 1992), presumably the vesicles may contain substances related to glycosaminoglycans. It is presumed, by analogy to otoconial formation (Salamat et al., 1980), that the spherules contain precursor materials of the organic matrix of the otolith. As noted above, vesicles originated from the microvillous secretion of the transitional epithelium may probably be involved in subcupular meshwork formation. Dunkelberger et al. (1980) demonstrated, in their ultrastructural studies of the sacculus in *Fundulus heteroclitus*, that fibers of the subcupular meshwork were incorporated into the otoliths as an organic constituent. Considering this together with the results of the present observations, perhaps materials in the microvilli-originated vesicles, or spherules, are incorporated into the subcupular mesh-work, which then may become an organic constituent of the otolith matrix.

Similar secretory spherules at the apical end of microvilli of mineralizing epithelium were observed in the midgut posterior caeca of an amphipod, *Orchestia*, in the pre-molt stage (Graf and Meyran, 1983 ; Graf and Michaut, 1980). Ultrastructural cytochemistry and X-ray microanalysis demonstrated that calcium was transported through the epithelium by way of apical microvilli (Meyran et al., 1984). Similarly, calcium for otolith formation might be transported through the saccular epithelium of *O. niloticus* by way of the apical microvilli. Mugiya (1974) demonstrated, in an autoradiographic study of ^{45}Ca incorporation into the rainbow trout otolith, that Ca deposition was most active at the medial (inner) surface except the sulcus acousticus, the surface shown in the present study to be facing to the transitional epithelium. The SEM profiles of the spherules on the transitional epithelium revealed a resemblance to calcareous spherules in the molluscan mantle (Istin and Krischner, 1968 ; Istin and Girard, 1970 ; Saleuddin, 1970 ; Istin and Masoni, 1973 ; Roinel et al., 1973 ; Watabe et al., 1976). The calcareous spherules in shell-regenerating *Pomacea* are composed of amorphous calcium carbonate or vaterite crystals and the organic matrix which contains sulfated mucopolysaccharides and proteins (Watabe et al., 1976). Those spherules were considered to originate from

Golgi vacuoles. The transitional epithelium cells of the sacculus of *O. niloticus* showed well-developed endoplasmic reticulum and Golgi apparatus with numerous vesicles. This characteristic feature may indicate that the spherules originate from Golgi vesicles as in molluscs, although no prominent intracellular secretory vesicles were observed. Probably, the spherules enlarge rapidly as they leave the cell surface.

Secretory blebs found on the squamous epithelium cells may eventually be dissolved into endolymph because they are found mostly attached to, or only very close to the cell surface. The squamous cells generally contained few organelles, but sometimes fairly well-developed endoplasmic reticulum and some vesicles or electron-dense bodies in association with a Golgi apparatus. Perhaps these vesicles and electron-dense bodies are related to secretory materials in the blebs. Considering together with their dilated intercellular spaces, the squamous cells may be involved in humoral control of the endolymph.

The present study revealed the distribution and ultrastructure of mitochondria-rich cells in the teleost inner ear. Higher vertebrates maintain high potassium and low sodium concentrations in the cochlear endolymph contrary to low potassium and high sodium concentrations in plasma (Citron et al., 1956). This is also the case for teleostean saccular endolymph (Enger, 1964). The sensitivity of receptor cells have been considered to depend on this ionic situation (Tasaki, 1960). A portion of the squamous epithelium was occupied by a patch of mitochondria-rich cells. Their characteristic ultrastructure suggests that they function in active transport of ions. In the mammalian inner ear, some "dark cells" of similar features are known to exist in the utricle and ampullae, and are assumed to be engaged in ionic control of the endolymph (Dohlman and Ormerod, 1960; Lundquist et al., 1963; Dohlman, 1964; Kimura et al., 1964; Hamilton, 1965; Nakai and Hilding, 1968; Kimura, 1969). Probably, the mitochondria-rich cells may be involved in the ionic regulation of the endolymph. Fenestrae of the endothelium of capillaries beneath the cells may be a feature responsible for the fluid transport (Rhodin, 1962). Similar ion transporting cells have been found in the kidney (Rhodin, 1962), salt secreting glands (Bulger, 1963), ciliary body of the eye (Tormey, 1963), choroid plexus (Pease, 1956), and gills (Copeland, 1948) in various animals.

The distribution of mitochondria-rich cells in the ampullae, the sinus superior and the utricle was similar in both ostariophysid goldfish and non-ostariophysid tilapia, and also to that of dark cells in utricular organs in mammals (Kimura, 1969). However, their distribution in the sacculus and lagena was different between the two species according to the structural difference of the labyrinth. In mammals, dark cells are absent in the sacculus and the cochlea (Kimura, 1969). It is of interest to note that the utricle and the sacculus are completely separated sacs in tilapia, while those of mammals are joined by a duct called ductus utriculosaccularis (Bloom and Fawcett, 1975) through which endolymph can flow freely. In goldfish, mitochondria-rich cells were not found in the lagena, which was connected with the sacculus by a broad opening, a similar situation between the utricle and the sacculus in mammals.

In goldfish, mitochondria-rich cells were found in the basal wall of the transverse canal and the perimacular transitional epithelium of the sacculus. The sacculus of tilapia lacks the transverse canal, and the cells were observed at the lateral wall of the sacculus and lagena. This different distribution of mitochondria-rich cells may be attributed to the structural difference of the labyrinth between ostariophysid and non-ostariophysid fishes.

IV. ULTRASTRUCTURE OF OTOLITH CRYSTALS

The otoliths of teleosts are complex polycrystalline bodies which serve as statoliths in the inner ear (Carström, 1963). They are composed mainly of crystallized calcium carbonate in the form of aragonite and of a fibrous protein (Degens et al., 1969).

Dale (1976) observed many spherules attached to the surface of cod otoliths and proposed that they were 'building units' of the otoliths. Generally, it is assumed that the otolith grows most rapidly along the anterior-posterior axis and that the growth patterns are best expressed on the dorso-ventral sagittal plane (Pannella, 1974). Bingel (1981) reported that cod otolith revealed non-uniform growth as a result of preferential deposition of crystals on specific surfaces of the otoliths.

On the other hand, Watabe et al. (1982) investigated the ultrastructural characteristics of otolith organic matrix in killifish and tilapia and showed that the crystals of the accretion zone were embedded in organic matrix fibers similar to and continuous with fibers of the discontinuous zone. Thus the rate of calcium deposition is considered to be influenced by the rate of organic matrix that also affects the size of calcium crystals.

Many spherules observed on the transitional epithelium of sacculus in tilapia were suggested to contain precursor materials of the otolith (Chapter III). It is questioned whether the otolith grows through direct apposition of these spherules over its surface as building units. This study was conducted in an attempt to characterize the ultrastructural surface texture of the otolith, and to propose a possible process of crystal (otolith) growth. To facilitate comparisons with previous descriptions (Dale, 1976; Bingel, 1981), otoliths of tilapia were observed without fixation or after fixation with different fixatives for various lengths of time.

1. Materials and methods

For scanning electron microscopy (SEM), adult *Oreochromis niloticus*, approximately 10cm in total length, were used. Rearing conditions were the same as in the TEM and LM studies of the previous Chapter.

SEM observations of saccular capsules were performed according to the same proce-

dures as described in Chapter III. Otoliths were observed by SEM after the following treatments. Otoliths (sagittae) dissected out from adult fish under a binocular dissecting microscope were washed in 99% ethanol and stored dry for a short time. They were placed in the primary fixative for various lengths of time from 1 min to 7 days. As primary fixatives, cacodylate or phosphate buffer solutions each adjusted pH at 7.4 were used for a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde (Karnovsky, 1965) adding 4% cetylpyridinium chloride (CPC) to stabilize glycosaminoglycans (Watabe et al., 1982). After fixation they were washed overnight in the cold buffer solution and dehydrated in ethanol.

After dehydration the capsules and otoliths were dried by the CO₂-critical point method using amyl acetate as an intermediary fluid. The specimens were placed on aluminium stubs, coated with gold in vacuum, and observed by a scanning electron microscope.

2. Results

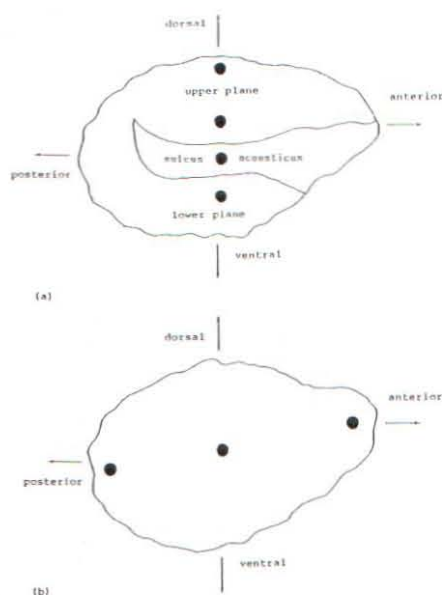
The saccular otolith (sagitta) of adult tilapia is an oval stone flattened medial-laterally (Fig. 24a, b). It lies vertically in a way that the sulcus acousticus of the medial side is in contact with the gelatinous layer of the otolithic membrane on the sensory epithelium. The contour of the sulcus acousticus is in accordance with an extent of the sensory epithelium or the macula which looks like an elongated footprint in shape (Fig. 24c).

Ultrastructural changes of calcium carbonate crystals of the otolith surface when treated with differently buffered fixatives for different lengths of fixation time were studied with different portions of the sagittal otolith. Scanning electron micrographs were taken at different portions indicated in Text-fig. 3.

The crystals were variable in size and shape with different portions of the same otolith. In intact, non-fixed otoliths, both the inner (medial) and the outer (lateral) surfaces showed crystals which seemed to be growing, as a whole, from the center to the otolith margin. In the central portion of the sulcus acousticus crystal needles were approximately 5–8 μm in width (Fig. 25a). Acicular crystals were about 3 μm in width on the upper and lower portions of the inner surface (Fig. 25b, c). At the outer surface spherical crystals about 1–2 μm in diameter occupied the central area (Fig. 25d). The anterior margin of the outer surface showed large acicular crystals about 10 μm in width (Fig. 25e).

The effects of the phosphate or cacodylate buffered fixatives on the surface feature of crystals could not be detected by fixations up to about 2 days, but clear differences were observed between treatments with the two fixatives after fixation for 4–7 days.

After fixation with the cacodylate buffered fixative for about 6 h (Fig. 26), a granular appearance began to appear on the surfaces of all the crystals. After fixation from 12 hours to 2 days (Fig. 27), crystal grains of about 0.1 μm in diameter became



Text-fig. 3. Otolith surfaces of *Oreochromis niloticus* to show the portions (●) selected for SEM observations of the crystal structure.

- (a) : inner surface
(b) : outer surface

clearer with time, and membranous structures appeared in and between the crystals. After fixation for 4 to 7 days (Fig. 28), the fibrous structures in and between crystals became more remarkable, and the crystal grains were also more obvious.

After fixation with the phosphate buffered fixative for 4 to 7 days, calcareous spherules of $5 - 7 \mu\text{m}$ in diameter, composed of groups of probably matured hydroxy-apatite platelets in a highly ordered hexagonal array, were observed on and around the areas of the sulcus acousticus (Fig. 29a, b) and on the opposite outer surface. On the anterior margin of the outer surface, small clusters of crystals ranging $0.1 - 1 \mu\text{m}$, possibly apatite crystals in an early phase, were observed.

3. Discussion

The main component of teleostean otoliths is calcium carbonate in the form of aragonite which is polycrystalline in nature (Irie, 1955, 1960 ; Carlström, 1963 ; Mugiya, 1974). The needle-like crystals of aragonite are oriented the long axis nearly perpendicular to the outer margin of the otolith (Irie, 1955 ; Degens et al., 1969).

Crystals on the surface of the otoliths of tilapia showed the same feature as reported by Bingel (1981) in cod otolith ; the size and shape of crystals varied in different portions of the otolith surface. This indicates that the growth of otolith is not uniform with directions. The size of crystals of the inner surface facing the macula

were larger than those on the outer surface facing the bulky lumen. Therefore, the otolith seems to grow more actively at the inner surface. This interpretation of otolith growth is in accord with the results of calcium-45 incorporation in rainbow trout otoliths in which much ^{45}Ca was shown to deposit on the inner surface (Irie, 1960 ; Mugiya, 1974). The shape of the sulcus acousticus of tilapia sagitta nearly corresponded to the contour of the sensory epithelium. Considering that the gelatinous layer overlying the macula region is in direct contact with the sulcus acousticus where the crystal growth is less active than the other portion of the otolith inner surface (Mugiya, 1974), the cells of the sensory epithelium are more likely to be involved in the formation of gelatinous layer rather than in the otolith growth.

On the other hand, the anterior and posterior margins of the outer surface of otolith showed larger acicular crystals in comparison with other portions. Perhaps this finding may indicate that the otolith grows more rapidly in the anterior and posterior directions.

According to Dale (1976), the macula side of the cod otolith is strewn with uniform spherules which are embedded into the otolith as its 'building units'. However, as Dale used phosphate buffer solution for fixation and storage, the spherules could have been formed artificially on the surface of the otolith as calcium phosphate crystals in the form of hydroxyapatite crystal (Meyer et al., 1972) . Similar hydroxyapatite crystals were observed in this study when using a phosphate buffer solution for the fixation of otolith. In the present observation of non-fixed tilapia otoliths, crystals were seen on the inner surface in various sizes and shapes. When the otoliths were fixed with cacodylate buffered 2.5% glutaraldehyde and 2.0% paraformaldehyde, the crystals were seen to be gradually decalcified with a longer fixation time, leaving grains of about $0.1\text{ }\mu\text{m}$ with membranous structures in and between crystals of the otolith surface. Probably the grains are crystal subunits (crystallites) of a single aragonite crystal and the membranous structures are intercrystal matrix.

V. MORPHOMETRIC ANALYSIS OF SECRETORY SPHERULES IN THE SACCULUS

The otoliths of teleosts contain an organic matrix composed of glycosaminoglycans, lipids (Mugiya, 1968) and proteins known as otolin (Degens et al., 1969). Mugiya (1968) suggested that the organic matrix of otolith is produced and secreted at the macula region into the otolith fluid (endolymph). As stated in Chapter III, spherules found on the transitional epithelium of the sacculus in tilapia are considered to contain precursor materials of the organic matrix of otolith. On the other hand, Tanaka et al, (1981) and Mugiya et al.(1981) suggested that the daily increments are formed by diurnal changes in otolith forming activities determined by the internal biological clock which is entrained by the photoperiod as an external time cue.

In this study, determination of daily changes of the secretory activity was attempted by quantifying the spherules on the transitional epithelium of sacculus in tilapia.

1. Materials and methods

Twenty adult tilapia *Oreochromis niloticus*, approximately 10cm in total length, were used as materials. They were kept in 60 liter freshwater aerated aquaria under a 12L-12D photoregime (light phase, 08:00-20:00). Water temperature and food supply were the same as described in Chapter III. Animals were acclimated to the photoperiod at least two weeks and then 5 fish were sacrificed at each 2:00, 8:00, 14:00 and 20:00 h. SEM specimens of the inner ear were prepared by the procedures described in Chapter III. The relative amount of secretory spherules on the perimacular transitional epithelium was determined by SEM observations with the three partitioned area shown in Text-fig. 4. The relative amount of secretory spherules was represented by the space factor which was a percentage proportion of total spaces occupied by the spherules in a unit area of 50 μm square (Text-fig. 5). About 5-10 unit areas were observed for a fish.

2. Results

Tilapia showed a diurnal rhythm in quantity of secretory spherules in the three different perimacular regions (anterior, middle and posterior). (Text-Fig. 6).

In the anterior perimacular region the space factor was the lowest (5.41%) at 8:00 h, and then increased significantly ($p < 0.01$) to 15.07% at 20:00h.

On the middle region the value was the lowest (0.39%) at 2:00h and increased linearly to the highest at 20:00h (6.19%). This value was significantly higher ($p < 0.01$)

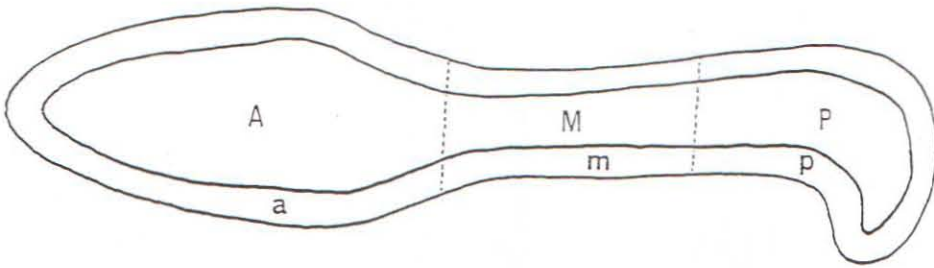
than that at 2:00h.

In the posterior region the space factor was the lowest (5.05%) at 8:00h, and then increased significantly ($p < 0.01$) to 11.08% at 20:00h.

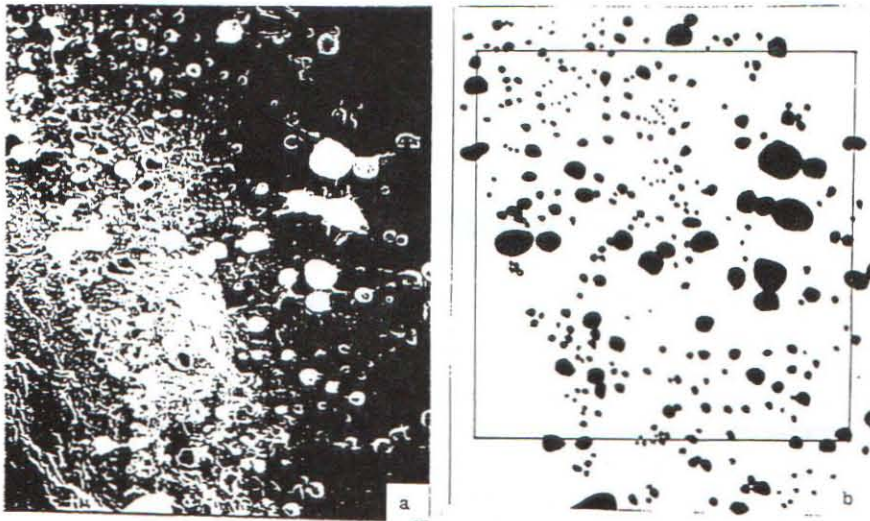
In comparisons between the anterior, middle and posterior perimacular regions, the anterior region showed the largest quantity of secretory spherules at any time of the day. Typical SEM photographs of the anterior region with numerous secretory spherules are shown in Fig. 30b, d. The posterior region followed the anterior region and the middle region was the least active. Typical SEM photographs of the middle perimacular region with small numbers of secretory spherules are shown in Fig. 30a, c.

3. Discussion

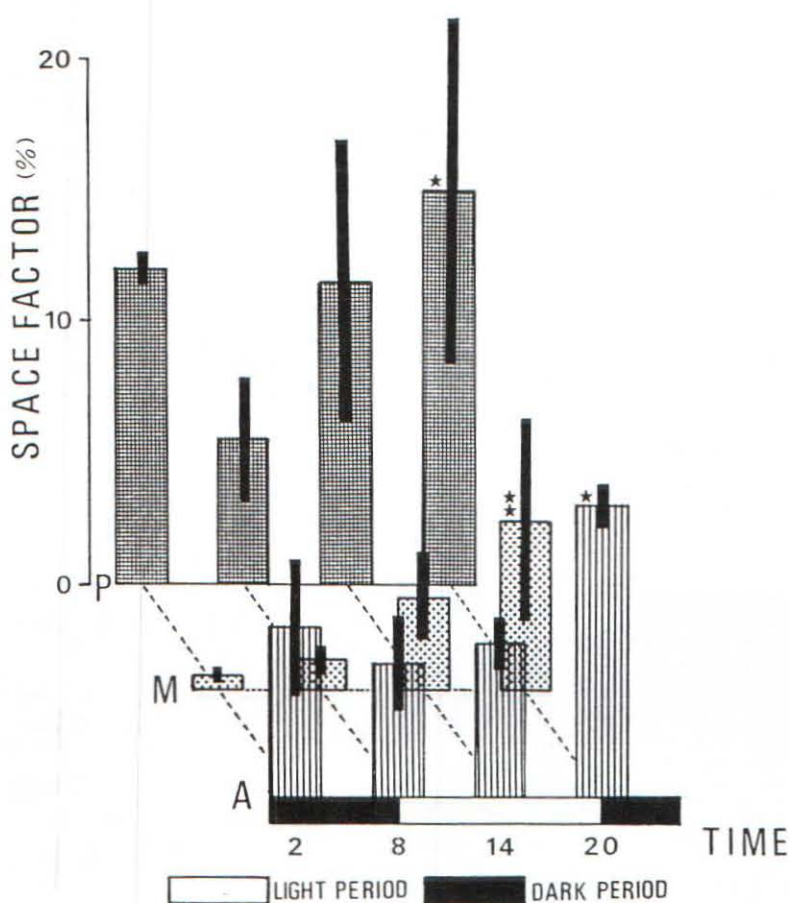
Diurnal quantitative changes of secretory spherules on the transitional epithelium were confirmed by SEM. The numerical density of the spherules showed a clear tendency that it is high in the afternoon and low in the morning on any of the anterior, middle



Text-fig. 4. Schematic representation of the saccular macula and the perimacular region (transitional epithelium) in tilapia. A, anterior part of the saccular macula ; M, middle part of the saccular macula ; P, posterior part of the saccular macula ; a, anterior part of the perimacular region ; m, middle part of the perimacular region ; p, posterior part of the perimacular region.



Text-fig. 5. Diagrams of the secretory spherules on the perimacular region.
 (a) : SEM photograph of secretory spherules.
 (b) : Copy of the photograph of (a). Black spots indicate areas covered by secretory spherules. Space factor was measured with the encased area ($50\mu\text{m}$ square).



Text-fig. 6. Daily changes of the space factor of the secretory spherules on the saccular transitional epithelium in tilapia. Vertical bars indicate SD of mean for 20–30 units. A, anterior part of the transitional epithelium; M, middle part of the transitional epithelium; P, posterior part of the transitional epithelium. ★Significantly different ($p < 0.05$) from the value at 8:00h. ★★Significantly different ($p < 0.01$) from the value at 2:00h.

and posterior perimacular regions. Mugiya et al (1981) and Mugiya (1984) found in goldfish and rainbow trout, respectively, that otolith formation slowed down or stopped at sunrise and resumed after 3 hrs. According to Tanaka et al (1981), the discontinuous zone of otolith increments was formed around the time of light-on under a regime of 12 L–12D photo-condition (light phase, 08:00–20:00) in tilapia.

If the contents of the spherules are organic materials with or without calcium necessary for otolith growth, and if the deposition of organic matrix and the growth of aragonite crystals on the otolith surface are almost concurrent events, the quantity of spherules on the perimacular region should be related to the growth rate of otolith. The secretory spherules were shown to be most abundant at the anterior perimacular

region, less at the posterior region and least at the middle region throughout the day. The anterior and the posterior buds of the outer otolith surface showed larger acicular crystals, and this was considered to indicate that the otolith grew more rapidly in the anterior and posterior directions (Chapter IV). Therefore, the growth rate of otolith can be correlated to the quantity of secretory spherules on the perimacular region. The observed diurnal rhythm of spherule secretion of the perimacular cells may have a causal relation with the daily increment formation.

Based on the morphological studies of Watabe et al. (1982) and Campana and Neilson (1985), Mugiya (1987) proposed three alternative hypotheses that might account for the formation of the bipartite structure of otolith increments: 1) both organic matrix and calcium depositions vary diurnally but in opposite phases, 2) calcium deposition varies diurnally, while matrix deposition does not, and 3) calcium deposition is constant, while matrix deposition varies diurnally. All these would result in the formation of alternate zones in which calcium or matrix deposition is predominated. In rainbow trout otolith, depositions of calcium and organic matrix were shown to be in opposite phases with each other; the rate of calcium uptake by the otolith varied in accordance with a diurnal rhythm of serum calcium concentration (the peak at 16:00h and the nadir at 22:00h), while glutamic acid uptake peaked at night (22:00h) against an almost constant level during the daytime (Mugiya, 1987). The present results revealed that the amount of spherules on the perimacular region changed diurnally (the peak at 20:00h and the nadir at 8:00h). It is needed to know whether calcium in addition to organic matrix precursors is contained in the spherules and how long time is required for the spherules from secretion at the transitional epithelium to deposition at the otolith surface.

VI. ELECTRON-PROBE X-RAY MICROANALYSES IN THE SACCULUS AND AMPULLA

Otoliths in bony fishes consists of crystalline calcium carbonate in the form of aragonite (Morales-Nin, 1987) and a small amount of organic matrix (Degens et al., 1969; Mugiya, 1968). Little has been known about the mechanism of the otolith growth, particularly on the cellular contribution because the otolith grows in a humoral environment out of contacts with any cellular structures.

Numerous spherules found on the transitional epithelium of the sacculus in tilapia were suggested to contain precursor materials of the organic matrix of otolith (Chapter III). The spherules were shown to occur by secretory activities of the transitional epithelium cells in a diurnal rhythm (Chapter V).

To investigate chemical characteristics of the spherules, electron-probe X-ray microanalyses (EPMA) were performed in comparison with those of the sacculus and ampulla in tilapia.

1. Materials and methods

Adult tilapia, *Oreochromis niloticus*, approximately 10cm in total length, were used as materials. The rearing conditions and the sampling schedule were the same as the study for the diurnal changes of the secretory spherules on the transitional epithelium (Chapter V).

The head was cut sagittally and fixed with 5 % glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 3 hrs, washed overnight in the same cold buffer and dehydrated through a graded series of ethanol. The post-fixation with the secondary fixative was not performed. Surrounding bones and cartilages were removed carefully in 70% ethanol until the entire labyrinth was loosened from its bony cradle. The membranous saccular capsule was opened with a razor blade and the otolith was pulled gently off the sensory macula. The ampullae were opened and the crista ampullaris were exposed in the same way. Following dehydration, the capsule and otolith were critical-point dried, placed on a carbon stub, and coated with carbon in vacuum. Otoliths were treated with the primary fixative containing 4 % CPC for a long time to decalcify and visualize their organic fibers in the same study for the surface texture of otolith crystals (Chapter IV).

EPMA was conducted with a Hitachi S-2300 scanning electron microscope equipped with a Horiba EMAX energy dispersive X-ray spectrometer at 20kV. The working distance was 20mm and the integration time for detection was 100 seconds.

A Hitachi X-650 SEM equipped with a S-6091 wavelength dispersive X-ray microanalyzer also was used to determine the amount of calcium in selected areas of specimens. It was operated at 25kV, in a working distance of 15mm and a detecting time of 20 sec.

Specimens were placed on stubs as flat as possible, and detected characteristic X-rays from specimens as much as possible by controlling the working distances of the SEM. For quantitative analysis of calcium, the wavelength dispersive X-ray microanalysis was performed according to the spot to spot method taking the otolith of tilapia as the standard sample and determined the relative counts for calcium.

2. Results

1) Elementary analyses

(1) Sacculus

Sensory epithelium : Secretory extrusions were seen as round bodies measuring 1.5–3 μ m in diameter among bundles of sensory hairs of the saccular epithelium (Fig. 31a). EPMA of the extrusions yielded three major peaks which were identified as $k\alpha$ emissions of phosphorus, sulphur and calcium, respectively (Fig. 31b). The highest peak was of sulphur.

Transitional epithelium : Spherical vesicles scattering over the perimacular transitional epithelium (Fig. 31c) were smaller ($0.5-0.6\ \mu\text{m}$ in diameter) than those on the sensory macula (Fig. 31a). Analysis of the spherules showed peaks in the order of calcium, sulphur and phosphorus (Fig. 31d). The relative proportion of calcium was higher in the spherules of the transitional epithelium than in the extrusions of the sensory epithelium.

Squamous epithelium : Secretory blebs of various shapes occurred on the squamous epithelium cells in the sacculus (Fig. 32a). Examinations of the blebs gave similar results with spherules on the transitional or sensory epithelium in that phosphorus, sulphur and calcium were the major elements contained (Fig. 32b). Moreover, the highest peak was of phosphorus.

Otolithic membrane : SEM observations of the gelatinous layer revealed a honey-combed structure with numerous holes of about $5\ \mu\text{m}$ in diameter (Fig. 32c). The analysis of the gelatinous layer gave only peak of sulphur (Fig. 32d). On the other hand, subcupular meshwork showed a finely textured fibrous structure with amorphous materials (Fig. 33a). Analysis of the subcupular meshwork showed prominent peaks for phosphorus, sulphur and calcium (Fig. 33b). The peak of calcium was the highest, and followed the peaks of phosphorus and sulphur in turn. The pattern of peaks was different from that of the spherules on the transitional epithelium in that the sulphur peak was very low.

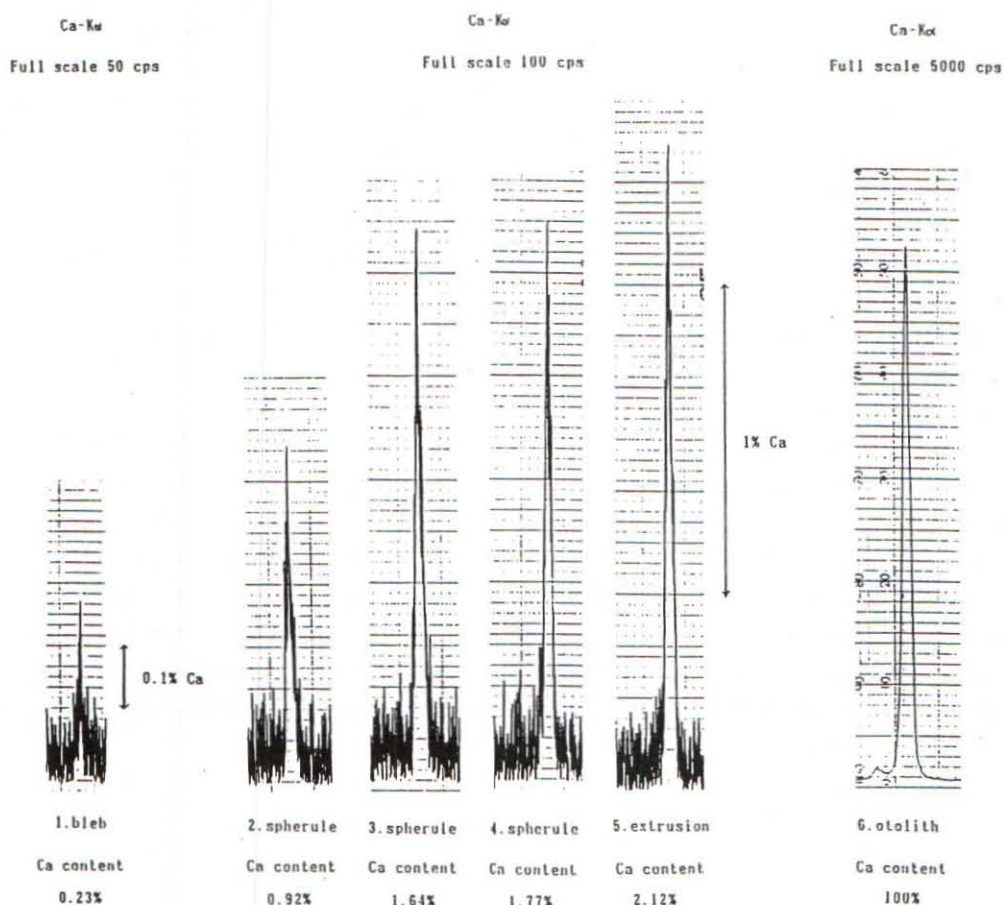
Otolith : SEM profiles of otolith surface after fixation with the primary fixative for 7 days showed fibrous structures in and between crystals (Fig. 33c). The analyses of both the fibrous structures gave almost the same pattern of a big peak for calcium with very small peaks for phosphorus and sulphur (Fig. 33d).

(2) Ampulla

Sensory epithelium : Cytoplasmic extrusions of $1-5\ \mu\text{m}$ diameter were on and among the hair bundles of the sensory cells of the anterior crista ampullaris were analysed (Fig. 34a). A prominent peak of sulphur and small peaks of phosphorus and calcium were also found. The calcium peak was much lower than the phosphorus peak (Fig. 34b).

Squamous epithelium : The surface view of epithelium adjacent to the sensory epithelium of crista ampullaris scarcely showed secretory spherules (Fig. 35a). The cell surface emitted three major peaks in the order of sulphur, phosphorus and calcium, a pattern similar to that of the cytoplasmic extrusions (Fig. 35b).

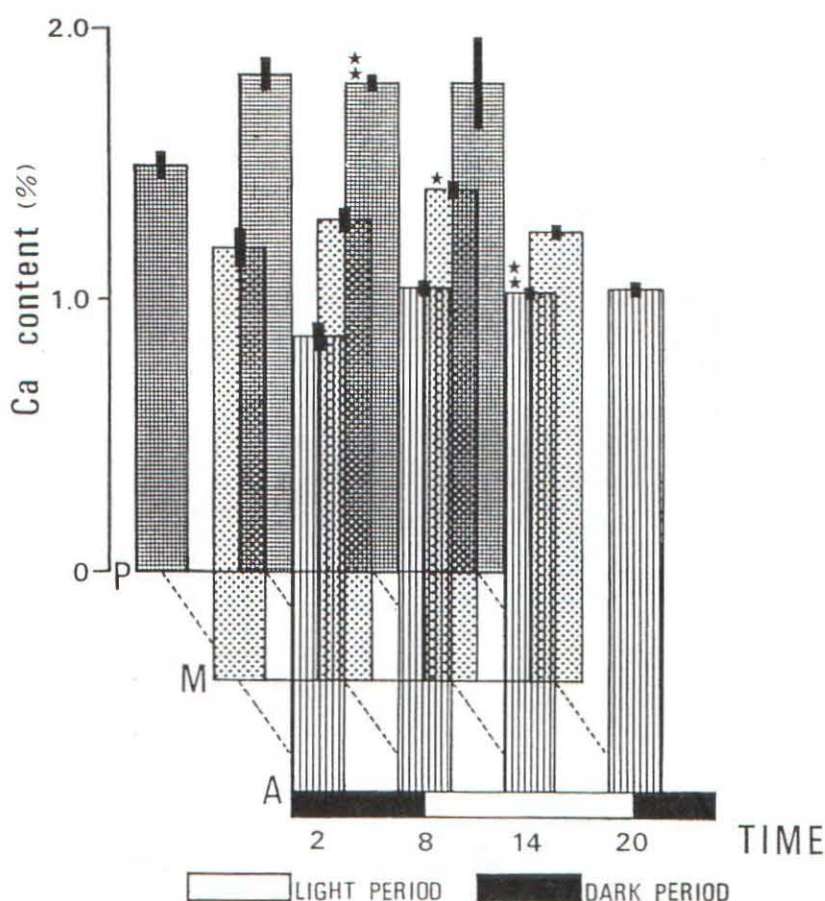
Cupula : The cupulae are gelatinous bodies located above the cristae, about $70-80\ \mu\text{m}$ in height (Fig. 34c). Prominent phosphorus, sulphur and calcium peaks were seen in that order (Fig. 34d). The spectrum showed almost the same pattern as that of the cytoplasmic extrusions on the sensory epithelium of the crista ampullaris.



Text-fig. 7. Relative amounts of calcium measured on the secretory structures of the sacculus taking the otolith value as 100%. The calcium contents of blebs on squamous epithelium, spherules on the transitional epithelium and extrusions on the sensory epithelium were 0.23%, 0.92–1.77% and 2.12%, respectively.

2) Quantitative analysis of calcium

The relative amounts of calcium in various secretory structures on the saccular epithelium were determined from the relative counts of spot to spot analyses, taking the otolith as the standard material (Text-fig. 7). Calcium of secretory extrusions on the sensory epithelium amounted to about 2.12% of the otolith. The calcium contents of spherules on the transitional epithelium were from 1.77% to 0.92% reducing as distant from the sensory epithelium. Blebs in the squamous region showed only 0.23% calcium compared to the otolith.



Text-fig. 8. Daily changes of the amount of calcium in secretory extrusions on three portions of the sensory epithelium. Vertical bars indicate SD of mean for 20–30 samples. A, anterior; M, middle; P, posterior.

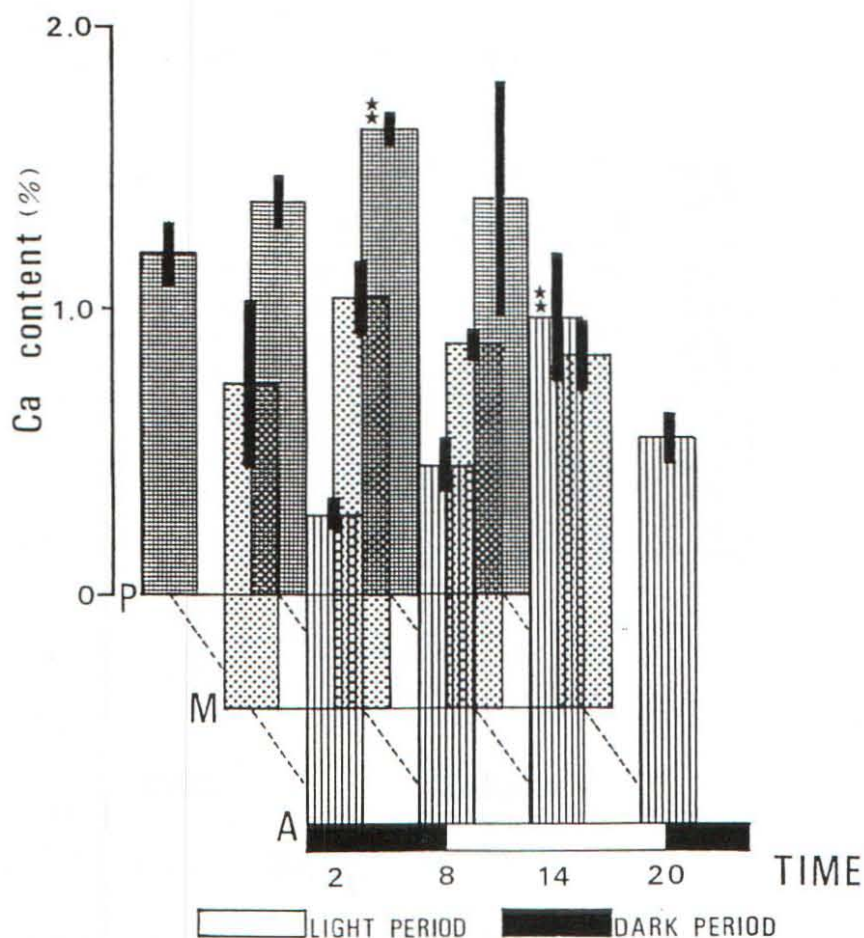
★Significantly different ($p < 0.05$) from the value at 2:00h.

★★Significantly different ($p < 0.01$) from the value at 2:00h.

3) Diurnal quantitative changes of calcium

(1) Sensory epithelium

Diurnal changes of the relative amount of calcium of secretory extrusions at different portions (anterior, middle and posterior, see Text-fig. 4) of the sensory epithelium were determined. It was the lowest at 2:00h, and constantly high after 8:00h at any portion (Text-fig. 8). The mean values of calcium contents (1.80–1.83%) of the three portions at 14:00h were significantly higher ($p < 0.01$) than those at 2:00h (1.49–1.67%). Comparing the three portions at 8:00h and 20:00h, the values of the middle portion were lower than those at the anterior and posterior portions.



Text-fig. 9. Daily changes of the amount of calcium in secretory spherules on three portions of the transitional epithelium. Vertical bars indicate SD of mean for 20–30 samples. A, anterior; M, middle; P, posterior.

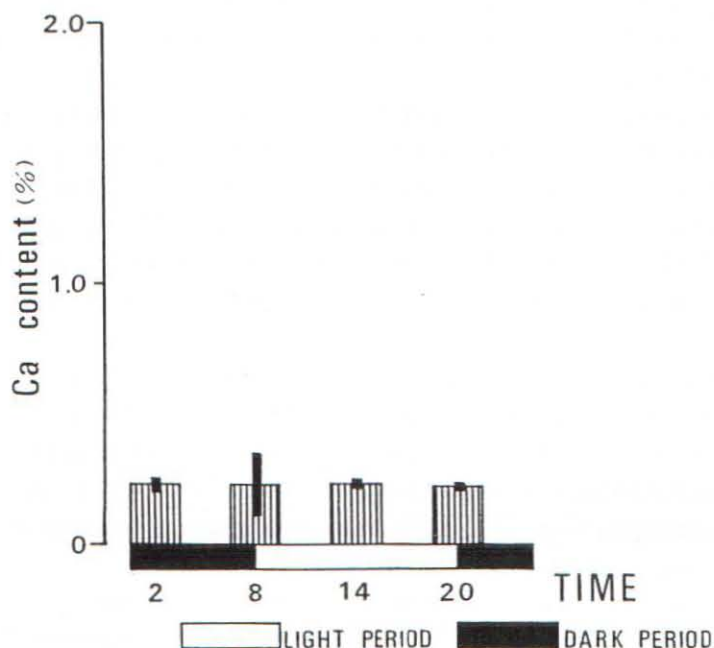
★★Significantly different ($p < 0.01$) from values at 2:00, 8:00 and 20:00h.

(2) Transitional epithelium

At the anterior and posterior perimacular portions, the calcium contents of secretory spherules were the highest (1.64–1.77%) at 14:00h, while significantly ($p < 0.01$) the lowest (1.08–1.20%) at 2:00h. On the other hand, the calcium content of spherules at the middle portion (1.14–1.44%) changed little all day long (Text-fig. 9).

(3) Squamous epithelium

Calcium contents of blebs were in a very low level (0.22–0.23%) at any time of the day compared with those of the secretory structures on the sensory and transitional



Text-fig. 10. Daily changes of the amount of calcium in blebs on the squamous epithelium. Vertical bars indicate SD of mean for 20–30 samples.

epithelium (Text-fig. 10).

3. Discussion

Elementary characteristics of the secretory structures on the saccular epithelium were shown by electron-probe X-ray microanalysis (EPMA). They contained phosphorus, sulphur and calcium among others. Phosphorus, sulphur and calcium were detected in all the secretory spherules on the saccular epithelium while only sulphur was confirmed in the gelatinous layer of the otolithic membrane. Sulphur in the gelatinous layer may represent sulfated glycosaminoglycans as its major component. In crista ampullaris, cytoplasmic extrusions were observed only on the surface of the sensory epithelium and not on the adjacent epithelium. These extrusions also contained phosphorus, sulphur and calcium. Different from the gelatinous layer of the sacculus, however, the cupula of the crista contained all these three elements. As neither the otolith and the subcupular meshwork is present on the sensory epithelium cells of the crista, the cytoplasmic extrusions must be responsible for the formation of cupula (Chapter III). In the sacculus, the same situation can be applied to the cytoplasmic extrusions of the sensory epithelium cells as to the origin of the gelatinous layer. It is considered, however, that there would

be different processes in the formation of these gelatinous structures, that is, the cupula may be formed directly from cytoplasmic extrusions, but the gelatinous layer after some further modification.

The X-ray spectra of both the secretory spherules on the transitional epithelium and the subcupular meshwork showed a similar pattern except a lower peak of sulphur and a higher peak of phosphorus in the meshwork. It is possible to presume that some sulphur containing components are transferred to the gelatinous layer. Judging from the results of EPMA together with the morphological observations (Chapter III), the subcupular meshwork may have its origin in the secretory spherules on the transitional epithelium.

Otoliths of teleosts contain the organic matrix composed of glycosaminoglycans, lipids (Mugiya, 1968) and proteins (Degens et al., 1969 ; Asano et al., 1988 ; Baba et al., 1991). The X-ray spectrum of the otolith fixed for a long time represented a big peak of calcium and very small peaks of phosphorus and sulphur. Sulphur may represent sulfated glycosaminoglycans in the otolith. On the other hand, phosphorus may be a component of phospholipids derived from cytoplasmic membranous structures secreted by the saccular epithelium cell. The peak of phosphorus in the subcupular meshwork was much higher than that of sulphur. If the subcupular meshwork is incorporated into the otolith as its organic matrix, the uptake of phosphorus would be reduced compared with that of sulphur by some mechanism.

The relative amount of calcium in spherules was the highest on the sensory epithelium, next on the transitional epithelium and the lowest on the squamous epithelium. The quantity of calcium detected in blebs on the squamous epithelium was significantly much lower than those on the other saccular epithelium. The calcium content of the secretory spherules on the sensory and transitional epithelia clearly showed a diurnal change. On the other hand, the calcium content of blebs on the squamous epithelium showed almost the same level all the day. Therefore, calcium in the blebs on the squamous epithelium may not take an important part in the otolith growth.

The calcium contents of secretory extrusions were the highest at 14:00 and the lowest at 2:00 on all the three portions of macula. The calcium contents of secretory spherules were also the highest at 14:00 and the lowest at 2:00 on the anterior and posterior portions of perimacular region, but scarcely changed throughout the day on the middle portion. If calcium in the secretory spherules on the perimacular epithelium has the major importance for the otolith growth, there must be a phase difference between the diurnal change of calcium content of secretory spherules and the diurnal growth of otolith, because the otolith growth in tilapia under the same photo-condition is reported to stop at 8:00 and resume after 3hrs (Tanaka et al., 1981). Time course as to the liberation and deposition of calcium in the spherules, as well as of organic contents, should be investigated.

Shichiri(1985) reported that the otolith of goldfish is round in the early life stage but is gradually transformed to a similar shape of its sacculus, and wondered why

calcium ions in endolymph are not distributed uniformly by diffusion. In this respect, it is very interesting that the calcium content of spherules on the perimacular region was locally different according to the distance from the macula. This can be an explanation for a cause of the non-uniform growth of the otolith.

VII. GENERAL DISCUSSION

The functional morphology of the inner ear of teleosts has been studied by many workers (Flöck, 1964 ; Hama, 1969 ; Lewis and Nemanic, 1972 ; Saito, 1973 ; Dale, 1976 ; Popper, 1976, 1977 ; Jenkins, 1977, 1979 ; Coombs and Popper, 1982 ; Platt, 1983 ; Popper and Platt, 1983; Saidel and Popper, 1983). Most of the previous investigations, however, have been carried out from the neuro-physiological point of view and morphological aspects in relation to the mechanism of otolith growth are poorly understood. The results obtained in this study using tilapia have presented morphological information of the teleostean inner ear, particularly suggestive for elucidation of the mechanism of otolith (sagitta) growth that is important for application of the otolith microstructure in population studies of fishes.

The development of the fish otolith begins with the appearance of organic cores called primordia in the otic vesicle in the early embryonic stage (Sokolowski, 1986 ; Yamada and Shimizu, 1988). Calcification of the primordia follows and the otolith grows by apposition of organic matrix and its calcification. It is now postulated that alternate depositions of calcium-rich and organic matrix-rich bands cause the daily growth increments in the otolith (Watabe et al., 1982 ; Mugiya, 1987).

Environmental conditions such as photoperiod (Brothers et al., 1976 ; Taubert and Coble, 1977 ; Campana and Neilson, 1985 ; Jones, 1986 ; Secor and Dean, 1986), temperature (Brothers, 1981 ; Campana, 1984 ; Neilson and Geen, 1985), feeding (Campana, 1983 ; Neilson and Geen, 1985), and endogenous conditions such as growth (Geffen, 1982), blood calcium (Mugiya et al., 1981) and circadian rhythm (Inoue, 1982) may all fluctuate cyclically, and all may have potential to influence the otolith growth. However, physiological mechanisms which control the daily growth of otolith still remains to be solved.

The otolith (sagitta) in the sacculus is located above the macular sensory epithelium, attached to the intermediate otolithic membrane and surrounded by otolith fluid (endolymph). No cells directly attach to the otolith. Therefore, precursor materials of the organic matrix and calcium are considered to be secreted by or through the saccular wall into endolymph and deposited onto the otolith surface. However, little have been studied about this process ; even the problem which cells of the saccular wall are involved in the process have not yet been made clear.

The results obtained from the present observation indicated that the secretory spherules of the transitional epithelium are the most possible source of precursor

materials of the otolith. However, it can not be excluded that the cytoplasmic extrusions of the sensory cells are also related to the otolith growth. Baba et al. (1991) identified immunohistochemically calcium-binding glycoproteins extracted from otoliths in hair cells of the macula in walleye pollack. In tilapia it is considered that cytoplasmic extrusions on the sensory epithelium are related to the formation of gelatinous layer rather than otolith growth, because they occur limited on the macular region in the sacculus and also on the otolith-less crista. The possibility that they are involved in the otolith growth should be examined further.

Observations of the otolith surface showed that aragonite crystals are different in size and shape and grow different directions at different portions. At the anterior and the posterior parts of the otolith, particularly at the latter, crystals grow faster compared to those at the middle part, corresponding to the regional differences in quantity of perimacular secretory spherules and their calcium content. This finding suggests that the non-uniform otolith growth is dependent on the amount and calcium content of secretory spherules on the perimacular region.

The calcium content of the secretory spherules changed diurnally (the peak at 14:00 h and the nadir at 2:00h), and the amount of spherules on the perimacular region also changed diurnally (the peak at 20:00h and the nadir at 8:00h), in different phases of about 6 hrs. According to Tanaka et al. (1981), tilapia under a 24-h photoperiod of 12L-12D (light phase 8:00-20:00) stop the otolith growth at 8:00 and resume it after 3 hrs. If spherules observed on the perimacular region contain organic matrix and calcium both necessary for otolith growth, the change in amount of secretory spherules should reflect on the diurnal growth of otolith that induces a daily growth increment. Thus the morphometric results of the present study provide the daily increment theory with morphological evidence that is consistent with previous physiological studies (Mugiya et al., 1981 ; Mugiya, 1984 ; Mugiya, 1987). A time lag of 6 hrs between the diurnal changes in amount of spherules and of calcium content must be considered from the process of incorporation of matrix substances and calcium onto the otolith, that needs to be clarified further.

In preparing the specimens for EPMA, a liquid fixative was used and dehydration was made through a series of graded ethanol. These procedures must have caused most water soluble substances to be dissolved out. Therefore, only less soluble materials, e. g., in the form of calcareous concretions seemed to have remained in the specimen. On the other hand, it is possible that some calcium ions in endolymph were adsorbed on the secretory spherules during fixation, and that serious losses or translocations of tissue calcium could have occurred. It is necessary to take these technical limitations into consideration in explaining calcium dynamics from results obtained by EPMA.

Although little has been known about the transport of calcium by the saccular cells, it has been proposed that an elevated level of serum calcium plays a triggering role in otolith formation through a calcium-calmodulin system in the cells (Mugiya, 1984 ; 1986). In this study, calcium necessary for otolith growth was considered to be at least

partially contained in the secretory spherules. It is necessary to trace the actual pathway of calcium from serum to endolymph via the saccular epithelium cells. Also, the chemical nature of spherules on the epithelium cells should be studied further.

SUMMARY

1. Anatomical and fine structural investigations of the inner ear of *Oreochromis niloticus* were conducted to establish the morphological basis of otolith (sagitta) growth with reference to the daily increment formation. The sacculus as an otolithic organ was studied by light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and electron probe X-ray microanalysis (EPMA) in comparison with the ampulla as a non-otolithic organ.

2. The saccular wall was composed of a single layer of cells classified into three different types of epithelia, the sensory epithelium, the transitional epithelium and the squamous epithelium. A patch of mitochondria-rich cells was located in the squamous epithelium. The otolithic membrane and the otolith (sagitta) were located over the sensory epithelium of the macula.

3. The sensory epithelium of the macula was composed of hair cells and supporting cells arranged alternately. Apocrine-type secretory extrusions containing some vesicles and electron dense materials were extruding from the apical surface of the sensory and supporting cells into the saccular lumen. They were seen to collapse and release the vesicles into endolymph of the saccular lumen at the subcupular zone. Another type of secretion was vesicular dilations of the distal ends of microvilli of the supporting cells.

4. The periphery of the sensory epithelium was transformed into a thinner transitional epithelium, which gradually became to the extremely thin squamous epithelium at the lateral and opposite sides of the macula. The transitional epithelium cells in sacculus were characterized by microvilli at the apical surface, well developed rough endoplasmic reticulum, Golgi apparatuses, mitochondria and wide intercellular spaces. These features indicated a high activity of synthesis of proteins. Numerous spherules derived from dilations of the microvilli were found on the transitional epithelium and in the subcupular space.

5. Secretory blebs of an apocrine-type were always present on the surface of the squamous epithelium of the sacculus. After pinched off into the lumen, the secretory blebs seemed to break down and dissolve themselves in the endolymph.

6. The sensory epithelium of the crista ampullaris was morphologically the same

as that of the saccular macula showing hair cells and supporting cells. Similar secretory extrusions were observed on the cell surface, but no microvilli originated spherules were found. Cells contiguous to the sensory epithelium of the crista ampullaris were morphologically the same as squamous cells of the sacculus; there were no transitional epithelium cells bearing microvilli. The cupula above the crista was composed of numerous vesicular structures, into which electron dense cytoplasmic extrusions of sensory cells were protruded.

7. In particular regions of the squamous epithelium of the sacculus, mitochondria-rich cells were found in groups. They were characterized by a great number of mitochondria associated with intricate smooth endoplasmic reticulum. Distributions of mitochondria-rich cells in the inner ears of tilapia (*Oreochromis niloticus*) and goldfish (*Carassius auratus*) were compared by LM and TEM. They were always found in patches in the structurally isolated compartments, the ampullae of three semicircular canals, the sinus superior, the utriculus and sacculus in both species. The cells were also found in the transverse canal in goldfish and the lagena in tilapia. Fenestrated capillaries were distributed in the connective tissue beneath the patches of mitochondria-rich cells.

8. The otolithic membrane of the sacculus consisted of the upper gelatinous layer, which is in close contact with the otolith, and the lower subcupular meshwork. The gelatinous layer was a reticulated structure of a honeycomb-like architecture covering the area of the sensory epithelium. The subcupular meshwork was composed of a very loose network of fine fibers extending over both the sensory and the transitional epithelium.

9. Crystals on the otolith surface (sagitta) were different in size and shape with different portions, and showed larger growth at the anterior and the posterior ends of the otolith. Inter- and intracrystalline organic matrices were disclosed by treating the otolith with fixatives for more than 4 days.

10. Quantitative distribution of secretory spherules on the transitional epithelium was in accord with the growth gradient of the otolith. The quantity of spherules on a unit area under a 12L-12D photoperiod (light-on 8:00h) showed a diurnal change, the largest at 20:00h and the smallest at 8:00h on the anterior and posterior portions. On the middle portion the spherules were few all the day.

11. Elementary analyses by EPMA of the spherules, vesicles and blebs on the sensory, transitional and squamous epithelium cells of the saccular and ampullar epithelia showed that phosphorous, sulphur and calcium were contained in all the secretory structures. The gelatinous layer of the otolithic membrane showed only sulphur, while the subcupular meshwork of the otolithic membrane and cupula of the

ampulla showed all the three elements. On the other hand, otolith matrix fibers exposed after fixation for a long time showed a bulk of calcium with little phosphorus and sulphur.

12. The calcium content of the cytoplasmic extrusions of the sensory epithelium was the lowest at 2:00h and constantly high at the following hours. The calcium content of the spherules on the anterior and posterior portions of the transitional epithelium was the highest at 14:00h and the lowest at 2:00h, while there was little change on the middle portion. The calcium content of blebs on the squamous epithelium was very low and varied little throughout the day.

13. The microvilli-bearing cells of the transitional epithelium were concluded to play the most important role in the otolith growth through secretion of spherules which contained calcium and organic matrix precursors. The contents of spherules were assumed to be incorporated into the otolith via fibrous structure of the subcupular meshwork. On the other hand, apical extrusions of the sensory epithelium cells were considered to be involved in the formation of gelatinous layer.

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Explanation of figures

Fig. 1.

- a : Lateral view of the left membranous labyrinth of *Oreochromis niloticus*. The three calcareous otoliths, lapillus (LA), saggita (SA) and asteriscus (A) are contained in the utricle, saccule and lagena, respectively. From the utricle, the three semicircular canals, ductus semicircularis anterior (DSA), ductus semicircularis posterior (DSP) and ductus semicircularis lateralis (DSL) are extended. AA, ampulla anterior ; AL, ampulla lateralis ; AP, ampulla posterior ; SS, sinus superior.
- b : Transverse section through the middle part of the saccule, LM, toluidine blue. Saccular wall consists of sensory epithelium (M, macula), transitional epithelium (TE), squamous epithelium (SE) and mitochondria-rich cells (MC). ES, endolymphatic space ; OM, otolithic membrane ; SA, otolith (sagitta).

Fig. 2. Transmission electron micrographs of four different parts of the saccular wall illustrating characteristic features of the epithelium cells.

- a : Sensory epithelium composed of hair cells (HC) and supporting cells (SC). The cytoplasm of supporting cells is more electron-dense than that of hair cells. Note an electron-dense cytoplasmic extrusion (arrow) on the top of a supporting cell. G, gelatinous layer ; ICS, intercellular space ; SH, sensory hair ; SM, subcupular meshwork ; V, vesicular structure.
- b : Transitional epithelium composed of cylindrical cells. The cells gradually reduce the height with increasingly wide intercellular spaces (ICS).
- c : Squamous epithelium composed of very thin cell with finger-like projection (FP) between the neighboring cells. A difference in electron density of the cytoplasm is noted between the cells.
- d : Mitochondria-rich cells in the middle part of the squamous epithelium. They are about three times taller than the squamous cells. Note capillaries (C) below the cells.

Fig. 3.

- a : Transmission electron micrograph of the apical part of the sensory epithelium (SE), otolithic membrane and otolith (Ot) of the saccule. The otolithic membrane is composed of the upper gelatinous layer (G) and the lower subcupular meshwork (SM). Note electron-dense cytoplasmic extrusions (ECE) on the surface of the sensory epithelium.
- b : Higher magnification of the apical portion of the sensory epithelium. Vesicular dilations at the distal end of microvilli (arrows) are evident. Some ribosome-like particles (arrowheads) are seen in an electron-dense cytoplasmic extrusion (ECE). SH, sensory hairs.

Fig. 4.

- a : Electron-dense cytoplasmic extrusion (ECE) occurring near the apical junctional complex (arrow) of a sensory cell. The extrusion contains ribosome-like particles (arrowheads).
- b : Electron-dense cytoplasmic extrusion (ECE) containing vesicles (arrow) and membranous structures (arrowhead).
- c : Electron-dense cytoplasmic extrusion (ECE) collapsing with an opening (arrow) in the subcupular zone.

Fig. 5. Scanning electron micrographs of the saccular macula.

- a : Many cytoplasmic extrusions among the sensory hair bundles.
- b : High magnification of round cytoplasmic extrusions.
- c : High magnification of a rough-surfaced cytoplasmic extrusion.
- d : High magnification of a cytoplasmic extrusion having a few holes (arrows).

Fig. 6. Transmission electron micrograph of cells of the transitional epithelium showing well-developed rough endoplasmic reticulum (RER) and Golgi apparatus (GA), mitochondria (M) scattered in the cytoplasm and numerous microvilli (Mv) on the cell surface. Relatively wide intercellular spaces (ICS) are seen between the columnar cells.

Fig. 7.

- a, b: Transmission electron micrographs showing Golgi sacs (G) and Golgi vesicles (GV) in a transitional epithelium cell. M, mitochondria; N, nucleus; RER, rough endoplasmic reticulum.
- c: Transmission electron micrograph showing well developed rough endoplasmic reticulum (RER) in a transitional epithelium cell. M, mitochondria; N, nucleus.

Fig. 8.

- a: Transmission electron micrograph of the basal part of the transitional epithelium cells. Arrow, desmosome; Fr, free ribosome; ICS, intercellular space; M, mitochondria; RER, rough endoplasmic reticulum; White arrow, basement membrane.
- b: Transmission electron micrograph of a mitochondria unusually shaped with a deep invagination (asterisk) in a transitional epithelium cell.

Fig. 9. Transmission electron micrographs showing the apical part of transitional epithelium cells.

- a: Vesicular dilation at the distal end of microvilli. A vesicle having a few membranous structures appears to lie free in the lumen (arrow).
- b: Vesicular dilation at the distal end of microvilli.
- c: Large vesicle containing many minute vesicles.
- d: Vesicles fusing together in the lumen (arrow).

Fig. 10. Scanning electron micrographs showing secretory spherules and microvilli on the transitional epithelium (perimacular region).

- a, b: Secretory spherules on the middle part of the perimacular region. Some spherules (arrows) seem to be formed by fusion of several small ones. Numerous microvilli are seen around the secretory spherules.
- c, d: Secretory spherules on the anterior and posterior portions of the perimacular region. The spherules are almost round in shape. Membranous structures covering the cell surfaces can be observed in (d), caused by fixation with a fixative containing CPC.

Fig. 11. Scanning electron micrographs of the surface of the transitional epithelium showing distribution of secretory spherules. Microvilli (Mv) and secretory spherules gradually decrease in number from the saccular macula (SM) to the perimacular region (a-f).

Fig. 12. Transmission electron micrograph of the squamous epithelium.

- a: Very complexed indentations within the cytoplasm and between the cells, occupying almost the entire cytoplasmic area.
- b: Some blebby structures in groups attached to the epithelial surface.
- c: Blebs containing vesicles and slightly electron-dense material (arrow).

Fig. 13. Scanning electron micrographs of blebs on the squamous epithelium.

- a: Oval-shaped bleb.
- b: Round-shaped bleb.
- c: Amorphous blebs.
- d: Linkage of several blebs.

- Fig. 14.** Scanning electron micrographs of the surface of the squamous epithelium.
- a : The texture of almost the entire area of the squamous epithelium. Numerous blebs are seen on the left half of the picture.
 - b : Left half of (a). Many blebs are seen on the surface, where microvilli are not so obvious.
 - c : Right half of (a). The cell boundaries are clearly delineated by microvilli.

- Fig. 15.** Transmission electron micrographs of the squamous epithelium.
- a : Well developed rough endoplasmic reticulum.
 - b : Many vesicles and electron-dense bodies (arrows) associated with Golgi apparatus.

- Fig. 16.** Scanning electron micrographs of the gelatinous layer, cut vertically and viewed from the lateral side.
- a : Survey view of the otolithic membrane (OM) and the macula (M). OSG, outer surface of the gelatinous layer.
 - b : Partially removed otolithic membrane (OM) and the underlying macula (M). SH, sensory hairs.
 - c : Sensory hairs protruding into the otolithic membrane, SH, sensory hairs ; OM, otolithic membrane.
 - d : Honeycomb-like structure of otolithic membrane. SH, sensory hair.

- Fig. 17.**
- a : Light micrograph of the crista ampullaris anterior (CAA) of the ampulla anterior (AA).
 - b : Transmission electron micrograph of the apical part of the crista ampullaris anterior. The sensory epithelium (SE) is composed of hair cells and more electron-dense supporting cells. Note numerous vesicles above the epithelium.
 - c : Higher magnification of the apical portion of the sensory epithelium. SH, sensory hairs. SC, supporting cell.
 - d : Higher magnification of the apical portion of the sensory epithelium showing an electron-dense cytoplasmic extrusion (ECE) occurring near the junctional complex (arrow).

- Fig. 18.**
- a : Scanning electron micrograph illustrating the surface texture of ampulla anterior (AA). CAA, crista ampullaris anterior.
 - b : Many cytoplasmic extrusions on and above the sensory hair bundles of the crista.
 - c : One end of the crista ampullaris anterior. Note a dense cluster of sensory hairs (SH) and squamous epithelium adjacent to the sensory epithelium.

- Fig. 19.** Transmission electron micrographs of the epithelium adjacent to the sensory epithelium of the crista ampullaris anterior.
- a : Cells adjacent to the sensory epithelium (SE). Note very few microvilli on the cell surface.
 - b : Flattened cells having infoldings in the cytoplasm. A capillary (C) is seen beneath the cells.
 - c : Large vacuole (arrow) and complexed indentations are noted in the cytoplasm.

- Fig. 20.** Scanning electron micrographs showing sensory and adjacent epithelia of the crista ampullaris anterior.
- a : Cupula (C) above the sensory epithelium. The sensory area is clearly delineated from the non-sensory area. SH, sensory hairs.
 - b : No secretory spherules and a few microvilli were seen on the epithelium adjacent to the sensory area.

Fig. 21.

- a : Light micrograph of the ampulla anterior (AA) of tilapia. Note mitochondria-rich cells (arrows) located near the crista ampullaris anterior (CAA).
- b : Light micrograph of the sacculus (S) and the utriculus (U) of tilapia. Mitochondria-rich cells (arrows) are seen in the lateral walls of the sacculus and the utriculus. A higher magnification of the encased area is shown in Fig. 22b.
- c : Light micrograph of the middle portion of the sacculus (S) of goldfish. Mitochondria-rich cells (arrows) are seen in the transitional epithelium of the macula (M).
- d : Light micrograph of a section through the right and left sacculi (S) and the transverse canal (TC) of goldfish. Note mitochondria-rich cells (arrows) in the basal wall of the transverse canal.

Fig. 22.

- a : Transmission electron micrograph (TEM) of mitochondria-rich cells in the ampulla anterior of tilapia. Note large vacuoles (arrow) and the nucleus (N) invaginated in the cytoplasm. Medullated nerve fibers (NF) are seen in the connective tissue. EC, epithelial cell.
- b : TEM of two mitochondria-rich cells in the middle portion of the utriculus of tilapia. The cell boundary is not evident. N, nucleus.
- c : TEM of mitochondria-rich cells in the middle portion of the utriculus of tilapia. Desmosomes (arrows) are apparent at the apico-lateral and baso-lateral regions. Dilated canaliculi of the smooth endoplasmic reticulum are seen near the cell boundary.

Fig. 23.

- a : Transmission electron micrograph (TEM) of a mitochondria-rich cell in the middle part of the utriculus of tilapia. Numerous glycogen granules are seen in the cytoplasm.
- b : TEM of a mitochondria-rich cell in the middle part of the utriculus of tilapia. The fenestrated endothelium (arrow) of a capillary is seen in the connective tissue.
- c : TEM of mitochondria-rich cells and an intervened epithelial cell in the perimacular epithelium of the sacculus of goldfish. Note many microvilli (Mv) on the epithelial cell surface. N, nucleus.
- d : TEM of mitochondria-rich cells in the transverse canal of goldfish. A few microvilli are seen on the cell surface.

Fig. 24.

- a, b : Scanning electron micrographs of a sagitta otolith from an adult *O. niloticus*, showing the medial (a) and lateral (b) surfaces of the otolith. The sulcus acousticus (SA, outlined in black) is found on the medial surface.
- c : Scanning electron micrograph of the saccular sensory epithelium of an adult tilapia. The macula (outlined in black) resembles the sulcus acousticus in shape (SA).

Fig. 25. Scanning electron micrographs showing different crystalline structures in the intact surfaces of a non-fixed otolith surfaces. Note the relatively smooth side faces of the calcium carbonate crystals in (a) and (e).

- a : Central portion of the sulcus acousticus
- b : Lower portion of the inner surface
- c : Upper portion of the inner surface
- d : Central portion of the outer surface
- e : Marginal portion of the outer surface

Fig. 26. Scanning electron micrographs of otolith surfaces fixed with a cacodylate-buffered fixative for 1–6h. Note crystal grains on the surfaces of individual crystals.

- a : Central portion of the sulcus acousticus
- b : Lower portion of the inner surface
- c : Upper portion of the inner surface
- d : Marginal portion of the upper inner surface
- e : Central portion of the outer surface
- f : Marginal portion of the outer surface

Fig. 27. Scanning electron micrographs showing the otolith surfaces fixed with a cacodylate buffered fixative for 12h–2days. Note membranous structures in and between the crystals and crystal grains on the surface.

- a : Central portion of the sulcus acousticus
- b : Lower portion of the inner surface
- c : Upper portion of the inner surface
- d : Central portion of the outer surface
- e : Marginal portion of the outer surface

Fig. 28. Scanning electron micrographs showing the otolith surfaces fixed with a cacodylate buffered fixative for 4–7days.

Note fibrous structures and crystal grains on individual crystals.

- a : Central portion of the sulcus acousticus
- b : Lower portion of the inner surface
- c : Upper portion of the inner surface
- d : Marginal portion of the upper surface
- e : Central portion of the outer surface
- f : Marginal portion of the outer surface

Fig. 29. Scanning electron micrographs showing otolith surfaces fixed with a phosphate buffered fixative for 4–7days. Note a highly ordered array of hexagonal platelets of hydroxyapatite crystals caused by artifact.

- a : Central part of the sulcus acousticus
- b : Marginal part of the sulcus acousticus

Fig. 30. Scanning electron micrographs of the perimacular region of the sacculus showing daily changes in amount of secretory spherules.

- a : Middle perimacular region at 2:00h.
- b : Anterior perimacular region with numerous secretory spherules at 2:00h.
- c : Middle perimacular region with numerous secretory spherules at 14:00h.
- d : Anterior perimacular region with numerous secretory spherules at 14:00h.

Fig. 31.

- a : Scanning electron micrograph of the sensory epithelium of the sacculus. Cytoplasmic extrusions were seen among hair bundles of sensory cells.
- b : Energy dispersive X-ray spectrum at a point (arrow) in Fig. 31a showing prominent peaks of P ($K\alpha$ 2.02KeV), ($K\alpha$ 2.31KeV) and Ca ($K\alpha$ 3.69KeV). X-ray counts were displayed as a function of X-ray energy in kiloelectronvolts.
- c : Scanning electron micrograph of the transitional epithelium. Round secretory spherules in various sizes were seen on the cell surface.
- d : Prominent calcium (Ca) and sulphur (S) peaks with a low phosphorus peak at a point (arrow) in Fig. 31c.

Fig. 32.

- a : Scanning electron micrograph of the squamous epithelium. Some secretory blebs of various shapes are seen on the cell surface.
- b : EPMA spectrum at a point (arrow) in Fig. 32a. Peaks ($K\alpha$) of S and Ca are prominent.

- c : Scanning electron micrograph of the gelatinous layer of the otolithic membrane.
- d : EPMA spectrum at a point (arrow) in Fig. 32c. Only a sulphur peak ($K\alpha$) is obvious.

Fig. 33.

- a : Scanning electron micrograph of the subcupular meshwork.
- b : EPMA spectrum at a point (arrow) in Fig. 33a. Prominent peaks ($K\alpha$) of phosphorus and calcium and a low peak of sulphur are noted.
- c : Scanning electron micrograph of otolith crystals after fixed for 7 days.
- d : EPMA spectrum at a point (arrow) in Fig. 33c. Peaks ($K\alpha$) of phosphorus and calcium are obvious. Note the vertical scale varied about 16 times as large since a turn at 3.10KeV.

Fig. 34.

- a : Scanning electron micrograph of the sensory epithelium of crista ampullaris anterior. The cytoplasmic extrusions (CE) were seen on and among the hair bundles (SH).
- b : EPMA spectrum at a point (arrow) in Fig. 34a. A peak ($K\alpha$) of sulphur (S) is prominent. Peaks of phosphorus (P) and calcium (Ca) are relatively small.
- c : Scanning electron micrograph of the cupula (C) of crista ampullaris anterior. Hair bundles are intruded into the cupula.
- d : EPMA spectrum at a point (arrow) in Fig. 34c. Peaks ($K\alpha$) of phosphorus (P), sulphur (S) and calcium (Ca) show a similar pattern to those of a cytoplasmic extrusion (b) on the sensory epithelium of the crista.

Fig. 35.

- a : Scanning electron micrograph of the epithelium contiguous to the sensory epithelium of crista ampullaris anterior. Secretory spherules were scarcely seen.
- b : EPMA spectrum at a point (arrow) in Fig. 35a. Peaks ($K\alpha$) of phosphorus (P), sulphur (S) and calcium (Ca) are prominent.

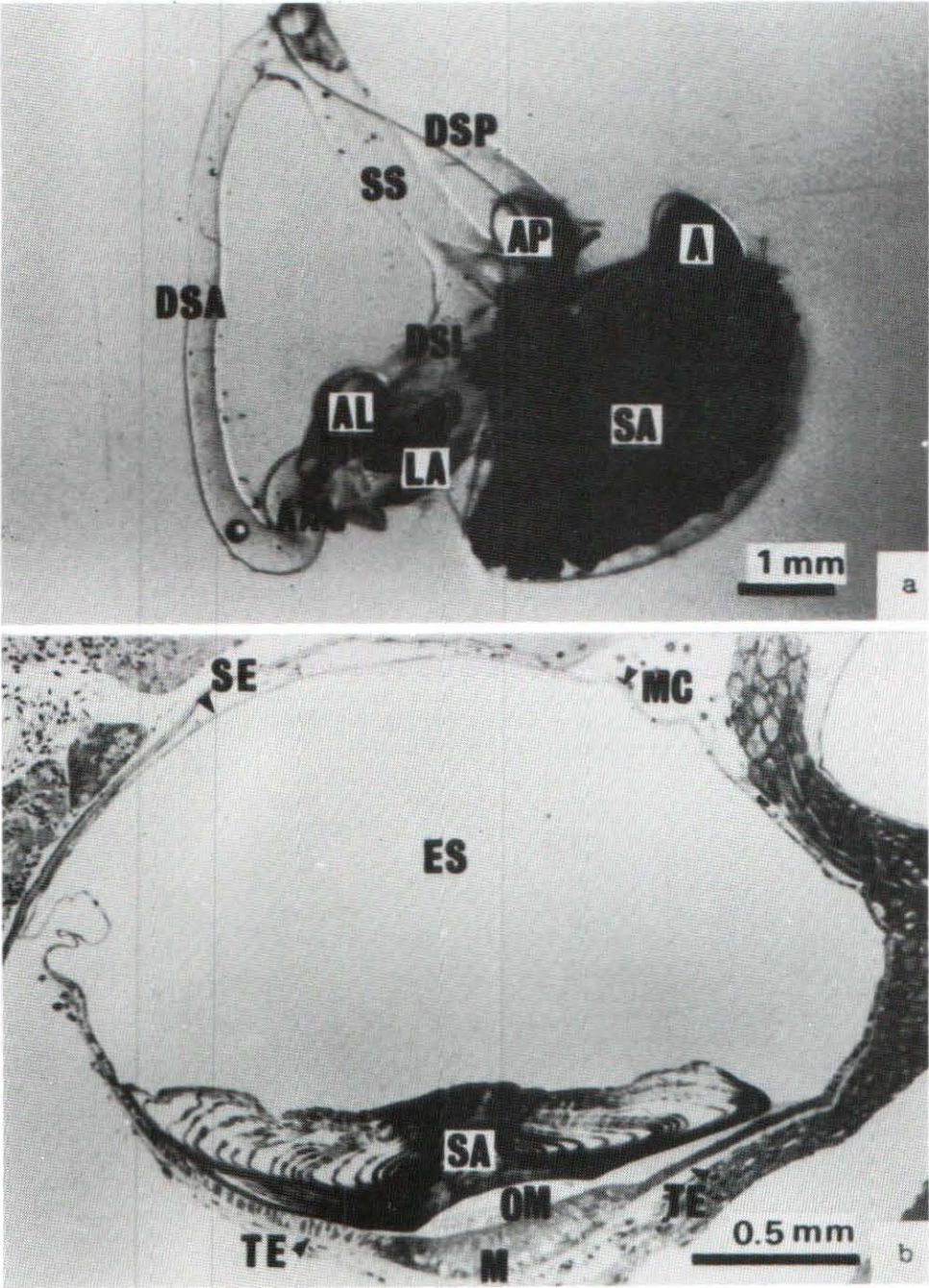


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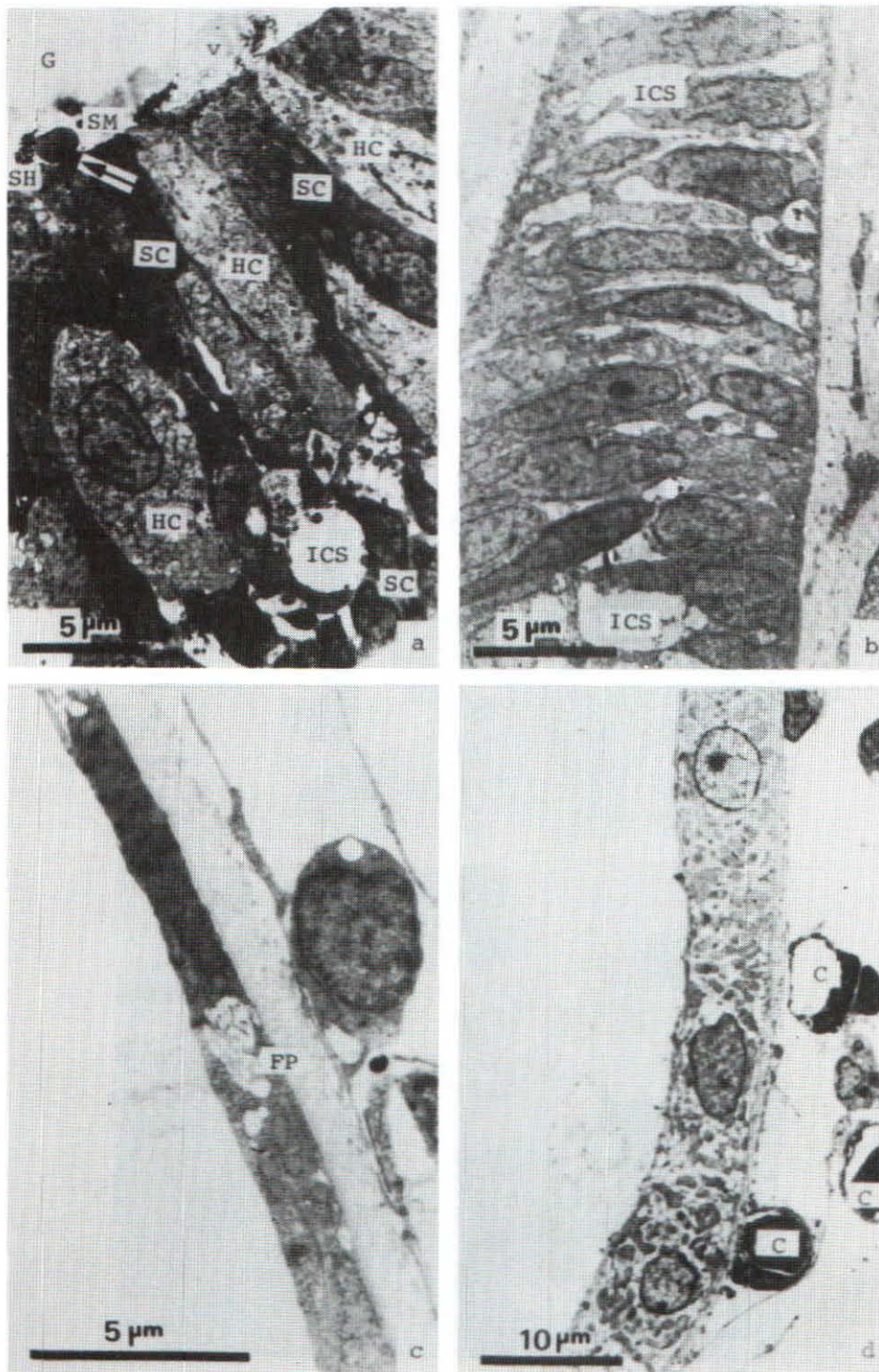


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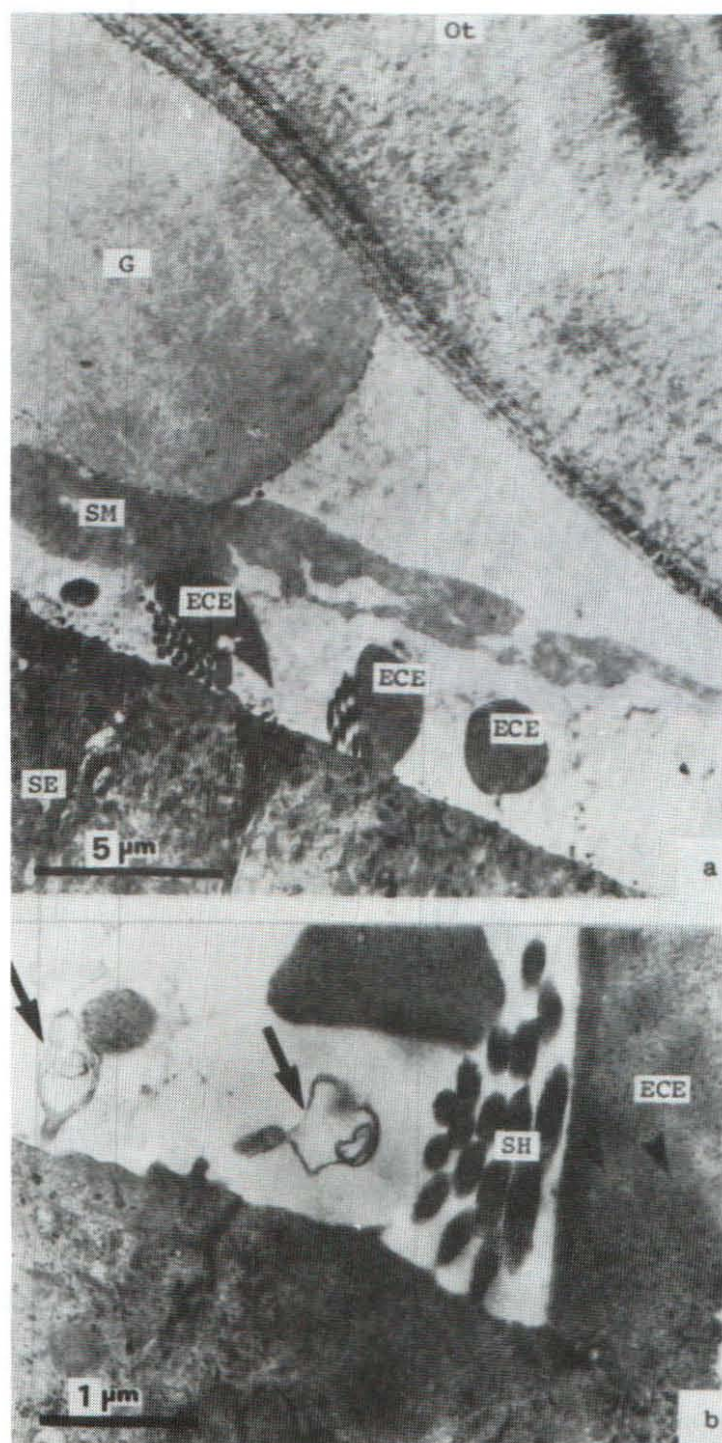


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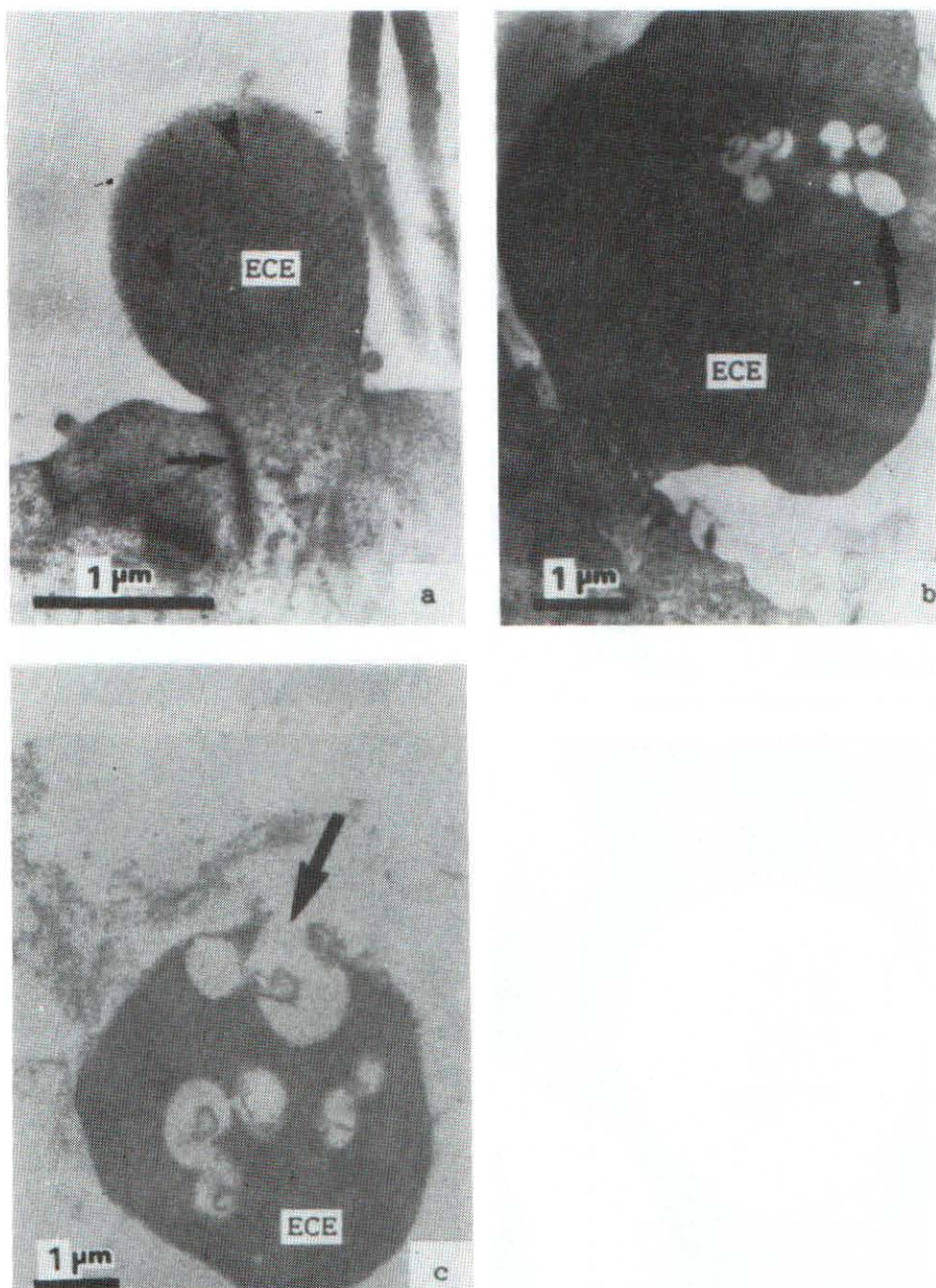


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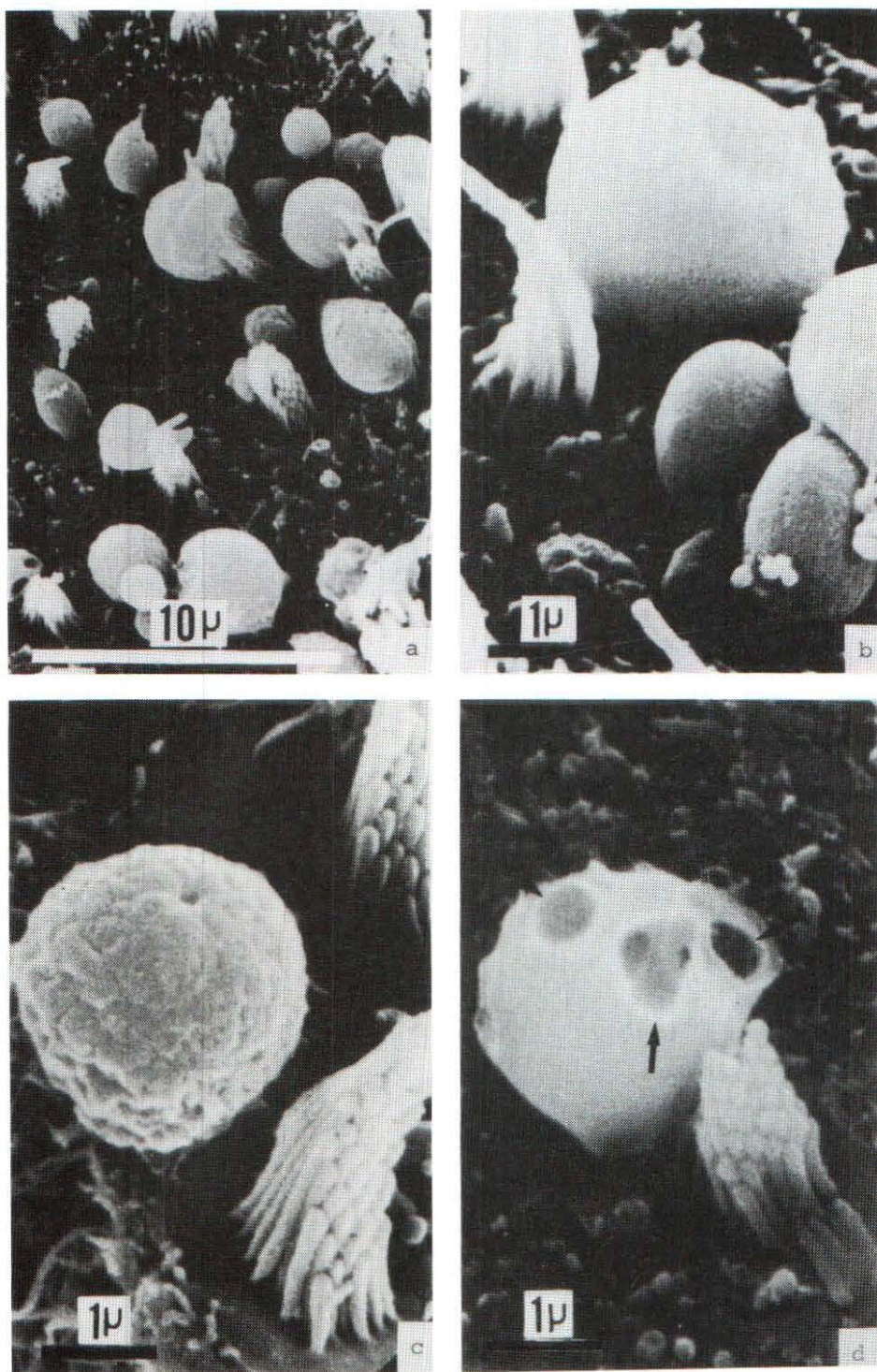


Fig. 5.



Fig. 6.

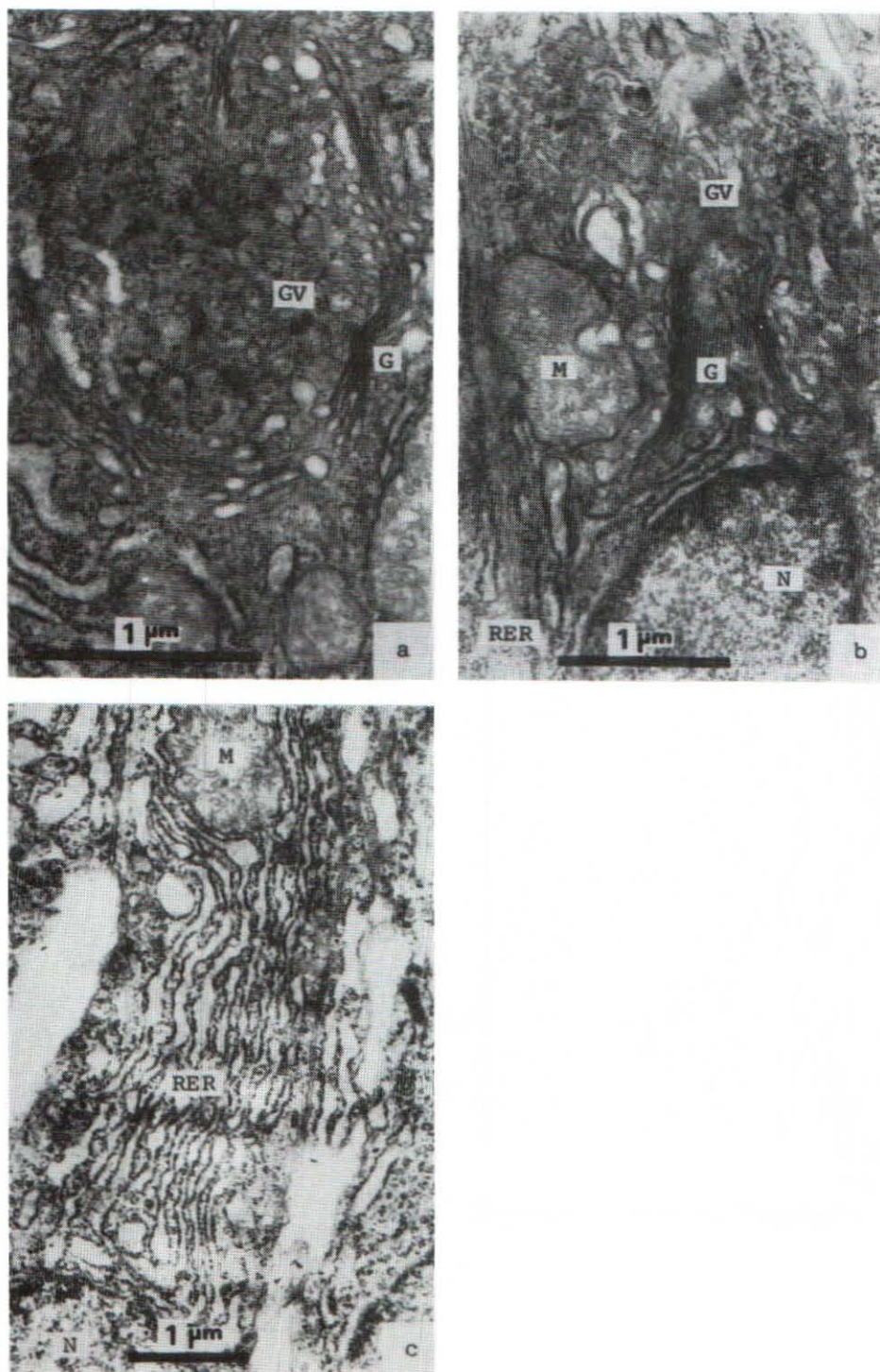


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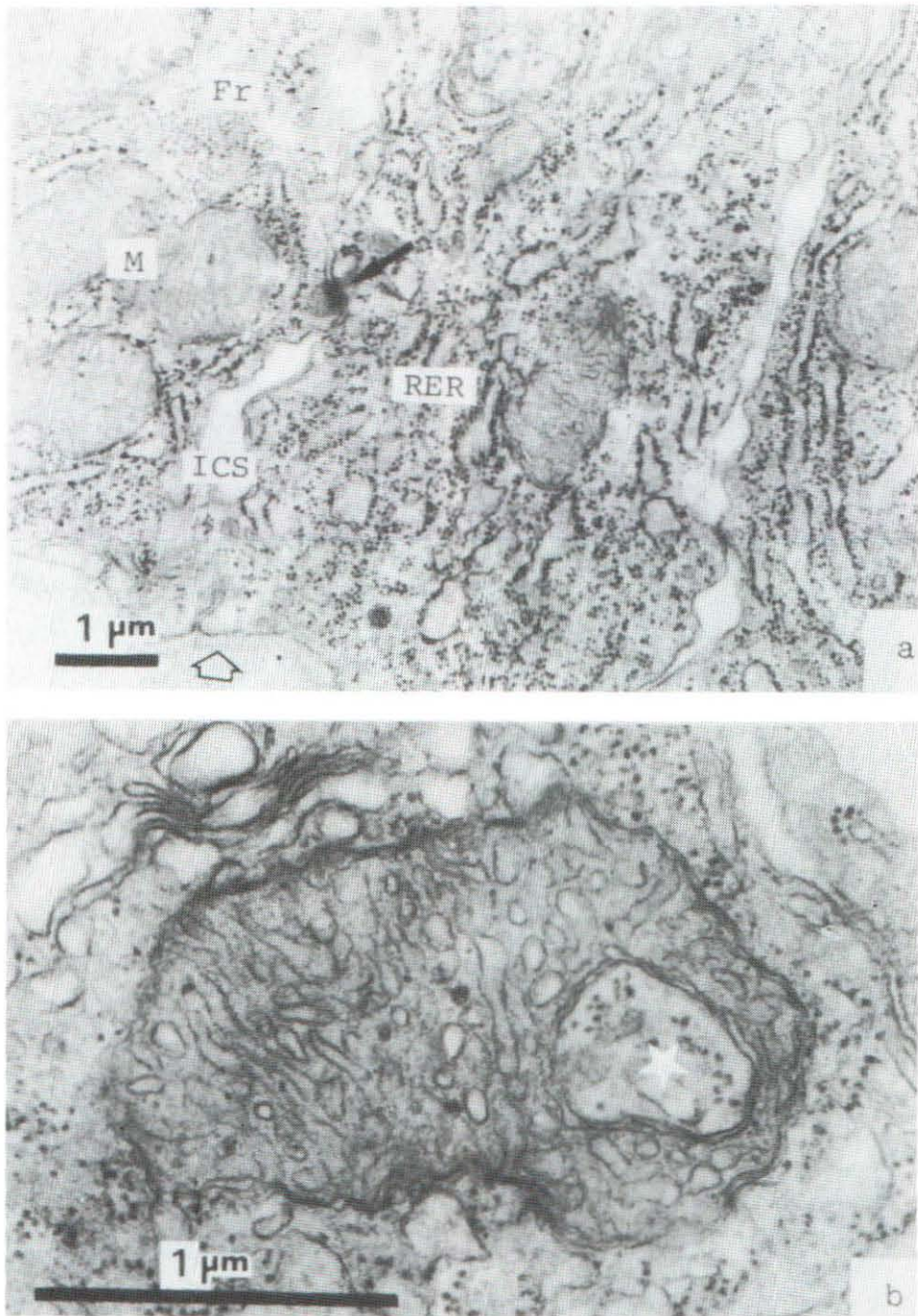


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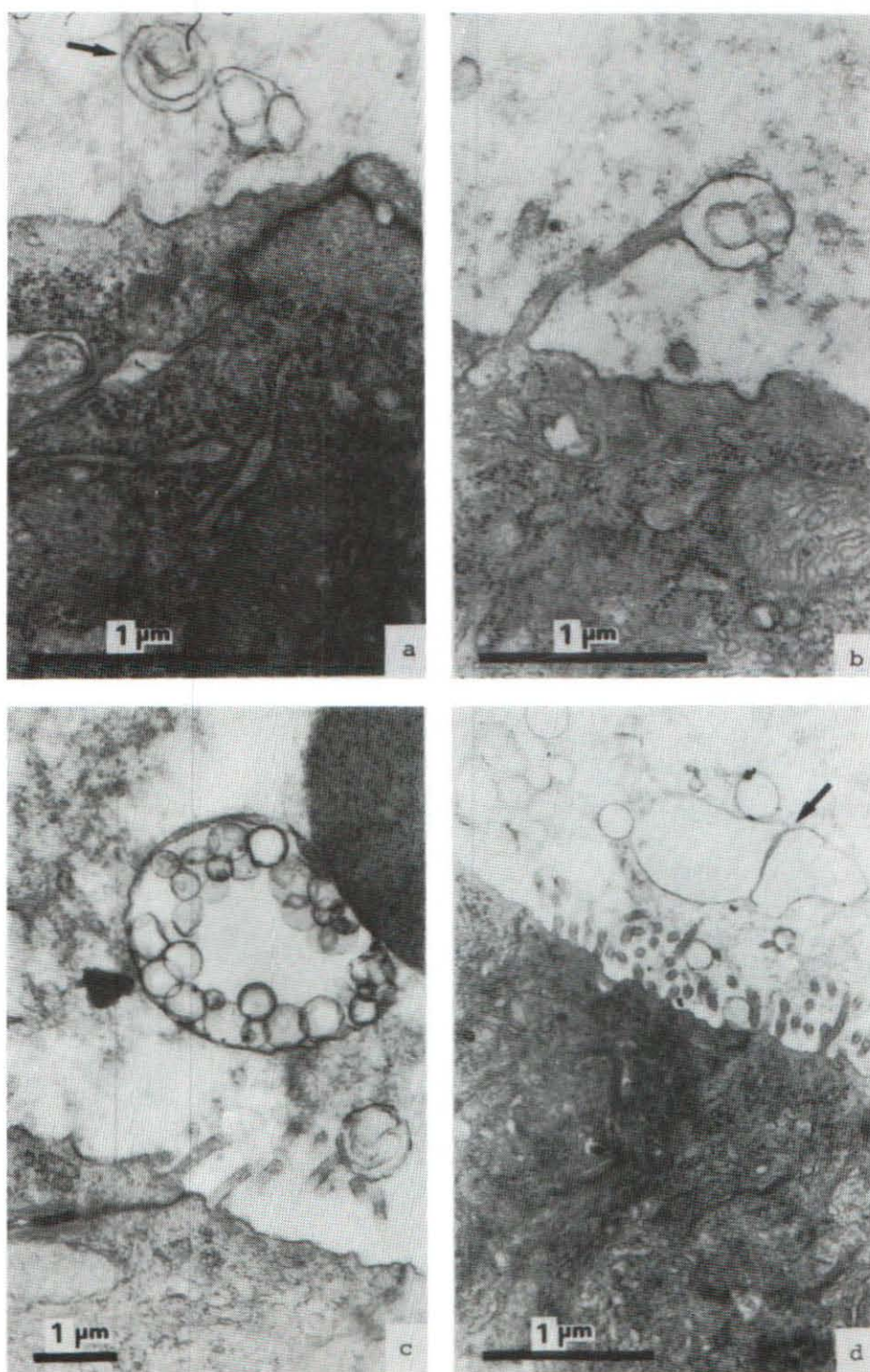


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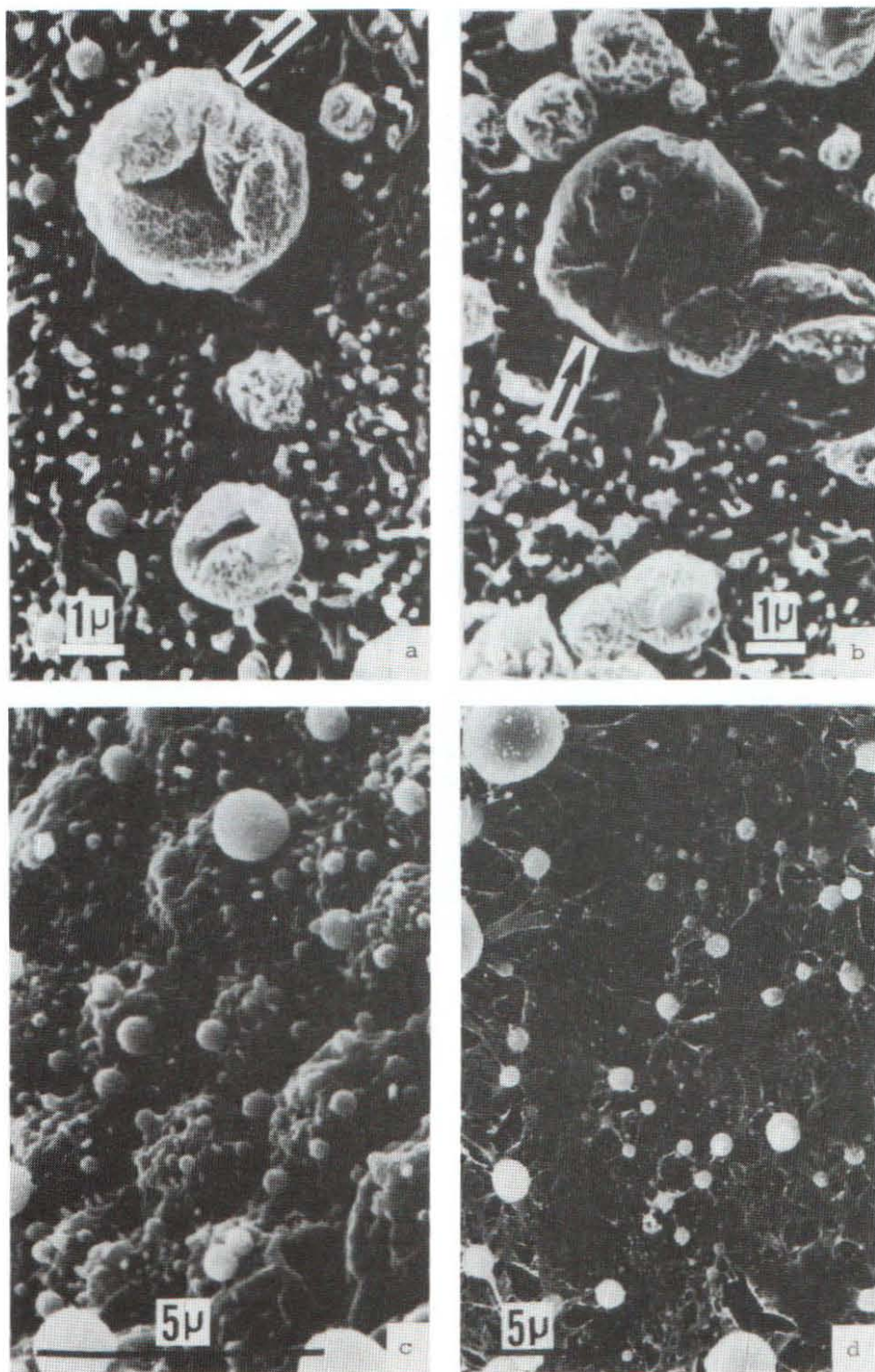


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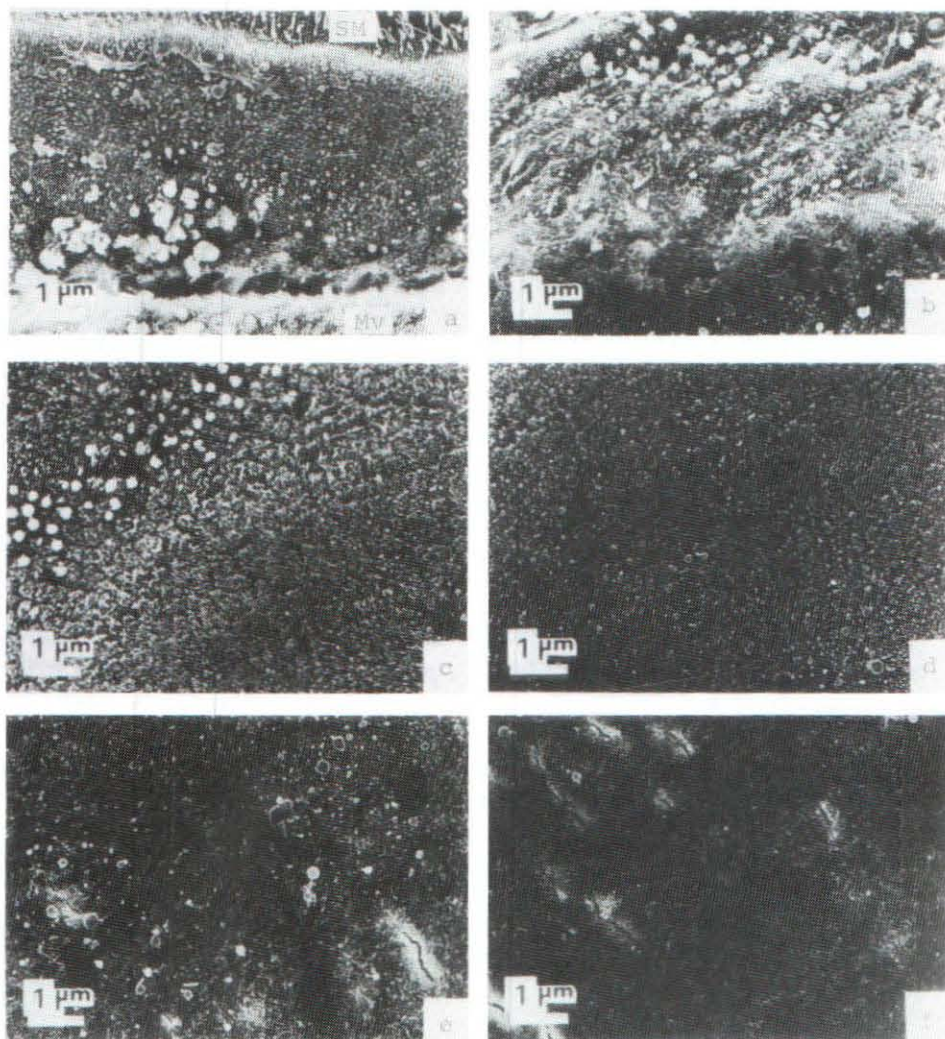


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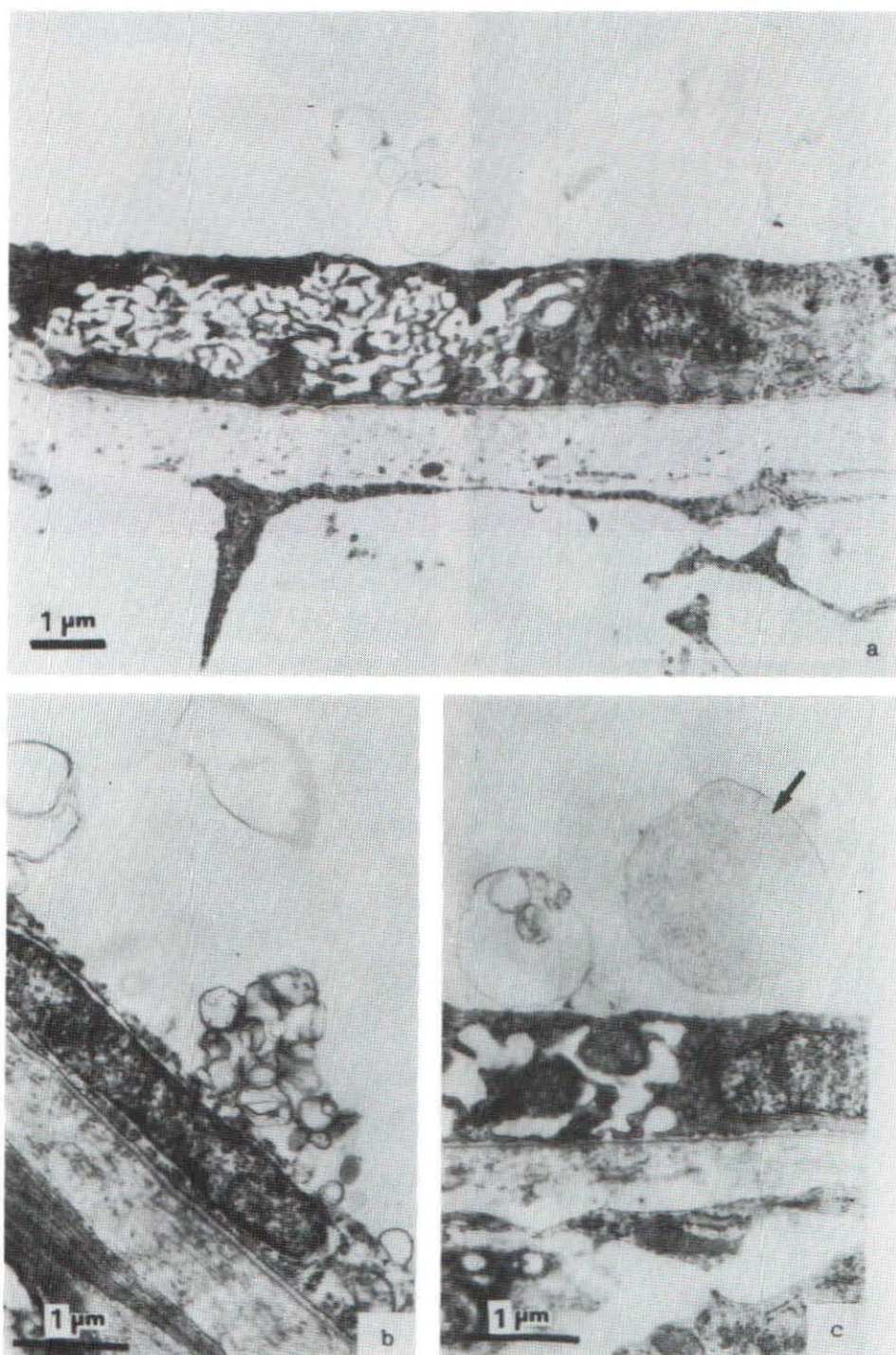


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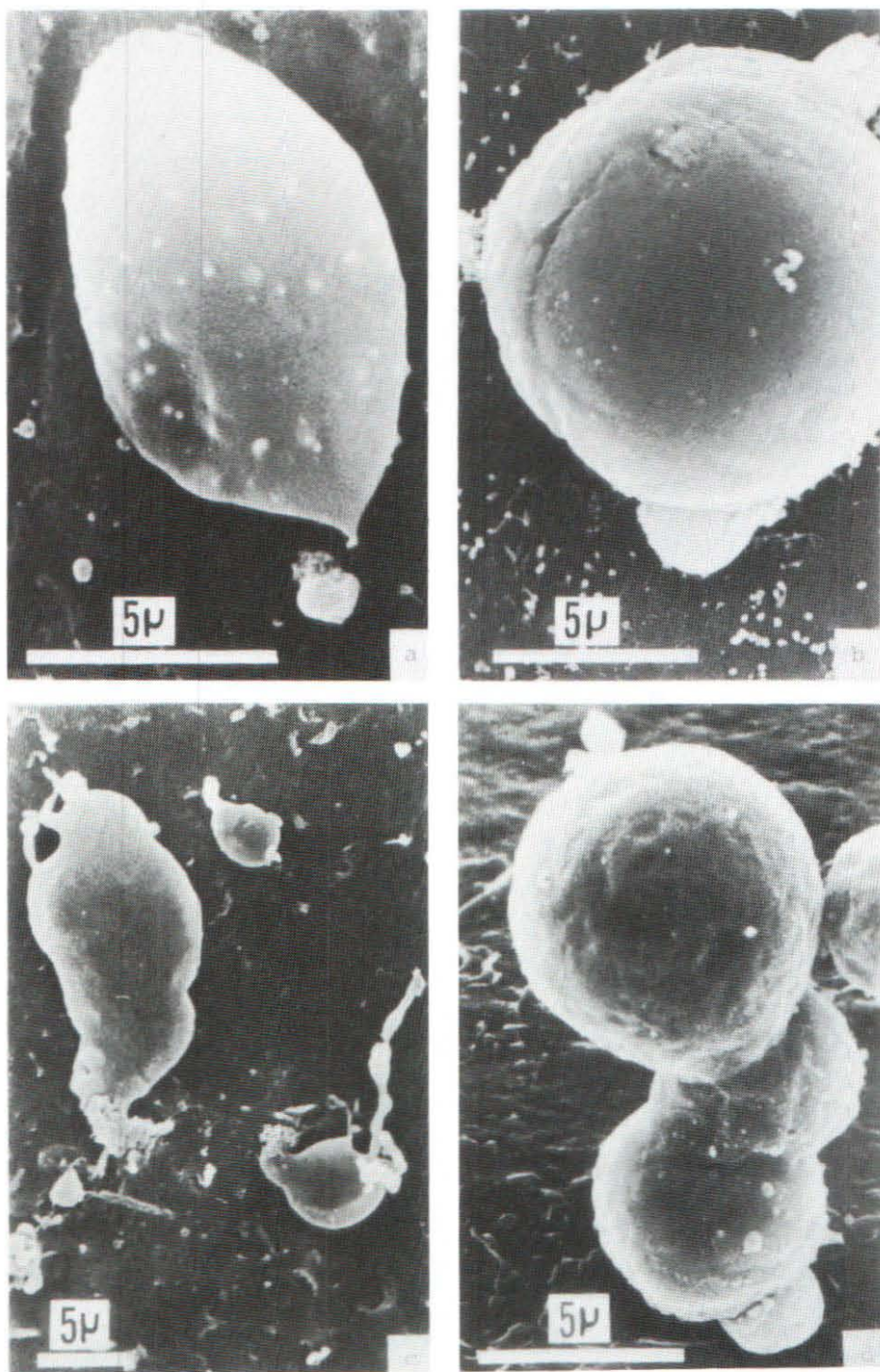


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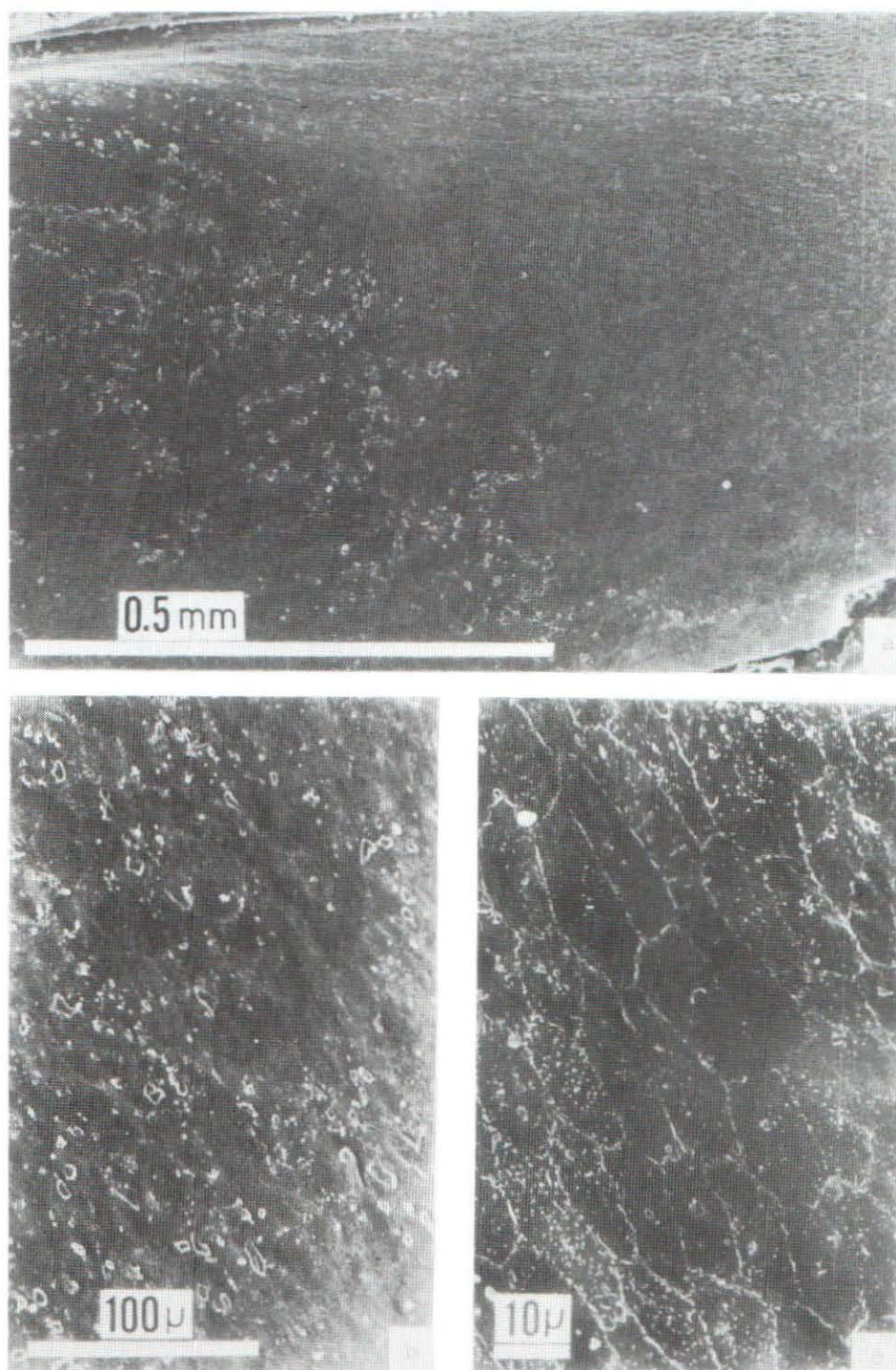


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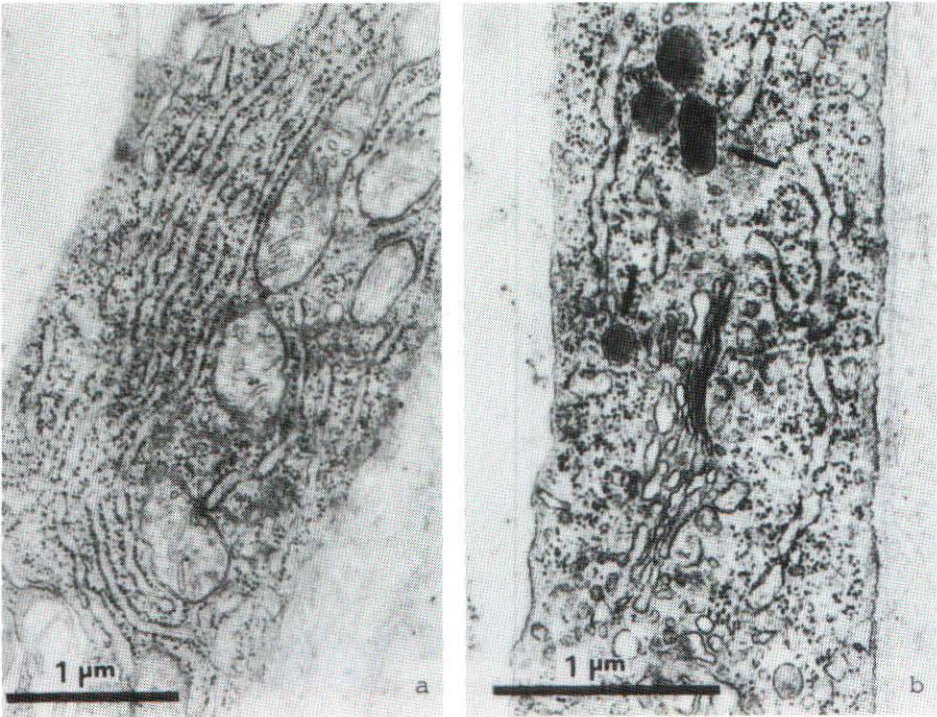


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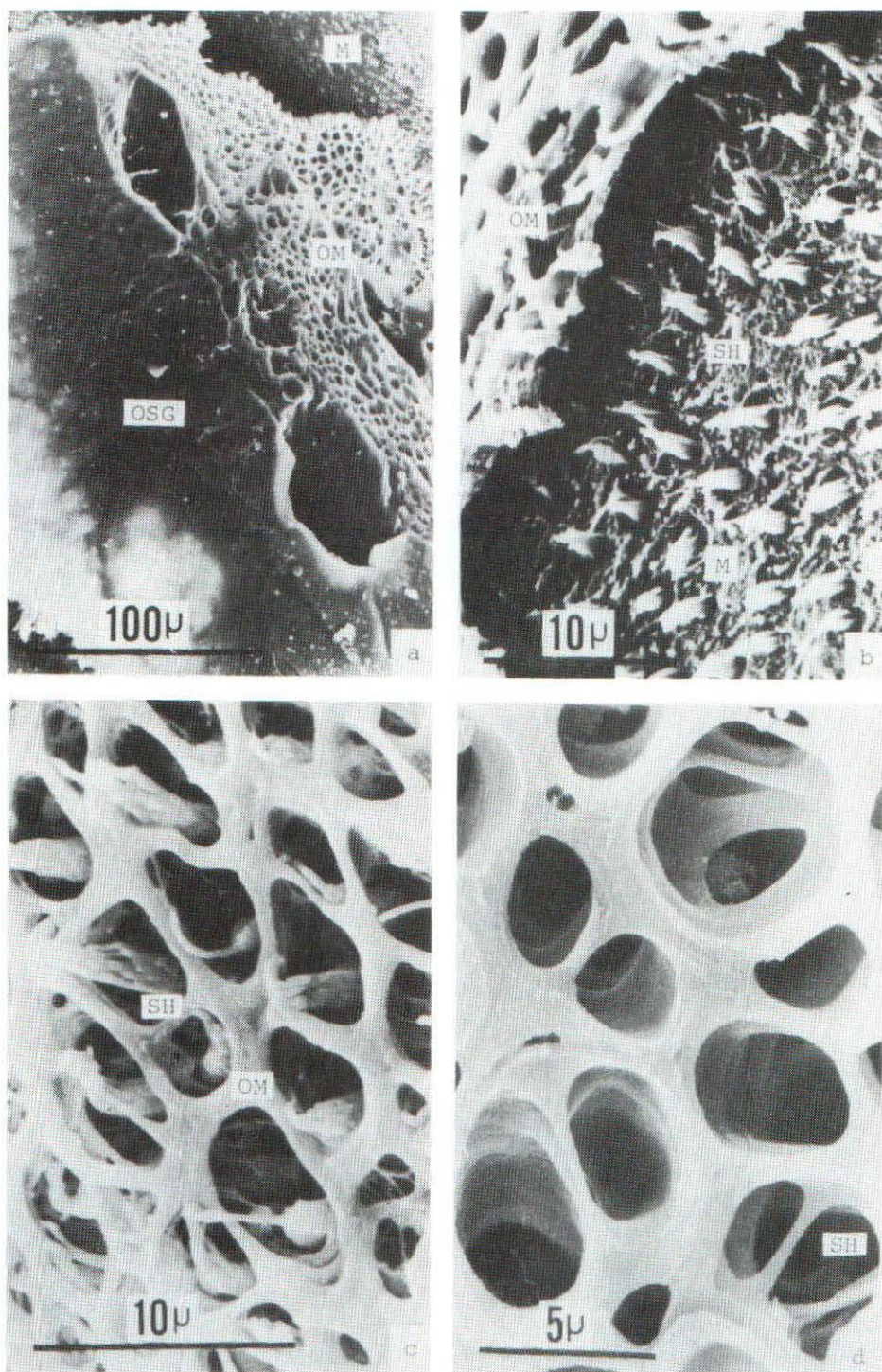


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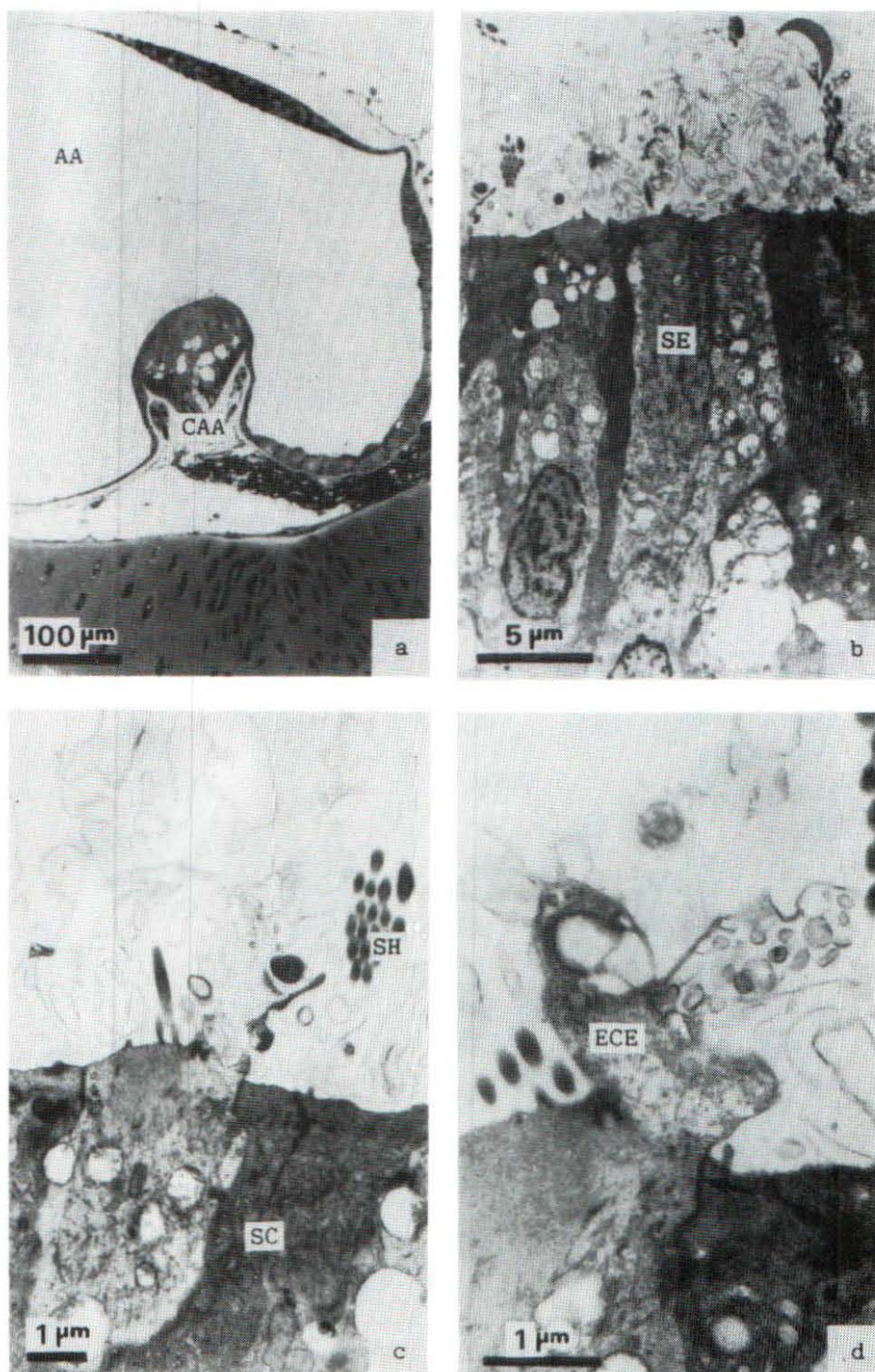


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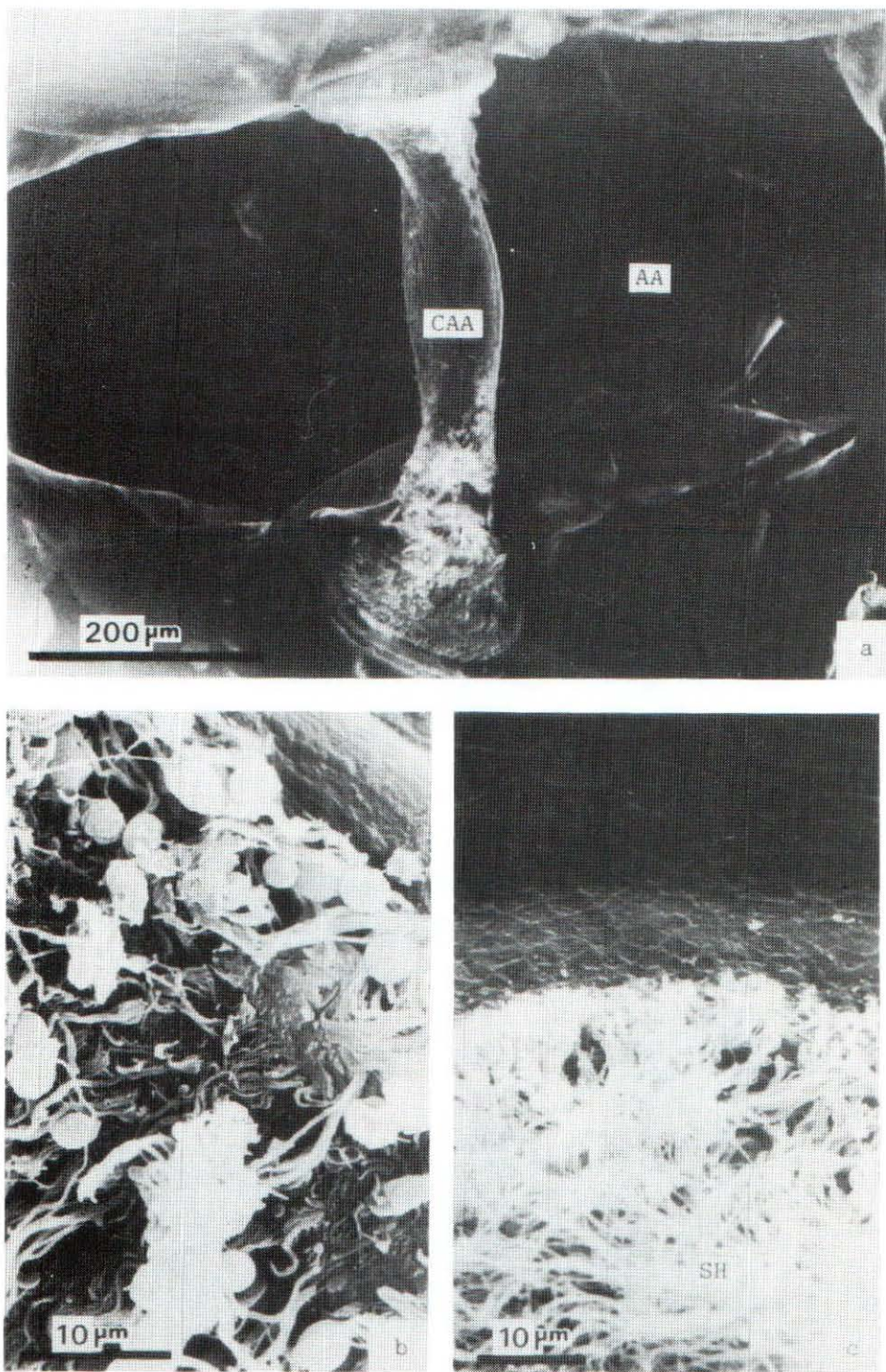


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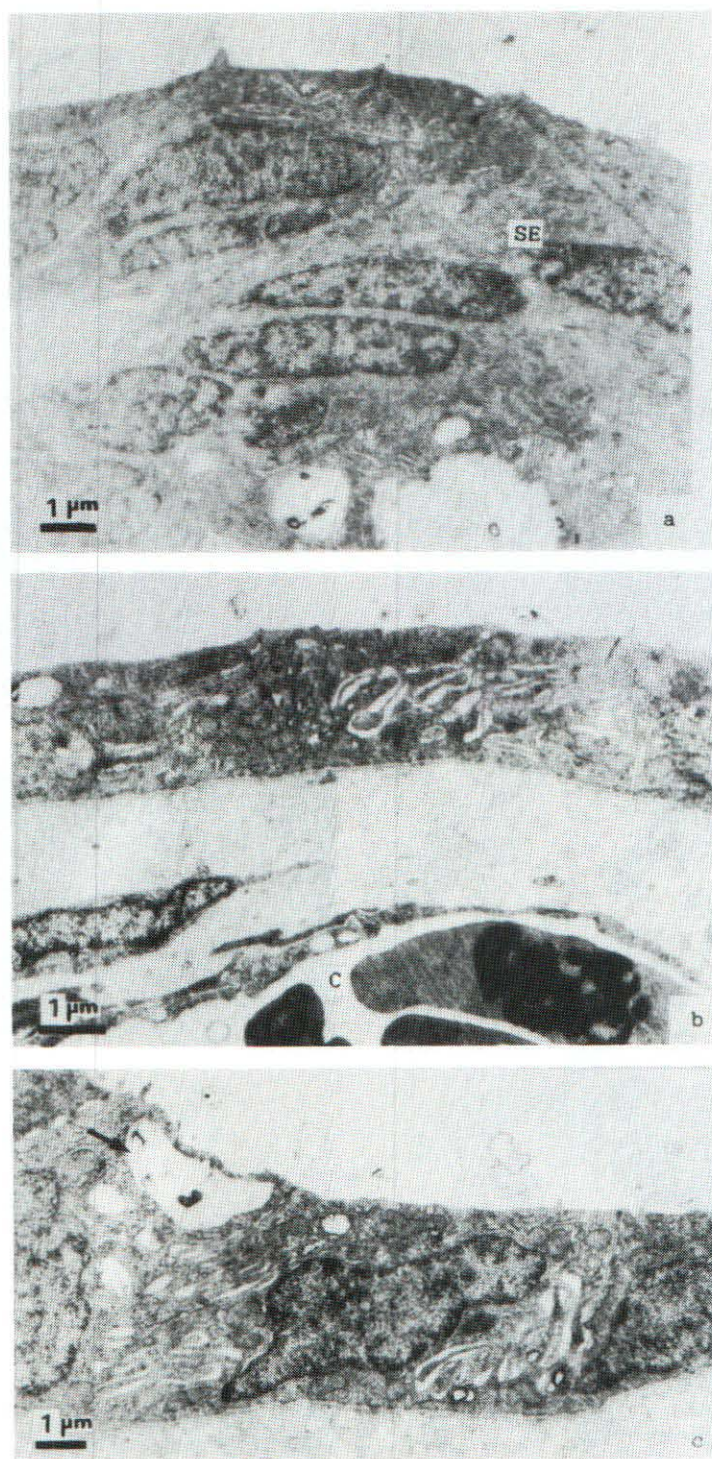


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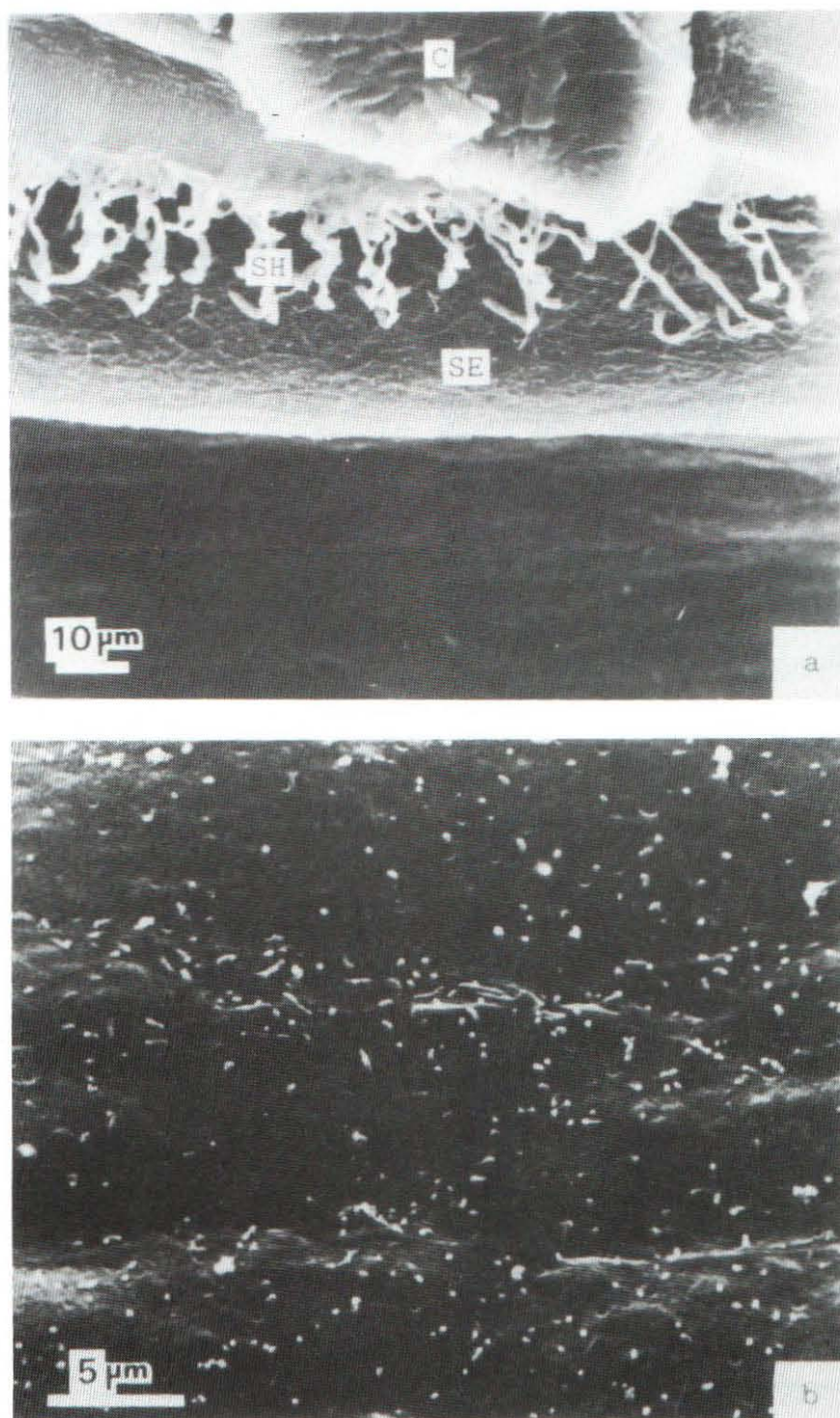


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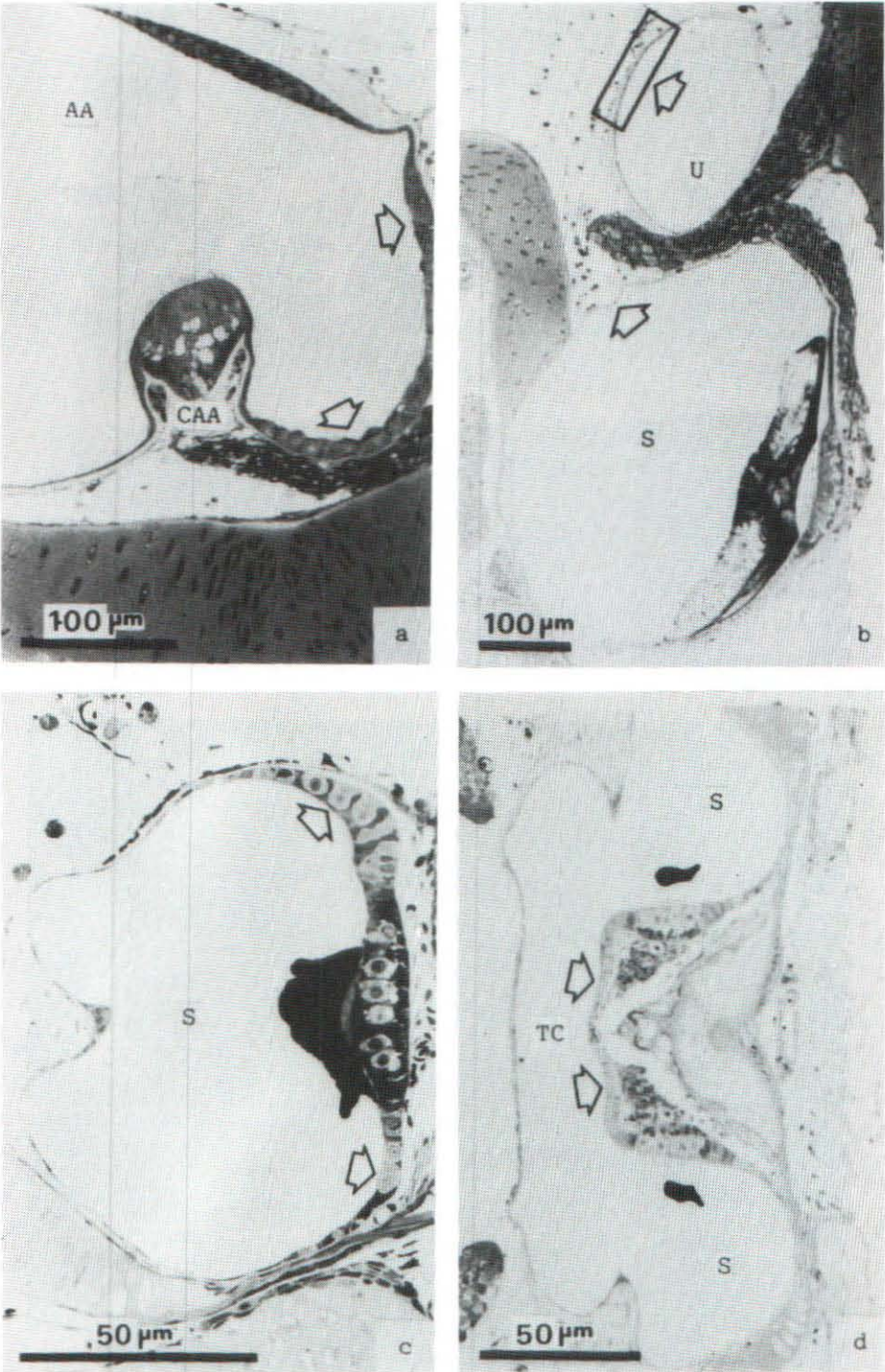


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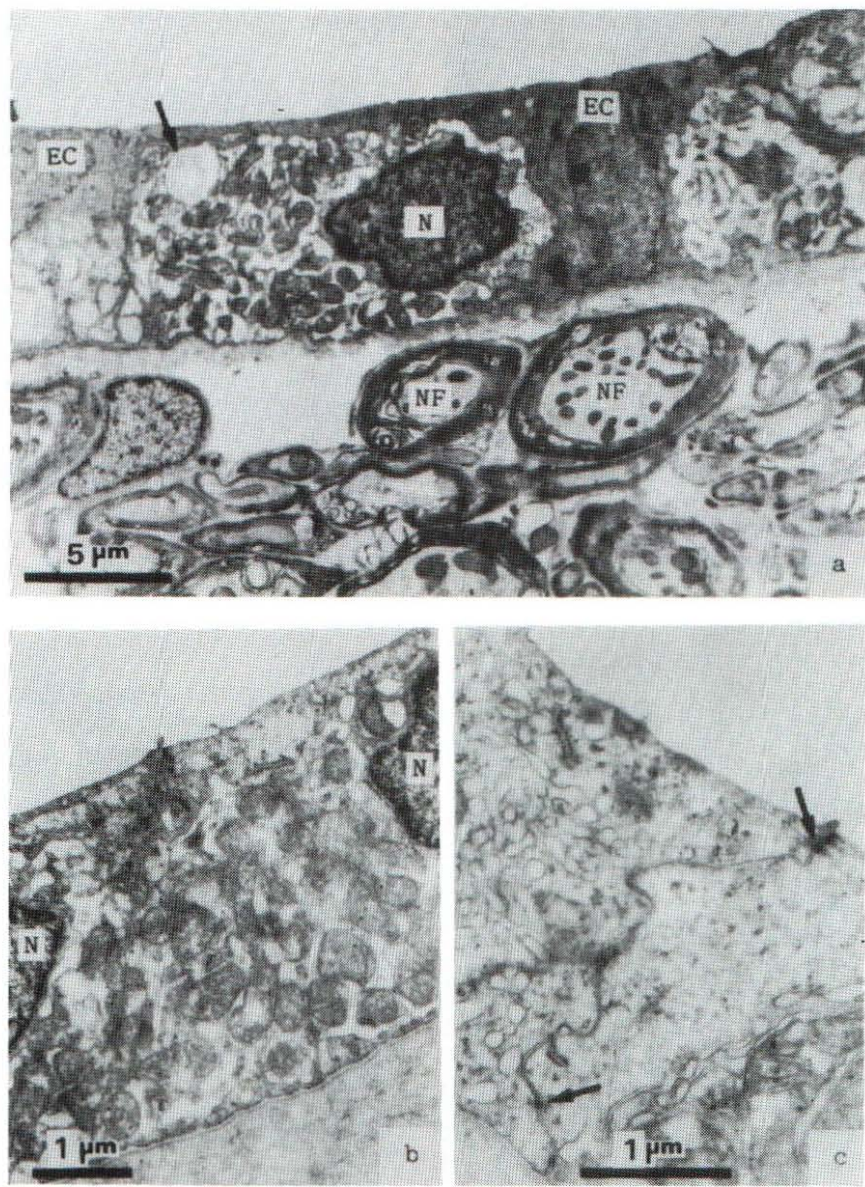


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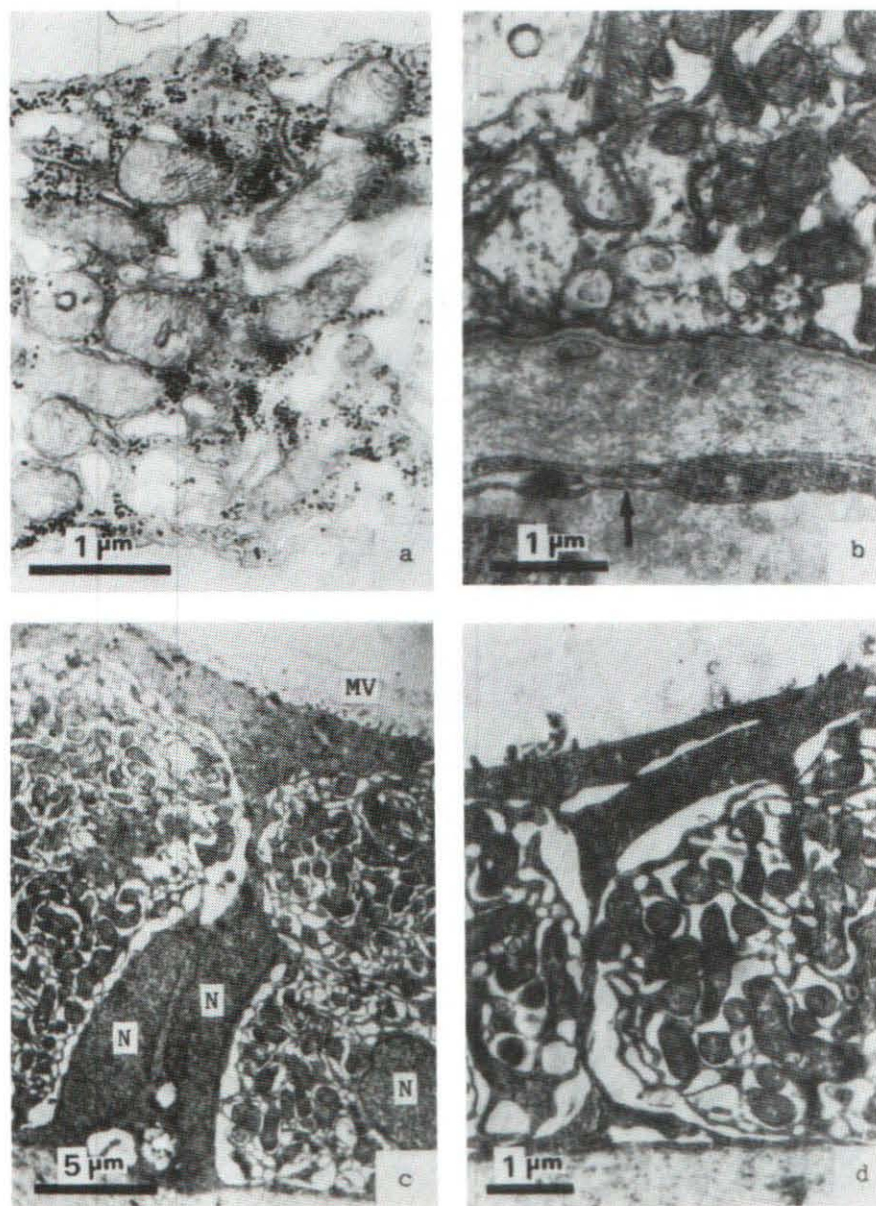


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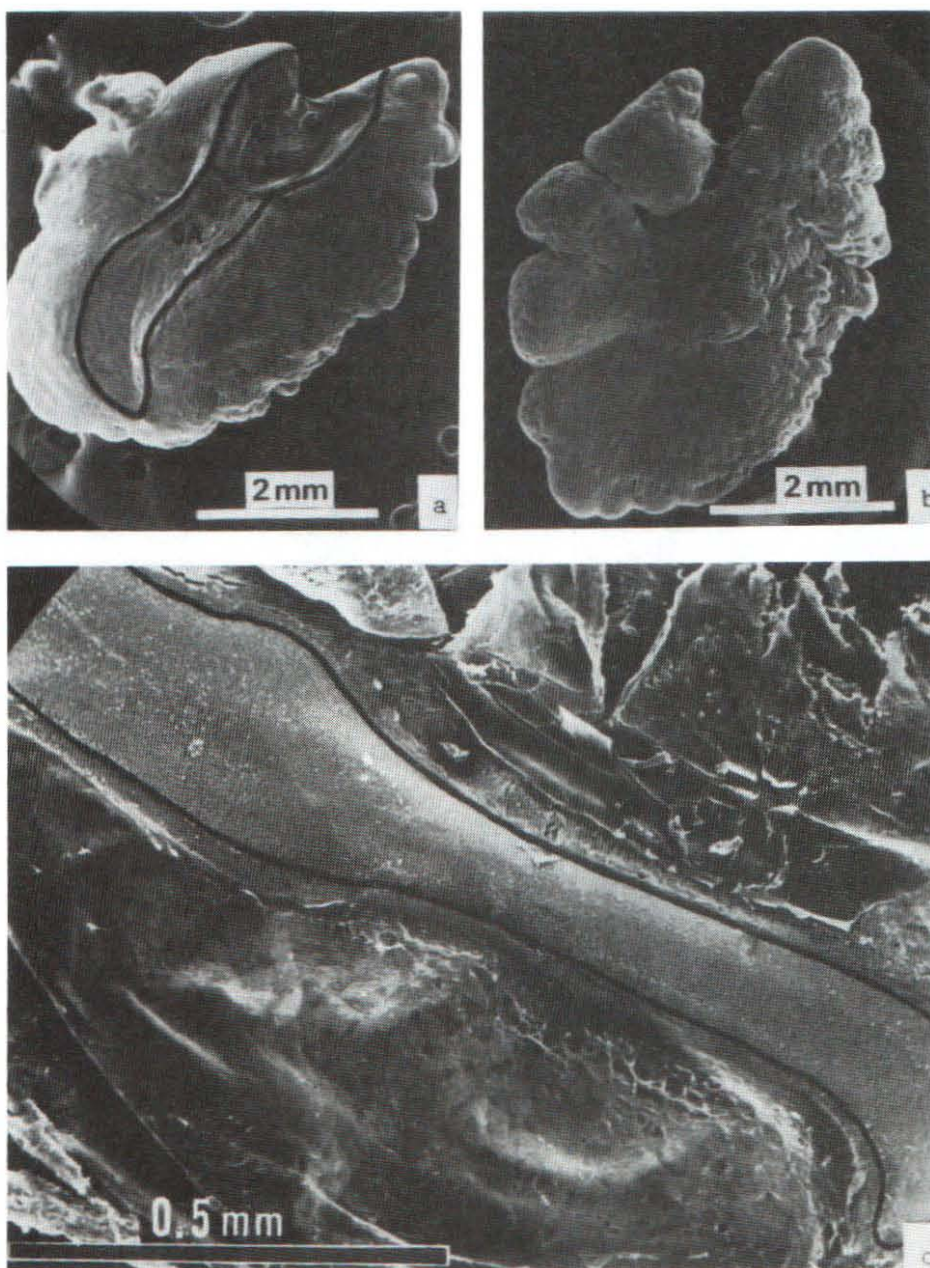


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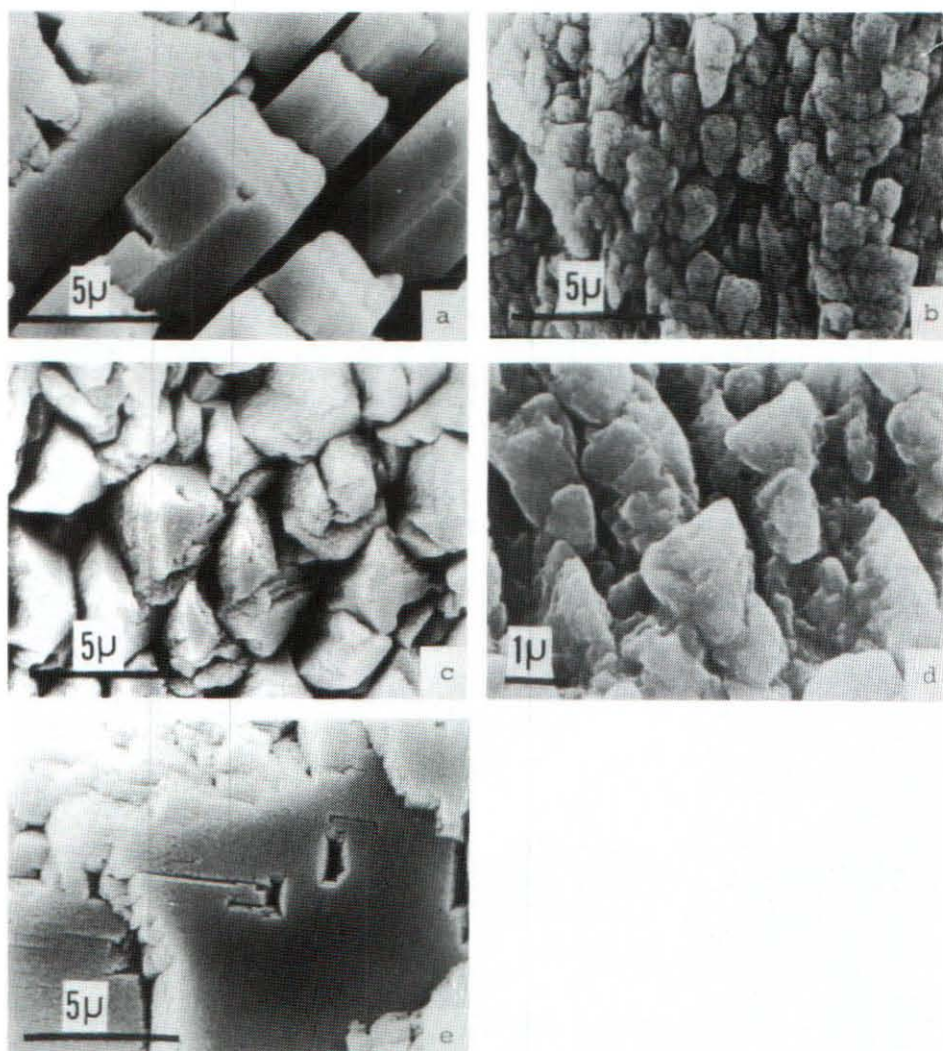


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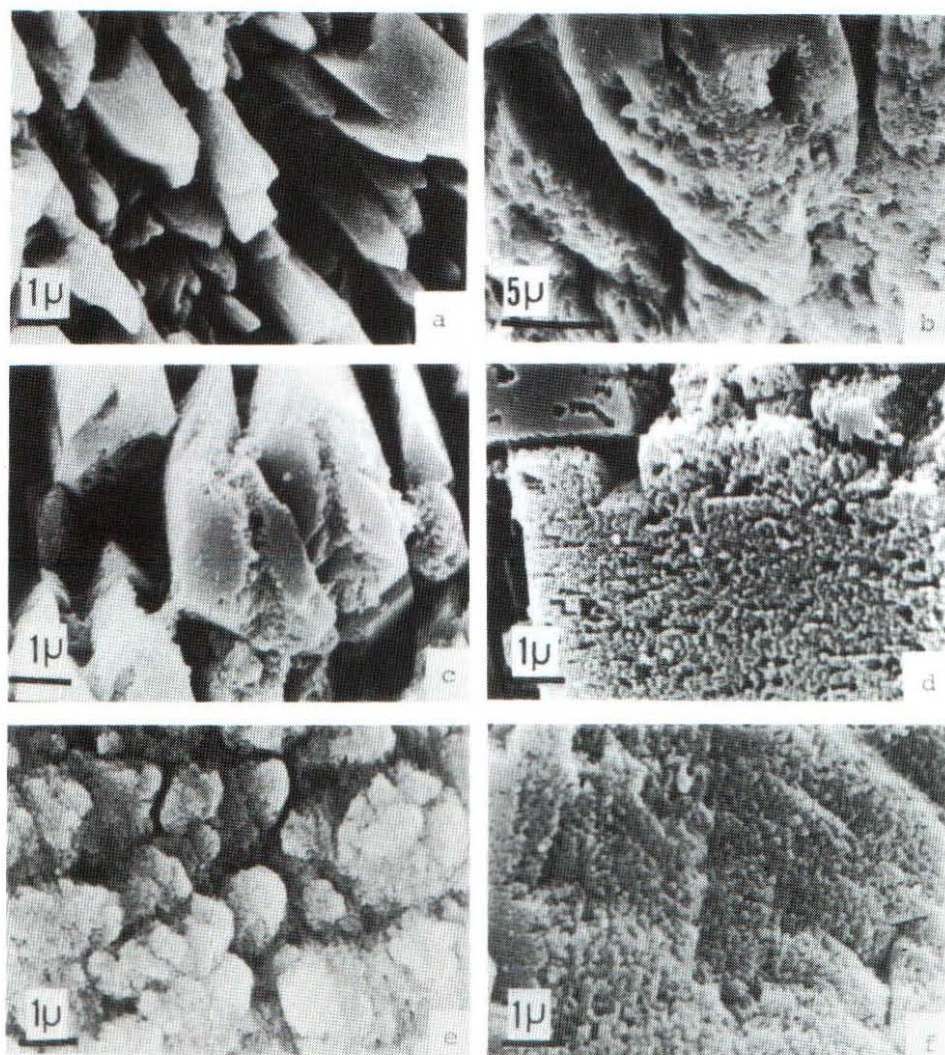


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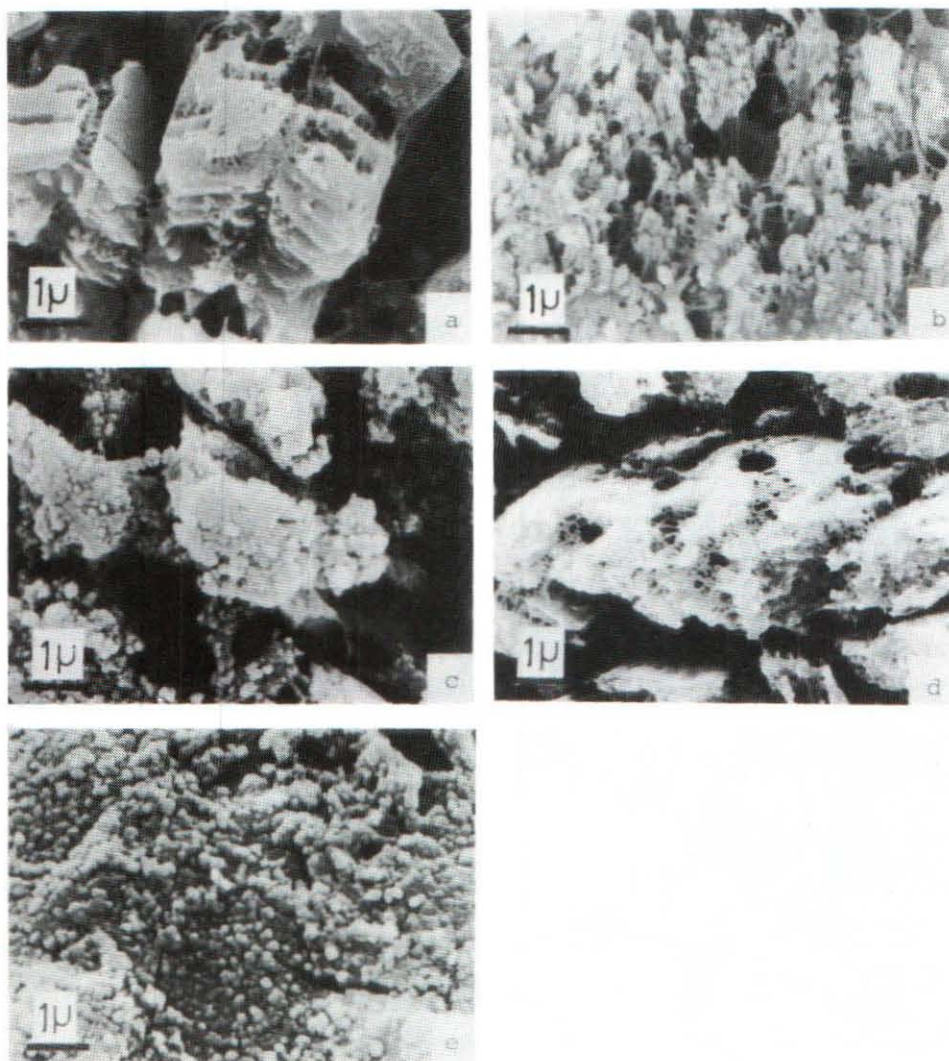


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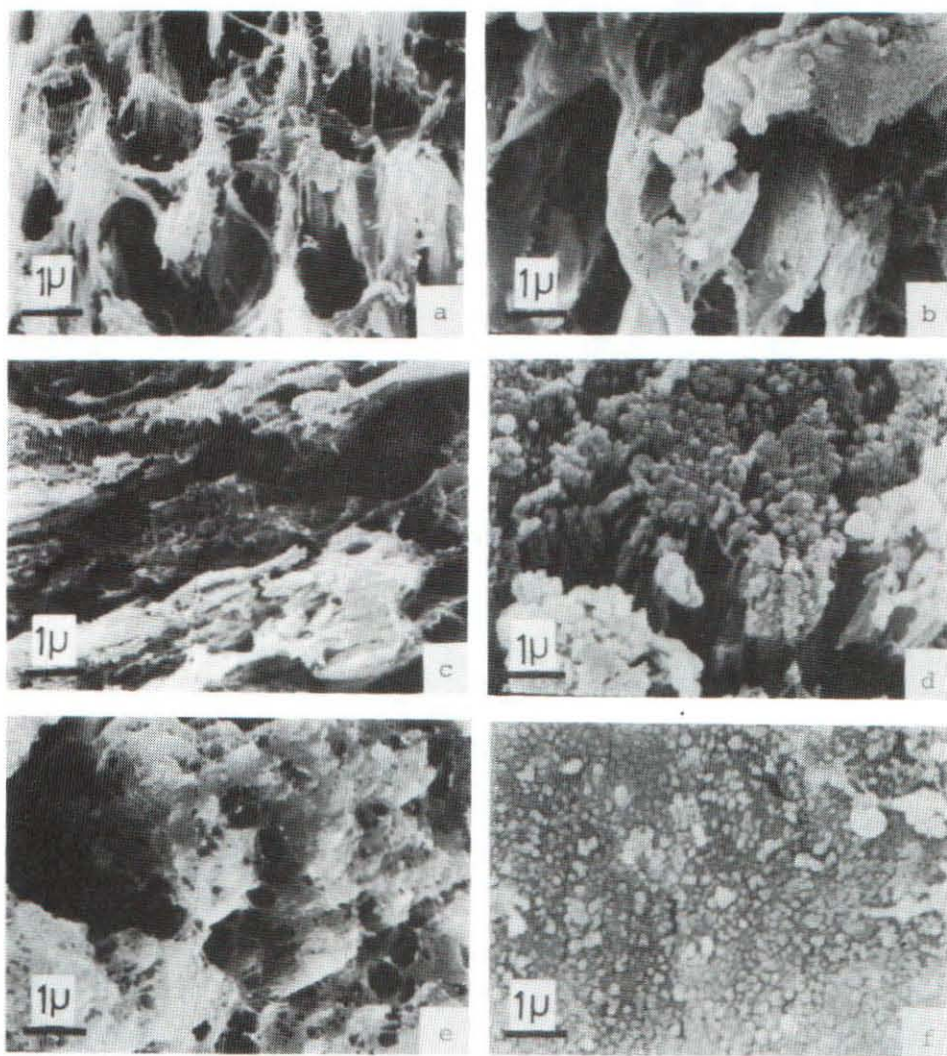


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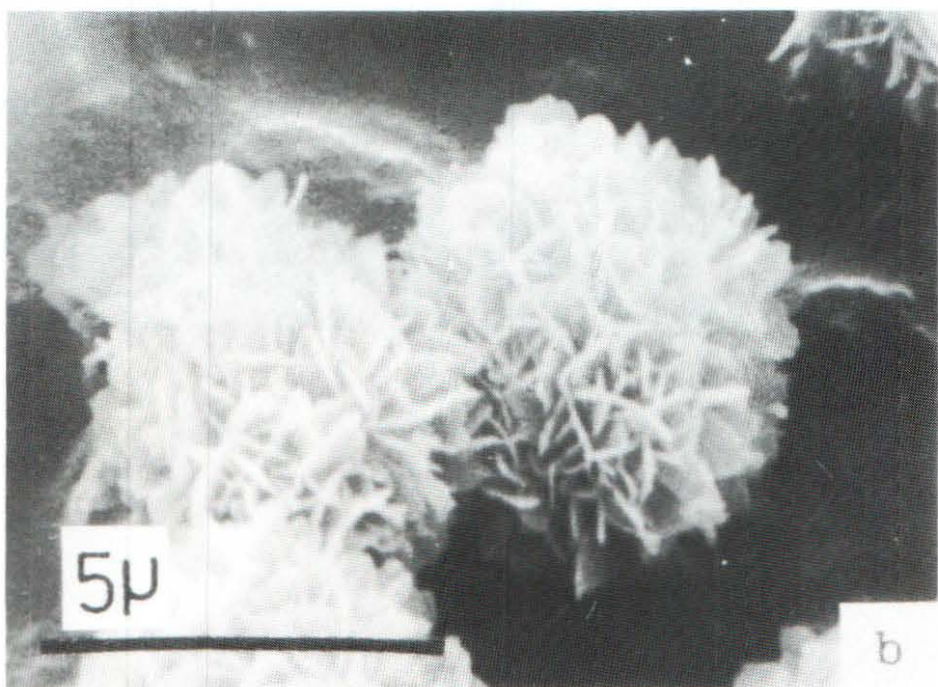
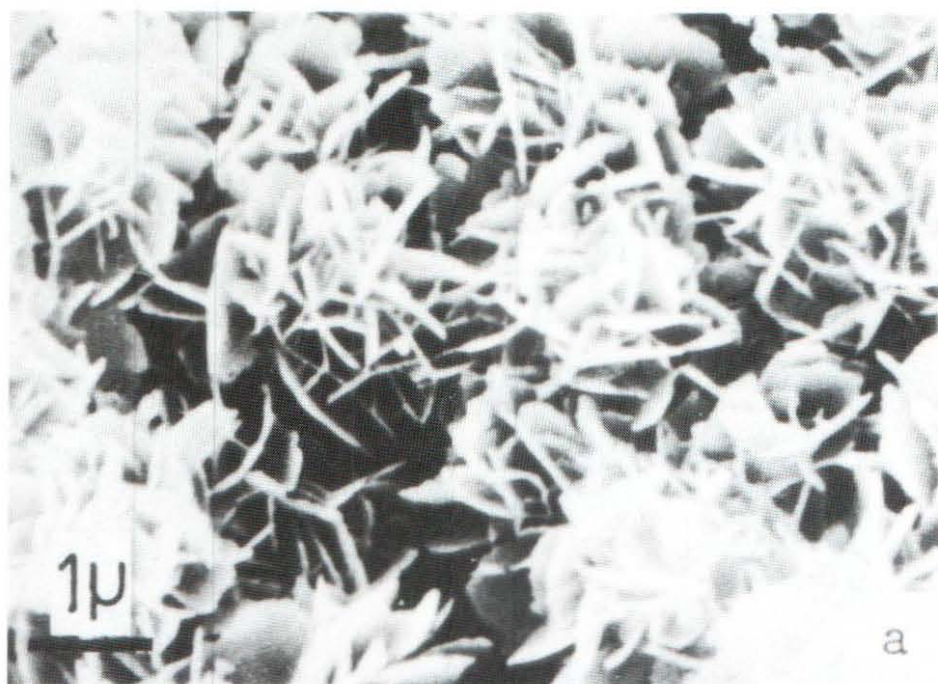


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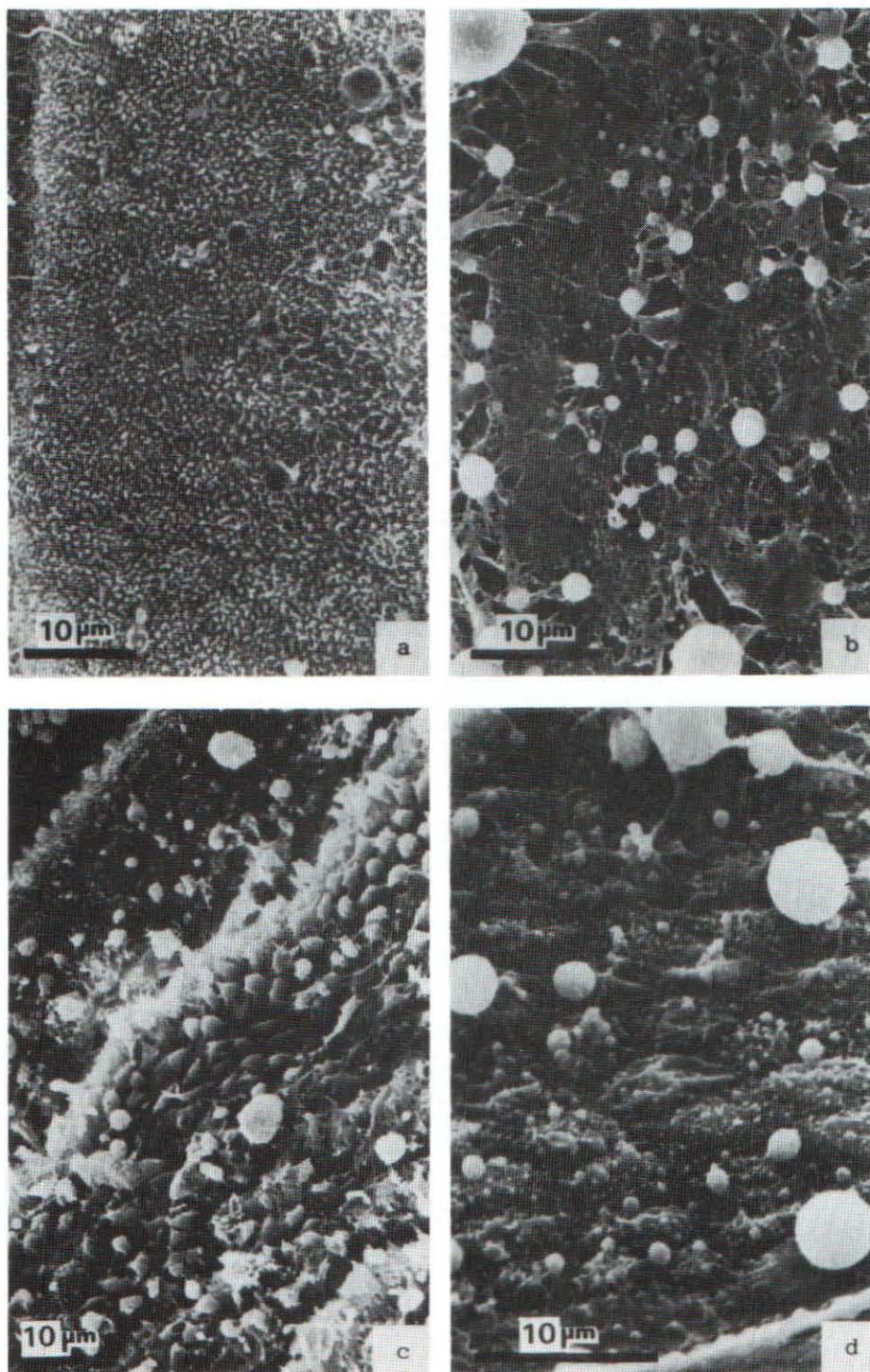


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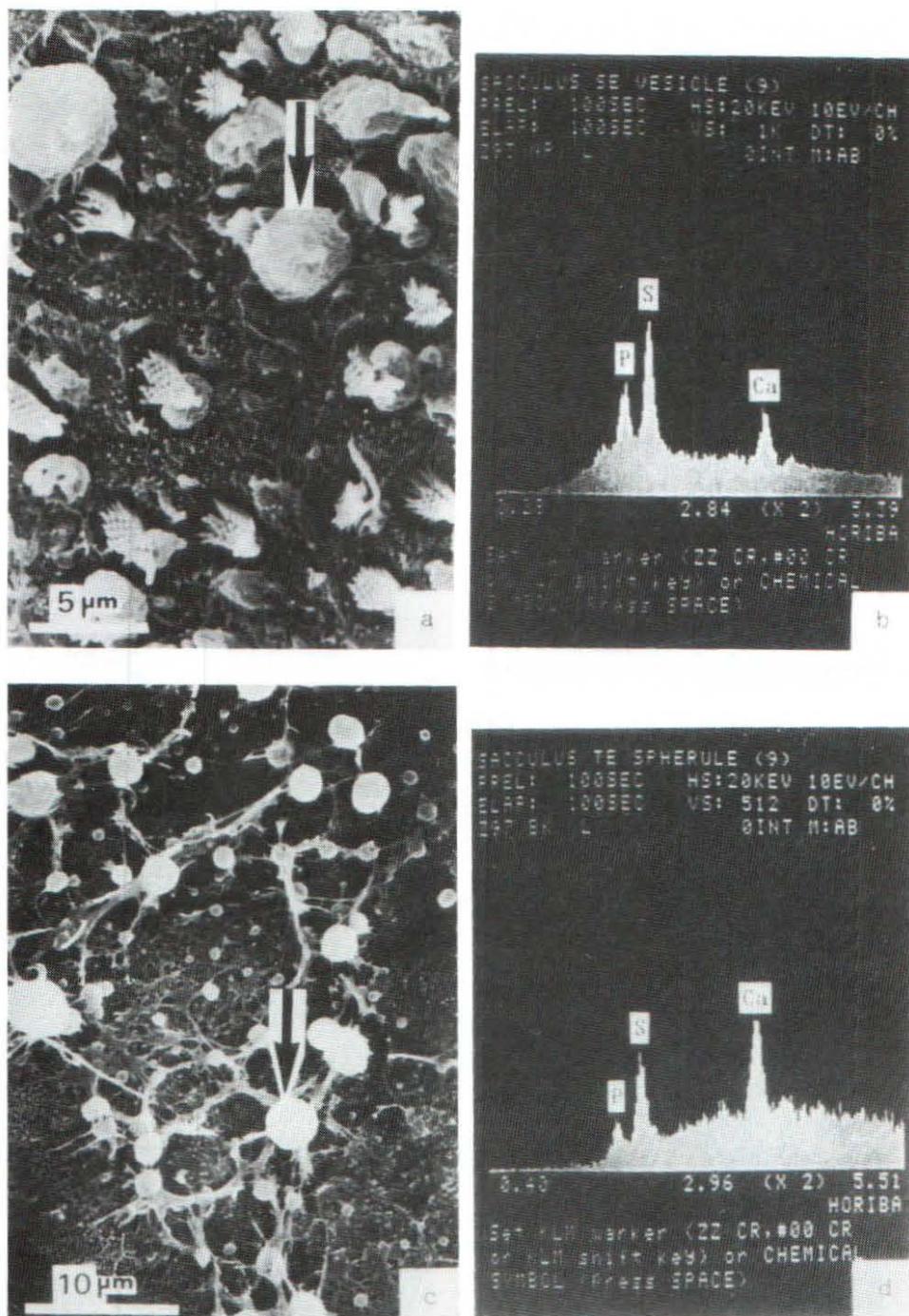


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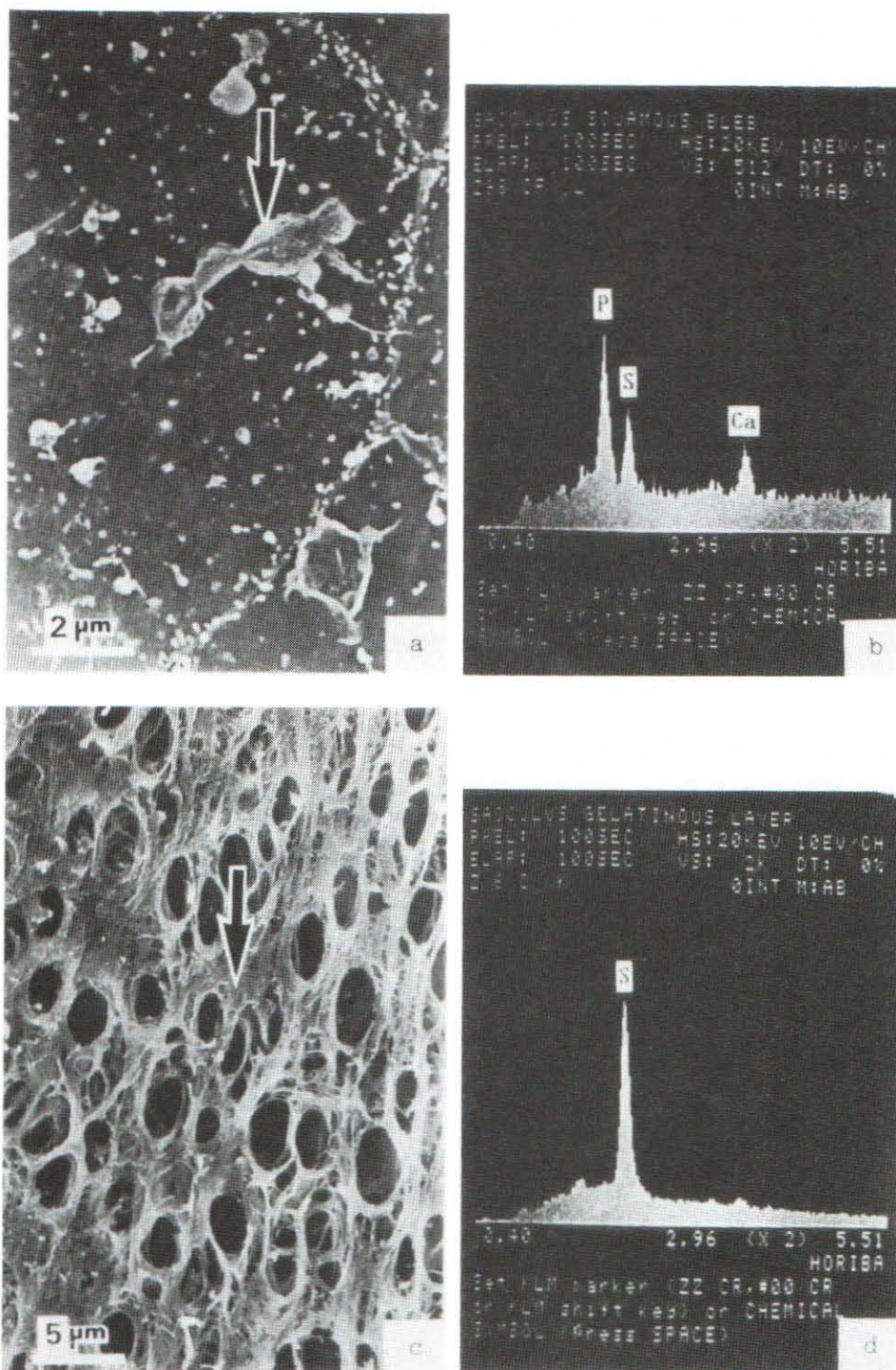


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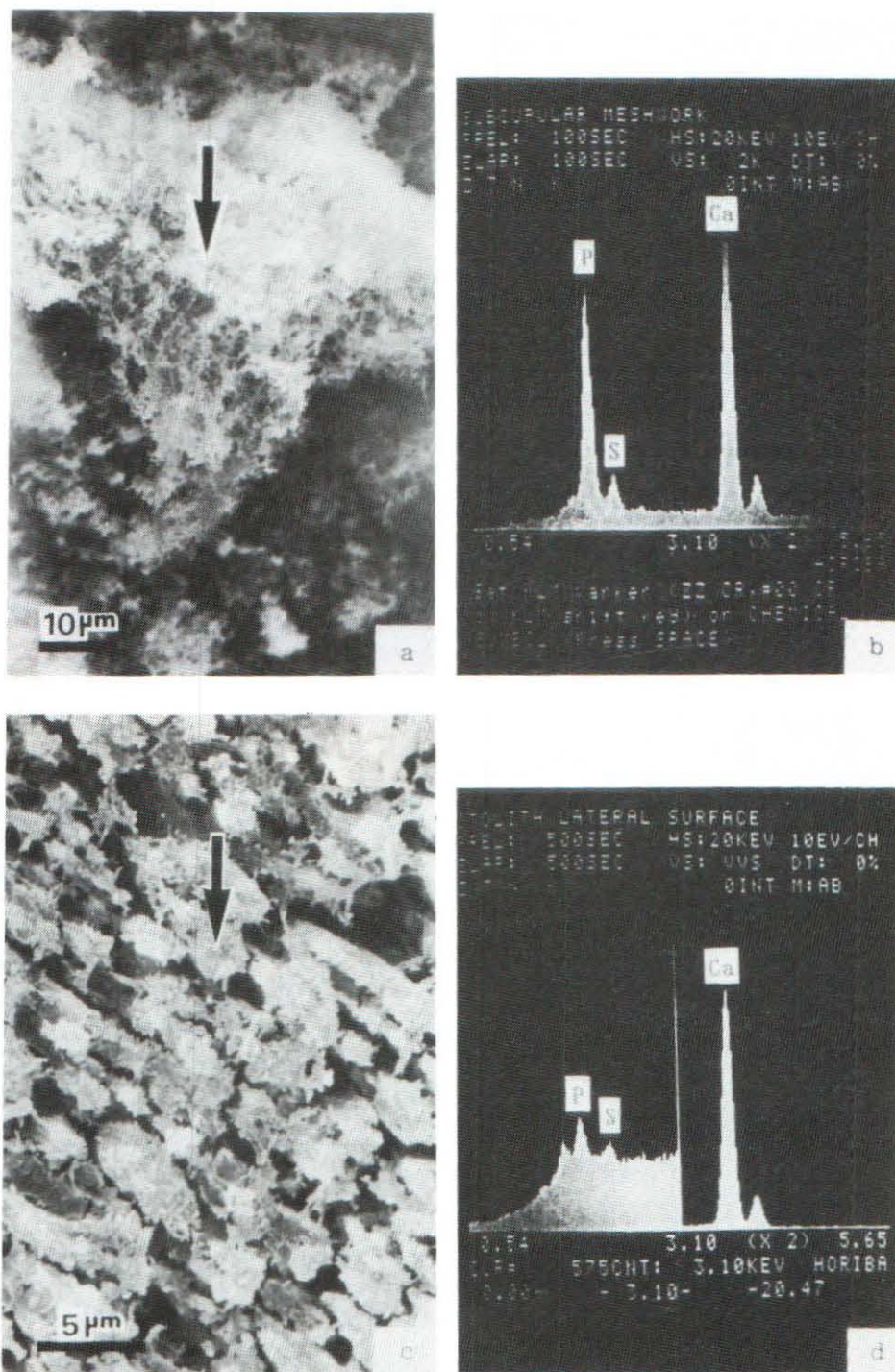


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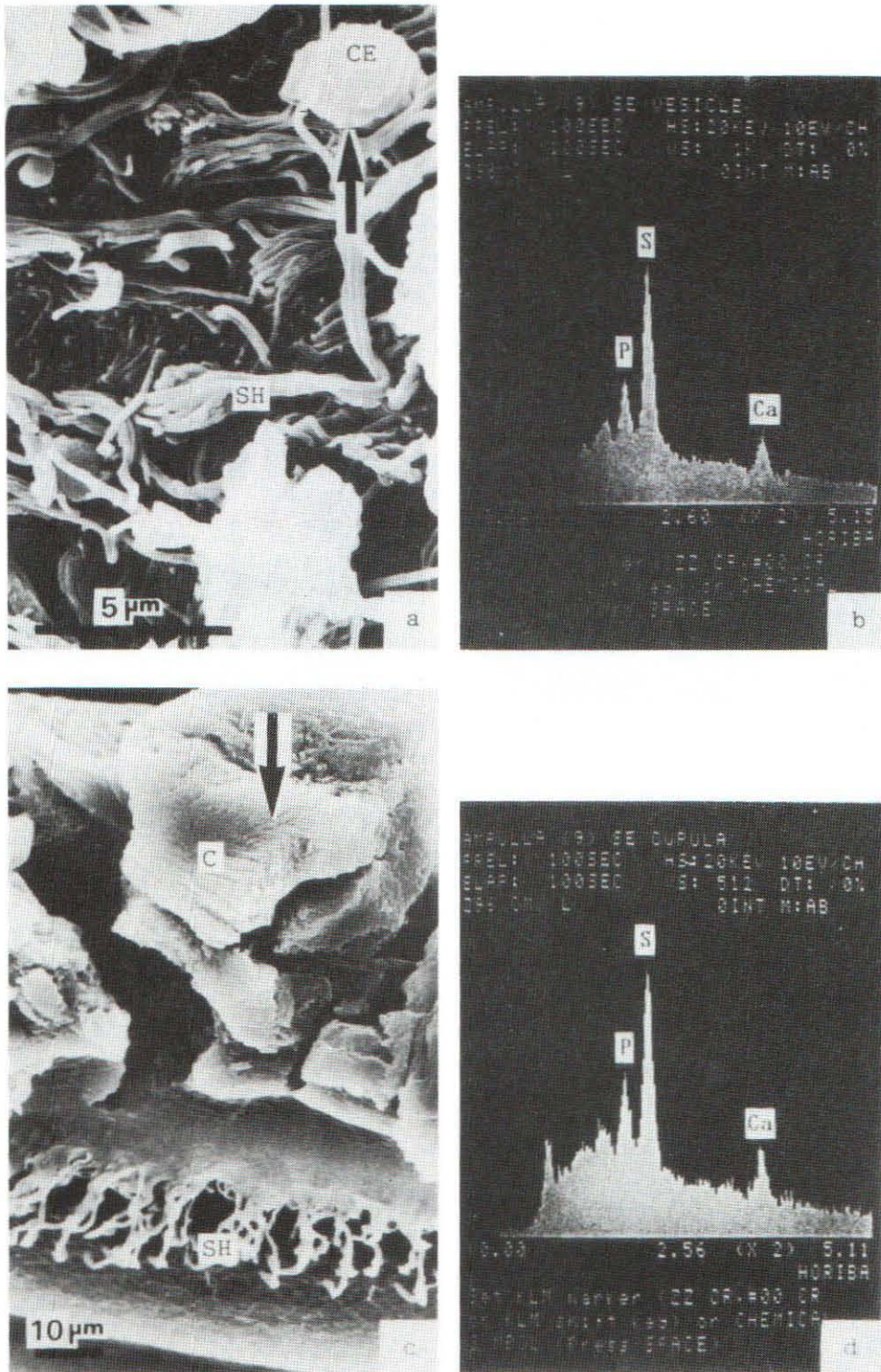


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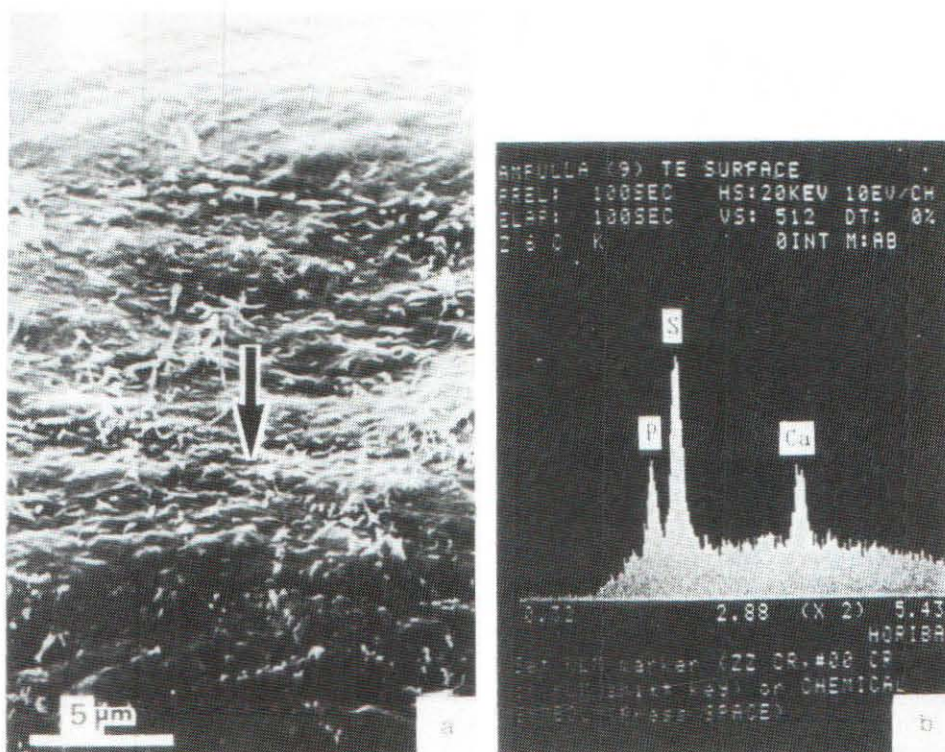


Fig. 35.

ティラピアの内耳、特に耳石器官と耳石の成長に関する 解剖学的、微細構造学的研究

齊 藤 節 雄

魚類の耳石は年齢形質として資源解析に重用されている。近年、多くの魚類の耳石に日周輪の存在が知られ、年齢構造を持たない熱帯性魚や生後1年未満の魚の日レベルでの齢査定の可能性を開くとともに、初期生活史に関する多くの生態的情報を提供するものと期待されている。しかし、日周輪や耳石の形成、成長機構に関する研究は極めて少なく、不明な点が多く残されている。

硬骨魚類の耳石は内耳迷路を構成する通囊、小囊及び壺囊の中に各々一個ずつ存在し、それぞれ礫石、扁平石及び星状石と呼ばれる。扁平石は通常最も大きく、齢査定におもに利用されている。

耳石は炭酸カルシウムを主成分とする高度に石灰化した組織で、基質として、蛋白質、多糖類、脂質などの有機質が含まれている。しかし、耳石は上述の囊内を満たす内リンパ液中で成長し、これと直接する細胞性構造を欠くことから、これらのカルシウムと有機質の由来や沈着経路についてはまだよくわかっていない。

本研究は、硬骨魚類の耳石の成長、特に日周輪の形成機構を、内耳迷路組織のなかで耳石をもつ小囊と、耳石をもたない三半規管膨大部との比較形態学的観察から解明することを目的に行った。

材料として日周輪の存在が証明されているティラピア (*Oreochromis niloticus*) の稚魚及び成魚を用いた。魚には養鯉用ペレットを任意に与え、水温 $27 \pm 1^{\circ}\text{C}$ 、12 L:12 D (8 時点灯、20 時消灯) の光周期条件下で飼育した。

光学及び電子顕微鏡 (SEM, TEM) による観察により、小囊は次の四種類の細胞群から成ることを明らかにした。即ち、感覚上皮、移行部上皮、扁平上皮の各上皮細胞及びミトコンドリアに富む細胞 (Mt-細胞) である。小囊壁の大部分は扁平上皮細胞から成り、その一部領域に Mt-細胞が密集して観察された。小囊壁体軸側には感覚上皮細胞と支持細胞から成る厚い感覚上皮 (感覚班) が存在し、これに隣接して移行部上皮細胞が薄い扁平上皮との間に存在する。感覚上皮細胞と移行部上皮細胞の上を耳石膜が被い、耳石はその上に載っている。

耳石膜はゼラチン層とクブラ下網状層の二つの異なる層からなる。ゼラチン層は耳石に接してクブラ下網状層の上方に位置し、感覚上皮域に対応している。ゼラチン層は、蜂の巣状の穴を持ち、感覚細胞の感覚毛がその穴に嵌入している。クブラ下網状層は細い繊維からなるゆるい網目状構造を呈し、感覚上皮域と移行部上皮域を覆っている。

感覚上皮の表面には、電子密度の高い細胞質の突出及び微絨毛の先端からの小胞の放出が多数観察された。また、クブラ下の空間 (クブラ下網状層と感覚上皮及び移行部上皮との間の空間) で突出物が崩壊している像が観察された。

移行部上皮細胞は、その表面に多数の微絨毛を持ち、細胞質中には粗面小胞体、ゴルジ装

置及びミトコンドリアが良く発達し、細胞間隙は比較的広い。これらの特徴は、蛋白質の合成、輸送を活発に行っている細胞の形態的特徴を示す。一方、微絨毛先端の膨出により形成される小球状分泌胞が細胞表面及びクプラ下の空間に多数観察された。これら感覚上皮及び移行部上皮の細胞表面に観察された小球状分泌胞や細胞質の突出物と、耳石及び耳石膜との位置関係から、耳石膜のゼラチン層は感覚上皮の突出物に、クプラ下網状層は小球状分泌胞にそれぞれ由来すると考えられた。

扁平上皮細胞の表面には、アポクリン型の分泌様式を示す多数の泡状分泌胞が見られた。扁平上皮細胞は非常に薄く、細胞質に乏しいが、時に、粗面小胞体やゴルジ装置が良く発達し、ゴルジ小胞や高電子密度物質を含んでいる細胞も観察された。これらの物質が泡状分泌胞の成分として内リンパ液側へ分泌されるものと考えられた。

Mt-細胞の細胞質は、ほとんどがミトコンドリアとそれを取り巻く滑面小胞体で占められ、細胞の基底部下には内皮に小孔を有する毛細血管が多数存在していた。骨鰐魚のキンギョと非骨鰐魚のティラピアの内耳におけるMt-細胞の分布を調べたところ、両種ともに三半規管膨大部、通嚢及び小嚢に密集して存在していた。またキンギョの横行管及び隔壁により独立しているティラピアの壺嚢にも存在が認められた。しかし、独立構造をとらないキンギョの壺嚢には観察されなかった。骨鰐魚と非骨鰐魚における分布の違いは、内耳組織の構造上の相違に対応していた。これらの形態及び分布の特徴から、Mt-細胞は内リンパ液のイオン調節に関与していると考えられた。

三半規管の膨大部稜は、形態学的に小嚢の聴班と同様に、感覚細胞と支持細胞から成る感覚上皮を形成する。しかし、移行部上皮に当たる隣接する上皮の形態は扁平上皮と同じく非常に薄く、微絨毛を有する細胞はほとんど観察されず、小球状分泌胞も存在しなかった。膨大部稜の上にはゼラチン状のクプラが被っていた。これらの観察から、耳石の成長にとって移行部上皮の小球状分泌胞が重要な役割を果していると考えられた。

小嚢の移行部上皮細胞の表面に観察された小球状分泌胞の量的日周変動を調べたところ、前方部と後方部では8:00に最も少なく、20:00に最も多かった。これに対して中央部では、2:00に最も少なく、その後直線的に増加し、20:00に最も多かった。この様に、いずれの部位においても明瞭な日周変動が認められたが、部位によりその周期性は多少異なった。

小嚢及び三半規管膨大部の細胞表面に認められた分泌物及び耳石膜と耳石について、局所X線微量分析(EPMA)による元素分析を行った。その結果、すべての分泌物からリン、イオウ、カルシウムが検出された。小嚢の耳石膜ゼラチン層からはイオウのみ検出されたが、膨大部のクプラからはリン、イオウ、カルシウムが検出された。両者とも感覚上皮の分泌物に由来すると考えられるが、ゼラチン層では、ある種の修飾機構の存在が示唆された。一方、耳石膜クプラ下網状層ではリンとカルシウムが顕著に多く、移行部上皮の小球状分泌胞にはイオウとカルシウムが多かった。長時間固定によりカルシウムを溶出させた耳石表面から検出されたのは、殆どカルシウムであり、相対的にリンとイオウは極く少量であった。クプラ下網状層は小球状分泌胞に由来し、耳石の有機基質として取り込まれると考えられたが、リンの取り込み量はイオウよりも相対的に少ないと思われた。小嚢感覚上皮の細胞質突出物中に含まれるカルシウム量は、2:00に最も低く、その後高い値で安定していた。移行部上皮の前部と後部における小球のカルシウム量にも2:00に最低、14:00に最高という明瞭な日周変化が認められたが、中央部では殆ど変化がなかった。一方、扁平上皮の小泡状分泌胞中のカルシウム量は非常に少なく、一日中殆ど変化しなかった。

微絨毛に由来する小球状分泌胞が耳石の成長に必要な有機質とカルシウムを含むと仮定すると、移行部上皮に認められた分泌胞自体、及びそのカルシウム含量の量的日周変動は、日周輪の形成に密接な関連を有し、その形成機構を説明する上で極めて重要な現象と考えられた。

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