THE DEVELOPMENT OF STATOCONIA IN MICE

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Dit proefschrift werd bewerkt in de Keel-, Neus- en Oorkliniek van de Universiteit van Amsterdam. (Hoofd: Prof. Dr. L. B. W. Jongkees)
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ACADEMISCH PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE UNIVERSITEIT VAN AMSTERDAM OP GEZAG VAN DE RECTOR MAGNIFICUS MR. A. D. BELINFANTE, HOOGLERAAR IN DE FACULTEIT DER RECHTSGELEERDHEID, IN HET OPENBAAR TE VERDEDIGEN IN DE AULA DER UNIVERSITEIT (TIJDELIJK IN DE LUTHERSE KERK, INGANG SINGEL 411, HOEK SPUI) OP DONDERDAG 12 JUNI 1969 TE 17 UUR

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VALENTIN BERNARDUS VEENHOF

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Promotor: Prof. Dr. L. B. W. Jongkees
Co-referent: Dr. J. James

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CONTENTS

Chapter I

INTRODUCTION

When Galen (131–201) for the first time used the word "labyrinth" for the system of holes and canals in the petrous bone, he probably did not anticipate how anatomists and physiologists would go astray for centuries before any clear notion was obtained with regard to the form and function of this organ. This was due to the fact that exploration of the petrosal bone for morphological, physiological or histological investigation was impossible by the simple means available in those days.

It was only in the sixteenth century that any attempt was made to analyse the anatomy and function of the labyrinth. In this period, Riolanus (1577–1667) localised the auditory sense in the cochlea as the first of the three sense organs of the labyrinth. In the meantime, the anatomical knowledge of the canal system increased continuously and also the statoliths were regularly studied and described. Among others Klein (1740) described the auditory organ of fishes. Illustrations of the otoliths are presented in his papers. Scarpa (1797) gave beautiful illustrations of fish otoliths enveloped by a membranous sac. Comparetti (1791) was the first to describe a calcareous substance in the human labyrinth.

The discovery of the auditory sense organ in the labyrinth for a long time misled many explorers with regard to the function of the canal system and the statolith organs. In the last century the following functions were attributed to the canals and the otolith organs:

magnification of sound (Müller 1837, Cagnaird Latour 1833, Krieger 1840).
sound perception (Chevalier 1830).
noise perception (Helmholtz 1870, Wundt 1880, Bruckner 1888, Hensen 1886).
perception of sound direction (Preyer 1887).

The confusion about all these hypotheses is also revealed in the oldest names given to equilibrium vesicles and equilibrium stones, i.e. (respectively) otocysts and otoliths or "hearing stones". This terminology was later changed to: statocysts and statoliths under the influence of the discovery of some aspects of the function of these organs. Whether this terminology is correct for all animal species remains an unsolved problem, which is the reason why both names are still in use. In this thesis both will be used, for two reasons: first, because the words of certain authors will otherwise be misquoted, and, secondly, because in

that way we will not be forced to introduce new terms such as "statoconia sac" instead of the commonly used "otolith sac".

Another reason why the functional importance of the organ of equilibrium remained obscure for such a long time is found in the fact that neither normal statokinetic equilibrium, nor the calamity of dysfunction of statokinetic equilibrium leads to a conscious mental perception of the seat of the peripheral sense organ as is the case in disturbances of vision, hearing, taste, smell or touch.

Flourens (1824, 1828, 1842), when describing his classical experiments, extensively summarized the disturbances of equilibrium which appeared after the destruction of parts of the labyrinth in his experimental animals. Not until fifty years later were these experiments interpreted in the right way. (It must be remembered that Flourens was performing these experiments in order to reach a better understanding of the localization of the auditory function in the labyrinth by cutting various canals of the labyrinth.)

After many speculations about the function of the semicircular canals, whether in connection with the initially ignored findings of Flourens or not, Goltz (1870) became the first to consider the semicircular canals as the seat of a separate sense organ, namely, the organ of equilibrium, whose function is the perception of linear and rotatory accelerations.

In 1888 Rudinger gave a description of the position of the macula utriculi and the macula sacculi as being nearly perpendicular to one another. In connection with this description, Breuer (1890) came to the conclusion that the otoliths must be responsible for the static functions of the organ of equilibrium.

The discoveries of Flourens, Goltz, Rudinger, Breuer and others drew the attention of the scientific world in the final decades of the nineteenth century. This resulted in an explosion of anatomical and physiological investigations of the labyrinth. From the very beginning, the perpendicular position of the semicircular canals in relation to each other was the focus of interest for the physiologists. The hypotheses about the importance of the canal system for the perception of rotatory accelerations was demonstrated successfully by means of experiments, although for a long time many authors maintained that this sense organ could also perceive linear accelerations.

As in the case of the canal system, attempts were made to explain the three-dimensional structure of the statolith organs in a simple scheme which, however, was based on the erroneous conception that every macula comprises a homogeneous, plate-like area of sensory epithelium. Werner (1928, 1930, 1933, 1936, 1939, 1940) demonstrated in a series of experiments that this view, which had been the basis of many physiological experiments and theories, was founded on an oversimplified view of macular histology. It had now gradually become clear that one should distinguish three

separate functional areas in the labyrinth of mammals and birds, namely:

1. sound perception in the cochlea.
2. perception of rotatory accelerations in the semicircular canals.
3. perception of linear accelerations in the utriculus and possibly also in the sacculus.

The combined position of the sense organs of hearing and of equilibrium in the labyrinth finds its origin in the joint development of the entire inner ear from the so-called otic vesicle; all the sense organs in the labyrinth have in fact a basic structural pattern in common. Essentially, each sense organ has the same two types of sensory cells, the same supporting cells, and the same extracellular structure over the sensory cells, the *sensularium*, which transforms an adequate stimulation into bending of the sensory hairs. The sensory cells in the labyrinth are *mechanoreceptors*, i.e. cells which are excited or inhibited when the sensory hairs are bent into another position by means of the moving sensularium, so giving rise to nervous impulses. Under physiological circumstances the sensularium and the architecture of the labyrinth prevent any other than the appropriate stimulation from bending the sensory hairs.

The more complicated structures from which the inner ear derives its name, labyrinth, are especially found in higher developed vertebrates such as mammals and birds. In general, one might say that the organs of these species of animals are distinguished by a very complex function of the organ, and by specialization of parts of the organ in one single facet of the total function of the organ. In contrast to this, one finds a more primitive architecture of the labyrinth in the less developed vertebrates. The simplest organ of equilibrium in this development, as found in many vertebrates, is the *statocyst*, which is an epithelial vesicle filled with fluid, whose wall consists completely or partly of sensory cells. This primitive organ, which determines the position of the animal in relation to the force of gravity, possesses two characteristic qualities which enable it to perceive precisely this kind of stimulation. These are: firstly, the form of the cyst, and, secondly, a little particle of solid matter with a very high specific gravity. This particle or statolith seeks the lowest possible position because of its heavy weight, whilst the globular form of the cyst is responsible for the fact that never more than one point will be the lowest "point". A change in position of the animal in relation to the direction of the gravitational force will result in another resting position for the statolith.

In the nineteenth century the phylogenetic development of the statocyst towards the labyrinth of mammals was studied in particular by Hasse and his coworkers. In his monograph Hasse (1873), when describing this development, explained the several degrees of specialization of parts of the labyrinth. In statocysts which are phylogenetically

somewhat more highly developed, the statolith is firmly connected to the hairs of the sensory cells. In this situation the globular form of the statocyst becomes unnecessary, because the lowest point of the cyst no longer serves any purpose for the determination of position in relation to the direction of gravity. The hagfish, *Myxina glutinosa*, a primitive vertebrate living in the mud of the sea bottom, has a tube-like expansion of the vesicular statolith organ, the *semicircular canal*, which contains a sensory area, the *crista*, at both ends. An extracellular organic mass, the *cupula*, is firmly connected to the hairs of the crista. Cupula and crista are set at right angles to the wall of the *ampulla*, the broadened end of the semicircular canal. Cupula and crista form a stop valve for the endolymph in the semicircular canal. This three-dimensional situation assures that only rotatory accelerations in the plane of the semicircular canals can bend the hairs of the sensory cells of the crista. The liquid column in the semicircular canal stays behind in relation to the rotating solid structures surrounding it, in accordance with the law of inertia. Under the influence of these forces of inertia, the cupula bends over and this again leads to a bending of the hairs of the sensory cells on the crista and to the stimulation of the sensory elements of the latter. The phylogenetically more highly developed animals possess more than one semicircular canal, so that, in the case of a completely three-dimensional structure of the canal system, circular accelerations can be perceived in any plane.

The statolith parts of the organ of equilibrium have also been subject to evolution. Bony fishes e.g. have three statolith organs in their labyrinths instead of one as found in primitive statocysts, i.e. the *utriculus*, the *sacculus*, and the *lagena*. In mammals the lagena has developed into the cochlea – the auditory sense organ – and the statolith of the lagena no longer exists.

Many questions remain open with respect to the histology of the statolith organs, especially with regard to the statoconia, which, phylogenetically, were formed very early. They are essential for the normal functioning of the maculae, and will be dealt with in greater detail in the following chapters.

Chapter II

LITERATURE

The origin, growth and maintenance of the statoconia belong to some of the largely unknown facts about the covering layer of the maculae in the otolith sac. The composition and shape of the statoliths have received much more attention from investigators of the labyrinth. In particular, the morphology of the otoliths in many species of fishes has been carefully studied and described. From these studies is became clear that every kind of fish has a very typical, species-bound, characteristic structure of the otoliths. The fishes of different species show very great differences in the shape of their otoliths. However, this great distinction in shape has not afforded any insight into the relationship between shape and function of the statoliths. Nor is much known about the origin, composition and structure of the organic matrix in which the statoconia are embedded.

Composition and structure of the hearing stones

The elements out of which the stones are build up are fairly uniform in the entire animal kingdom and consist of calcium salts in a crystalline state, mostly formed by the organism itself. Some species of animals, e.g. lobsters, deposit foreign bodies with a high specific weight into their superficially situated otolith organs, to make their macular sensularia heavier. These foreign bodies are chiefly grains of sand which are firmly fixed by a thin organic matrix. The statoliths formed by the organism itself may present as one big concrement of crystalline calcium salt, as in bony fishes, or they may consist of many tiny stones of microscopical dimensions per macula, as is the case in mammals. Breschet (1836) named the collections of tiny stones otoconia as distinct from otoliths, which name he reserved for the description of a single concrement of calcium salt per macula.

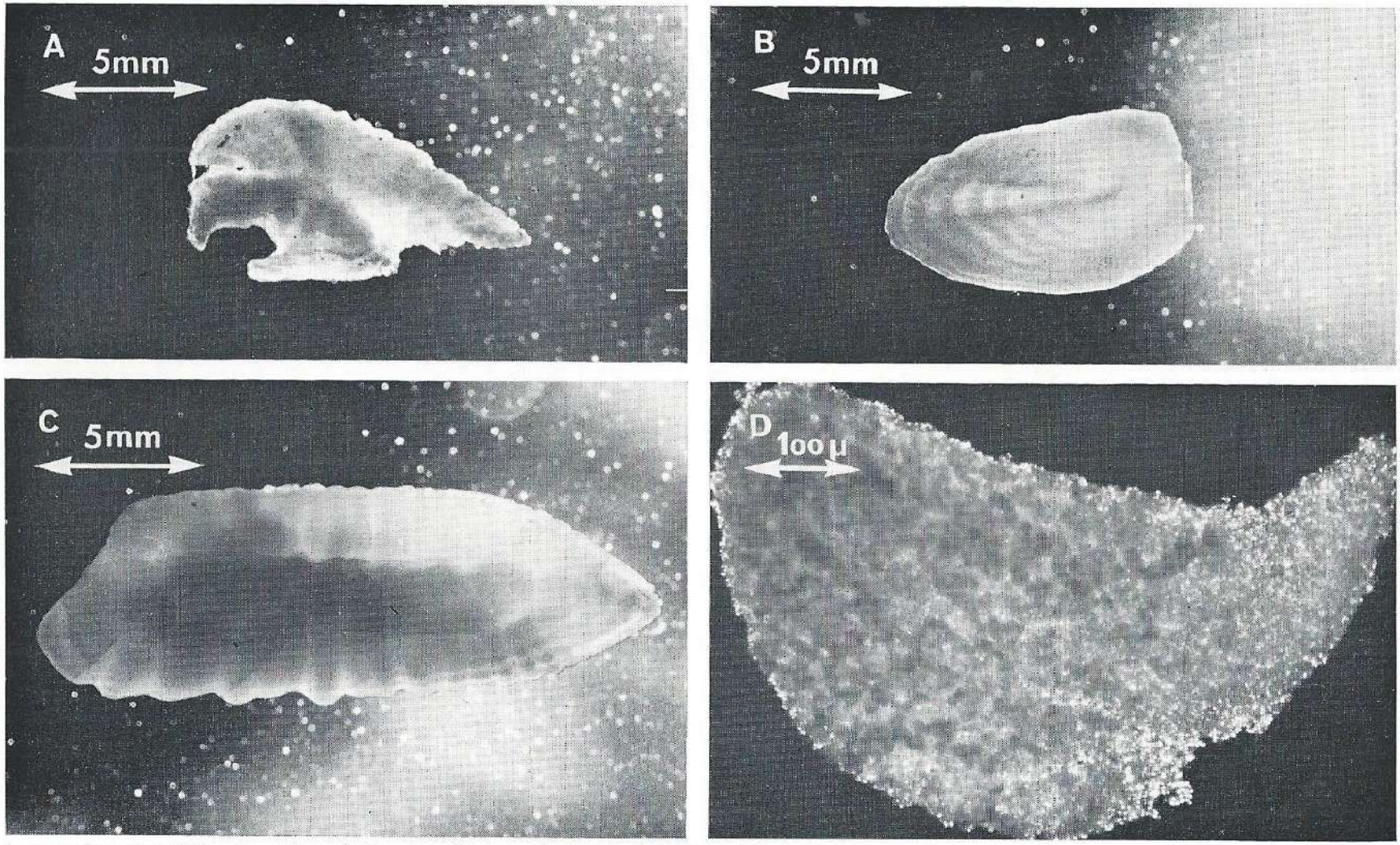
Krieger (1840) established that these big otoliths were composed of tiny crystalline rods of calcium carbonate. He described light and dark concentric layers in ground sections of fish otoliths. Later Reibisch (1899) discovered that these concentric layers consisted of one light and one dark deposit of calcium salt crystals per year. Since that time these "annual rings" have been used for the determination of the age of fishes (plate 1).

Crystalline calcium salts occurring in nature can be found in three forms. The phosphate salts *apatite* [$Ca_{10}(PO_4)_6F_2$] and *hydroxyapatite* [$Ca_{10}(PO_4)_6(OH)_2$] are found in bone and teeth. The carbonates *calcite*

and *aragonite* are both forms of $CaCO_3$, but they have a different crystal lattice. Aragonite has the least stable lattice of the two; it can change spontaneously into the calcite form. The crystalline structure of the statoliths of many sorts of animals has been examined. SANDER examined the otoliths of fishes at the request of HERZOG (1925). He found that they consisted of calcium carbonate crystals with an aragonite structure. BELONOSCHKIN (1931) on the other hand, when studying chicken embryos, thought that at least part of the crystals must have been built up from calcium phosphate. He came to this conclusion because he observed that, together with the development of the otoliths (at about the 7th day of hatching), there was also a strong increase of organic phosphate in the chicken embryo. HASTINGS (1935) examined the otoliths of *Amblystoma tigrinum* by means of x-ray spectrometry. It appeared that otoliths contained phosphate and carbonate salts of calcium, whereas 15,9% of the calcium salts had an apatite structure and 83,2% an aragonite structure. CARLSTRÖM, ENGSTRÖM and HJORTH (1953) examined the statoconia of man by means of x-ray diffractometry. They found a diffraction pattern that corresponded with that of calcite. The diffractogram further pointed to the presence of a small quantity of hydroxyapatite. These authors concluded that the statoconia might possess calcium salts in the apatite form, but did not exclude the possibility that addition of a tiny quantity of bone might have taken place during the preparation. SASAKI and MIYATA (1955) also applied the x-ray diffraction method and found in the statoconia of mammals a calcite diffraction pattern. In the statoliths of bony fishes they found a diffraction pattern mainly in accordance with an argonite structure. PARSONS and CARDELL (1963, 1965) examined statoliths by means of emission spectroscopy and x-ray diffractometry. The statoconia of birds and mammals appeared in particular to contain calcite. The statoliths and statoconia of reptiles, amphibians, and bony fishes chiefly showed an aragonite structure. In cartilageneous fishes, a quartz pattern was observed in statoliths which obviously contained grains of sand.

The formation of the hearing stones

About the formation of otoliths very little is known with certainty. Especially in the last century there were some rather extravagant opinions about this process. KRIEGER (1840), for instance, believed that the formation of the calcium corpuscules was based on excretion of calcium from the nerves which, he believed, get rid of their superfluous calcium salts in this way. He inferred this from the fact that "otoconia" were also found elsewhere in the body, e.g. in the peritoneum! KOKEN (1884, 1888), with somewhat more caution, suggested that excretion of calcium salts could be related to the action of the auditory nerve. KAWANO (1922) thought that calcium salts excreted from the macula would crystallize under influence of the endolymph. HERZOG (1925) found in chicken



A, saccular statolith of a pike of 6 years; B, saccular statolith of a plaice of 7 years; C, saccular statolith of a haddock of 7 years; D, saccular statoconial membrane of an adult mouse.

PLATE 1

embryos a sudden and relatively big increase of otoconia on the 7th day of hatching. He assumed the existence of an increase in the calcium content of the endolymph, whether by secretion, or from desintegration of cells, resulting in a saturation and crystallization of calcium salts inside the sensularium of the macula. DONADEI (1925) considered the structures which cover the sensory epithelia of the labyrinth as differentiations of the apical ectoplasm of the sensory cells. NISHIO (1926) found a rather sudden appearance of statoconia and their matrix in mouse embryos of 17–18 mm length. He was not able to show the pathway along which the otolith material is supplied; he suggested that it might be secreted by some parts of the utricular wall. To demonstrate the local increase of calcium salts in the endolymph he fixed mouse embryos with a mixture of Na-oxalate and formaline. In these preparations Nishio found calcium oxalate crystals in between the otoconia and the macula. LYON (1955), however, raised two serious objections against the technique of Nishio. In the first place the solution might be significantly displaced by diffusion before the calcium oxalate was precipitated. In the second place she stated that Nishio presupposed – probably wrongly – that no calcium phosphate and calcium carbonate would be dissolved and reprecipitated as oxalate. Looked at in this light, it is doubtful whether Nishio really demonstrated only free calcium ions in the submembranous space.

STRICKER (1928) thought that in the jelly-like mass over the macula an organic casting mould is created in which later the calcium salts are crystallized.

BELONOSCHKIN (1931) made frozen sections of chicken embryos. He found on the 6th day of hatching that the sensory cells were covered with a jelly-like mass, in which on the 7th day a large number of otoconia could be seen. This was in agreement with the findings and experiments of Herzog.

LYON (1955) investigated the development of otoconia in mouse embryos. Using alcohol fixation she found small calcium salt depositions on the 14th day of development and organised otoconia in 15 days embryos. When using other fixatives, such as formalin, Bouin and Susa, the first indication of otoconia was observed at a much later moment in the development. The author attributed this difference to the acidity of these fixatives, resulting in a demineralization of the otoconia, leading to their disappearance. Quite a different view from the above-mentioned authors is held by WOLFF. At the "Conference on the Neural Mechanics of the Auditory and Vestibular system" held in Bethesda, U.S.A. in 1959, Wolff made mention of otoconia, "*just emerging from these macular cells in what appeared to be a liquid state*". In other places in the same preparations he also found calcium crystals. He stated: "*perhaps true crystallization does not occur until the otoconium reaches the endolymph*".

GEUZE (1968) made an electron-microscopical study of the formation of stones in the statocysts of young water snails *(Lymnaea stagnalis)*

and also in adult snails 6, 12, 24 and 48 hours after having surgically opened the statocysts. The younger snails that were not operated upon appeared to build up statoconia in the apical parts of the supporting cells, after which statoliths were ejected and pushed out into the lumen of the cyst. Within 12 hours the statocysts that were operated upon showed a transformation of the cyst into an undifferentiated cell-mass. In the apex of these cells the vacuoles, which are formed around the statoliths, collected. The vacuoles (with intracellular fluid) of these cells flowed together and formed a new cyst cavity.

The new formation of a sensularium after removal of statoconia from the macula has been described before by WERNER (1933) studying the sections from HASEGAWA (1932). Werner described histopathological changes of the statolith sacs of frogs, rabbits and guinea-pigs after removal of the statoconia from the maculae by means of centrifugation. After 110 days he found the sensularium practically unchanged, lying away from the macula in the statolith sacs, whilst in the meantime a new sensularium was formed. In carps, VON FRISCH (1938) found a new formation of calcium concrements 2 months after removal of the otoliths. On the other hand JAMES (1962) did not find new statoconia in the sacculus of the rabbit 3 month after destruction of the otolith sacs, but he did observe a regeneration of the epithelial vesicle as such. The destruction of these otolith sacs was performed by PHILIPSZOON (1962) and this entailed destruction of the macula and the macular nerve of the utriculus and the sacculus; whereas Hasegawa and von Frisch kept the nerve intact and tried to save the macula from destruction as much as possible. The experiments of James and Philipszoon are, therefore, not entirely comparable with those of Hasegawa and von Frisch.

Growth and maintenance of the hearing stones

Hardly anything can be found in the literature about the growth and maintenance of the statoliths. We know that some of the invertebrate animals possess a connecting tunnel between the outer world and labyrinth cavity, through which grains of sand can be brought into the statocyst. It is known that in some species of lobsters the sensularium maculae, which is made heavier with sand, is shed and renewed together with the desquamation of the chitine armour (HENSEN 1863). The statoliths of bony fishes grow parallely with the entire animal during the whole of its life as shown by the annual rings mentioned above. How this happens is not yet clear.

PLATE (1924) believed that calcium-containing endolymph penetrates into the jelly-like sensularium and crystallizes into statoconia. By fusion of these statoconia, statoliths are formed. Plate postulated that the stato*conia* as they appear in various kinds of animals, have reached the peak of their evolution in the stato*liths* of bony fishes. It is not known whether the otoconia of birds and mammals are formed only once and

for all, or whether they are formed continuously during life, as is the case, for instance, in bony fishes. The only publication which took up this problem is BÉLANGER's (1960). He injected juvenile rats aged 3 and 9 days with $Ca^{45}Cl_2$ after which they were sacrificed at intervals of 4 hours or 4 days. Four hours after giving the isotope he found no activity over the statoconia on autoradiograms; after four days, however, he found a slight but clear incorporation of the radioactive calcium into the statoconia, indicating a slow rate of calcium uptake.

Structure and composition of the statolith matrix

As in the case of the cupula and the tectorial membrane of the cochlea, the statolith matrix consists of an organic substance whose origin, growth, structure and composition are unknown. RETZIUS (1881), VAN DER STRICHT (1921) and KOLMER (1926, 1927) considered the jelly-like mass of the tectoria to be an excretion product from the supporting cells of the sensory epithelium. AYERS (1891, 1898), WITTMAACK (1926), MYGIND (1952) and BORGHESAN (1952, 1959), however, are of the opinion that the matrix consists of a tissue derived from the sensory hairs, or from products of those hairs. DONADEI (1925) considered it to be a differentiation of the epithelial ectoplasm. This was disputed by WERNER (1940), who found that the matrix remained alive for months if separated from the sensory epithelium.

From electron-microscopical investigations by BAIRATI and IURATO (1957), SPOENDLIN (1957), ENGSTRÖM and WERSÄLL (1958) and IURATO (1960) it became clear that the cupula and the tectorial membrane of the cochlea are composed of submicroscopical fibres with a thickness of about 90 Å. These observations, however, did not solve the question of whether those fibres really exist in vivo. Spoendlin suggested that the fibres he observed in the tectorial membrane of the cochlea could be artefacts caused by coagulation and polymerization as a result of the histological techniques to which the preparations are inevitably exposed. Iurato did not share these doubts about the real existence of these fibres in vivo. He also found fibre-like structures in fresh preparations observed with the aid of the phase-contrast microscope. Furthermore, he did not observe either coagulation or precipitation when the sensularia were examined microscopically during fixation. The structure of the homogeneous layer in which the otoliths are embedded has not been studied with the electron microscope so far. It is, however, probable that its composition does not differ much from that of other covering membranes in the labyrinth.

Various investigators have tried to determine the chemical composition of the otolith matrix by means of microscopical histochemistry. In the handbook of von Möllendorff, KOLMER (1927) described a certain affinity of the otolith membrane for mucicarmine. WISLOCKI and LADMAN (1955) found a clearly positive staining of the otoliths with the PAS (Periodic-

Acid-Schiff) reaction, indicating the presence of sugars. LYON (1955) studied the development of otoliths in mice. In the covering membranes of the maculae she demonstrated the presence of mucopolysaccharides and glycogen, which are also found in the membrana tectoria cochlea and in the cupula. (cf Santi 1950, Zorzoli and Zorzoli 1954, Wislocki and Ladman 1954, Friberg and Ringertz 1956, Ferreri and Crifo 1956, Iurato 1960 and Bélanger 1961). In addition, Lyon demonstrated the presence of alkaline phosphatase in the otolith membrane. She drew attention to the fact that, during the embryonic development of statoconia in mice the same substances are found in the sensularia as during the formation of bone, i.e. alkaline phosphatase and protein-polysaccharide complexes.

It is obvious from the above review of the literature that many aspects of the statoconia are still completely unclear. During our investigations we have attempted to answer the following specific questions:

1. Where are the statoconia formed?
   a. entirely intracellularly,
   b. at first intracellularly, with secondary extracellular growth in the lumen of the otolith sacs or,
   c. entirely extracellularly in an organic matrix inside the otolith sac.
2. How is the supply of the calcium salts arranged?
3. Are the statoconia formed once and for all, or is there a continuous growth in the same way as in the case of the statoliths of bony fishes?
4. To what extent can the forming of statoconia be compared with bone formation?

We have tried to find an answer to these questions mainly by the study of histological preparations of the membranous labyrinth. To complete and support the histological investigations we also examined some micro-anatomical aspects of the vestibular apparatus.

Moreover, it seemed necessary in the course of our investigations to broaden the base, by adding data from electron microscopy and x-ray diffractography.

Chapter III

MATERIAL AND METHODS

This investigation was carried out on mouse embryos, juvenile and adult mice of the so-called RQ strain, supplied by the animal breeding farm of TNO (Toegepast Natuurwetenschappelijk Onderzoek) in Zeist. We chose this material in the first place because the heads of the mouse embryos and the juvenile mice seemed to be very suitable for histological sectioning without decalcification. In the second place, these (dark grey) mice had a rather intense pigmentation of the membranous labyrinth, which had certain advantages in the microdissection of the labyrinth. It was necessary to avoid any decalcification, because the examination was principally concerned with the statoconia which are mainly composed of calcium salts.

Another advantage of the avoiding decalcification of the material was that the rather loose organic matrix of the statoconia could be preserved more in its natural state. The progressive calcification of the skull of mice limited the investigation to juvenile mice.

The microdissection of the labyrinths of adult mice will be described together with the microanatomy in chapter IV. Anatomical studies of the labyrinth of the adult mice were made mainly to provide a three-dimensional control of the histological sections of embryos and juvenile mice.

The ultrastructure of the statoconial membrane was studied by means of an electron microscope.

Mouse embryos of 12 to 20 days old were used. The normal pregnancy lasted 20 days in this strain of mice. The period of pregnancy was counted from the moment of conception. This moment was confirmed as follows. First, female mice, kept separately, were left together with male mice for one night only. Then each female mouse was examined after mating for the presence of a so-called vaginal plug. The presence of such a plug proves that copulation had taken place within the previous 12 hours.

The juvenile mice in our examination were two, six, eight and sixteen days old. The last mentioned age was the limit of what could be cut on the microtome in the undecalcified state with the mounting and cutting technique used by us.

The fixation of the petrous bones was started as soon as possible after decapitation. In the youngest experimental animals the entire skull was fixed. In animals of 15 days gestation and older the petrous bones were first dissected. The skin was stripped from the skull, which was not too difficult because of the loose texture of the connective tissue between

skin and skull. After this, the skull was cut in a sagittal plane into two halves, after which the contents of the cranial cavity could be removed. Then both halves of the skull were immersed in the fixative. This whole procedure took only some tens of seconds.

It was impossible to use the classic acid fixatives. It is known, for instance, that after fixation in Bouin, statoconia in mice are only observed after birth, although they have been formed a long time previously (LYON 1955). Undoubtedly the decalcifying properties of the acid fixatives play a role in this disappearance. Thus we were forced to apply neutral, non-decalcifying fixatives.

Three fixation methods were tried:

I. Fixation in glutaraldehyde 2,5% in phosphate buffer (pH 7,1) for 3 hours with postfixation in a mixture of 40 parts of alcohol 70% and 1 part of undiluted formol for 2,5 hours.
II. The second method is a modification of the first and differs from it in that a postfixation of formol was used, buffered in the same neutral phosphate buffer of method I.
III. In the third method the prepared skull fragments were immersed in alcohol 80% at 4 °C.

After the fixation the skulls were dehydrated via the alcohol series, and embedded, via methylbenzoate with 2% celloidin, chloroform and toluol, in Ralwax (R. Lamb, London). The orientation of the petrous bone in the embedding was performed in such a way that the sections ultimately went through utriculus and sacculus at the same time. For this purpose, a cutting plane was chosen, perpendicular to the median plane, with an inclination of 120° to the base of the skull.

After the embedding, the preparations were cut into serial sections with a thickness of 6 μ. Every tenth section was stained with hematoxylin-phloxin. These sections were made in order to obtain a general impression about the quality of the fixation and the further treatment of the material. The series of sections which demonstrated coarse deformations or in which the plane of cutting deviated too much, were excluded from further investigation. At the same time these sections were used to determine in which regions of the series the utriculus and sacculus were situated and thus which sections could be used for further investigations.

In examining the sections stained with hematoxylin-phloxin, it became clear that the form of the sensularia in the membranous labyrinth was conserved best after fixation according to method III, that is, with alcohol 80% at 4 °C. Further investigations made it clear that the statoconia, especially with regard to the matrix, were also preserved best in the labyrinths treated by method III. For these two reasons we used only the labyrinths preserved by method III for further histochemical and histological investigation.

The following scheme gives us a survey of the number of skulls fixed according to method III which could be used. Likewise indicated in this scheme are those animals injected with radioactive Ca^{45}, and animals treated with tetracycline for subsequent investigation of calcium incorporation.

age in days	embryonic									neonatal			
	12	13	14	15	16	17	18	19	20	2 (22) *	6 (26) *	8 (28) *	16 (36)*
not treated in vivo	2	3	3	3	3	3	3	2	2	2		2	2
in vivo prepared with Ca^{45}		4	2	2		2		2		2	2	2	
in vivo prepared with tetracycline		2	2	2		2		2		5			2

* The numbers between brackets indicate the number of days from the conception.

Fig. 1.

Apart from the hematoxylin-phloxin stained preparations, the following histological and histochemical techniques were applied to those sections passing through the labyrinth. Each successive section was stained according to a fixed scheme, so that the different techniques could be compared in sections cut from a not too distant part from the same block.

A. STAINING TECHNIQUES FOR CALCIUM SALTS
   1. Von Kossa's method.
   2. Alizarin red-S.

B. OTHER TECHNIQUES FOR DEMONSTRATION OF CALCIUM SALTS
   1. Microincineration.
   2. X-ray contact microradiography.
   3. X-ray projection microradiography.
   4. Autoradiography.
   5. Fluorescence microscopy, after treatment of the animals with tetracycline.

C. DEMONSTRATION OF ALKALINE PHOSPHATASE with the technique of RUTENBERG (1966).

D. DEMONSTRATION OF CARBOHYDRATES AND RELATED SUBSTANCES.
   1. Staining with the Mc Manus (PAS)-method, with and without diastase treatment.
   2. Staining with alcian blue.
   3. Staining with toluidin blue.

A. Staining techniques for calcium salts

1. *Calcium staining according to von Kossa*

The VON KOSSA technique (1901) is a classical method for indicating calcium salts in animal tissues. Contrary to former opinions, this method is not specific for the detection of calcium ions. The staining is based on binding silver ions with phosphates and carbonates into insoluble salts. Nevertheless, this so-called substitution technique is a useful method in histological practice. We applied the following modification of the von Kossa staining method.

a. After deparaffinizing the sections are placed in aqua distillata.
b. Subsequently they are treated for one hour with 5% $AgNO_3$ in daylight.
c. Rinsing with aqua distillata.
d. Rinsing half a minute in $2\frac{1}{2}$% $Na_2S_2O_3$.
e. Rinsing in tap water for 5 minutes.
f. Counterstaining with 10 × diluted hematoxylin (the counterstaining with hematoxylin was not performed in all sections).
g. Dehydration and clearing, mounting in Eukitt.

2. *Alizarin red-S staining*

The alizarin red-S staining is a histological technique for demonstrating the presence of calcium salts in young growing bone. This staining technique was first applied to histological sections by DAHL (1952).

The staining of the sections was performed as follows:

a. After deparaffinizing the sections are placed in aqua distillata.
b. Staining with aqueous ($\frac{1}{2}$%) alizarin red-S for ten minutes.
c. Rinsing in aqua distillata for one minute.
d. Rinsing in differentiation-fluid for 5 minutes.
e. Rinsing in tap water for 10 minutes.
f. Dehydration and mounting in Eukitt.

Alizarin red-S = Alizarin Na-sulphate.

Dilute half a gram of alizarin Na-sulphate in 45 ml. aqua distillata.
Bring the solution up pH 6,5 with 25% NH_4OH and dilute 1 : 1 with aqua distillata.
This stock solution is stable for one month.

Differentiation-fluid.

$\frac{1}{2}$ ml. 5% acetic acid.
50 ml. alcohol 96%.

B. Other techniques for demonstration of calcium salts

1. *Microincineration technique*

The technique of microincineration (POLICARD 1938) is based on the principle that by heating microscopical sections all organic material is oxidized and only inorganic material remains. This technique can be used to show the presence of different mineral salts. The sections so treated are called spodograms. We used this technique to strengthen

the polarizing properties of the calcite-containing statoconia. Irregularities in the crystal structure may disappear on heating, and consequently the birefringent effect of the tiny stones as a whole increases.

A maximal polarization effect of the statoconia was achieved by heating the paraffin sections in an oven at a temperature of 450 °C. After cooling down the sections were enclosed in Eukitt without any further treatment.

2. *X-ray contact microradiography*

X-ray contact microradiography of the undecalcified and unstained sections was done on high resolution photographic emulsions. For this procedure the sections were stuck, with diluted glycerin-albumin, to Mylar polyester film type C, 3,8 μ thick (Du Pont de Nemours, 's-Gravenhage, Holland). This polyester film is further referred to as Mylar film. To stretch the film we fastened it between two teflon rings in the following way:

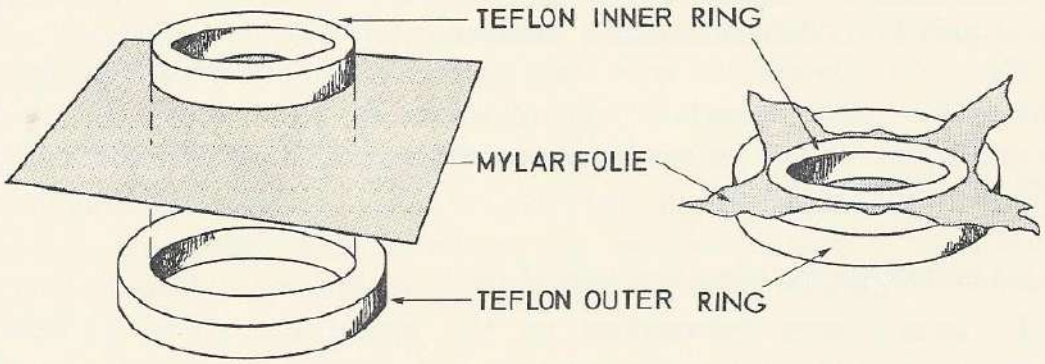


Fig. 2.

After deparaffinizing a microradiograph was made, for which Eastman Kodak 649–0 Spectroscopic film was used. A Philips CMR 5 apparatus served as x-ray source. The focus-film distance was 15 mm. The tube voltage and amperage were respectively 4 kV and 2 mA. The exposure time was 4 min. The films were developed in Kodak D 19 at 20 °C. The photographs were mounted and further treated as a microscopic section. The specimens were studied microscopically with magnifications up to 300 times.

3. *X-ray projection microradiography*

The x-ray projection microradiography was carried out on a TPD[1]) röntgen-projection microscope at the Anatomic-Embryologic Laboratory of the University of Amsterdam. A description of the principles and construction of the x-ray projection microscope is given by Ong Sing Poen (1959).

Boersma [2]) (1969) recently described some applications of this radio-

[1]) TPD = Technisch Physische Dienst TNO-TH, Delft Holland.

[2]) The use of x-ray projection microscopy for medical-biological purpose was recently introduced in our country.

graphic method in morphology. With the newest development of the "Delft microscope" it now is possible to produce x-ray microphotographs of objects in vacuum as well as under atmospheric conditions. Stained or unstained sections with a very wide range of thickness may be used, as well as still larger specimens. A resolution of less than 0,3 μ and a primary magnification on the photographic material of from 2 to about 150 times can be obtained.

Thin unstained sections from the same series as described on page 17 were put on Mylar film in the same way as mentioned in the previous paragraph.

The tube tension, strength of current, exposure time and target material were adapted to the specimens in order to obtain optimal results. Thin sections and dry objects were placed in vacuum; total mounts were photographed under atmospheric conditions.

Exposures were made on Gevaert Duplo Ortofilm. The films were developed in Kodak D 76 for 7 minutes by 20 °C.

The x-ray micrographs were then studied under a conventional light microscope using (secondary) magnifications up to 50 times. A larger magnification was of no use because in that case the grains of the film will obscure the details.

4. *Autoradiography after injection of* Ca^{45}

To obtain more information on the mechanism of the calcium metabolism of the statoconia, pregnant and juvenile mice were injected intraperitoneally with saline containing 0,4 μC α-radioactive calcium (Ca^{45}) per gram body weight. After 24 hours the embryos and the juvenile mice were decapitated; the heads were fixed and embedded as mentioned above.

Autoradiographs (Kodak AR 10 stripping film) were made of every fourth section. The exposure time was three weeks. After development (Kodak D 19) and fixation, the preparations from the older animals were decalcified through the stripping film with 5% EDTA[1]) (pH7), rinsed and stained with methylgreen-pyronin.

The other sections in these series were stained with the von Kossa method, alizarin red-S, the PAS technique, alcian blue or submitted to microincinaration.

The petrous bones of the pregnant mice injected with radioactive calcium were also used to investigate the calcium metabolism.

5. *Fluorescence microscopy, after treatment of the animals with tetracycline*

Tetracyclines, when present in the body fluids are adsorbed to every bone surface and are released if the concentration in the body fluids

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[1]) EDTA = Ethylene-diamino-tetra-acetate.

diminishes. In growing bone, however, the tetracycline is incorporated by new deposits of calcium salts and this prevents the antibiotic from leaving the bone (STEENDIJK 1964). The tetracycline deposits are manifested under the fluorescence microscope with excitation in proximal ultraviolet light as a yellow fluorescence.

To obtain the information on the binding properties in growing statoconia we performed two experiments.

In the first experiment adult mice at different stages of pregnancy and juvenile mice (fig. 1) were injected intraperitoneally with oxytetracycline in a dose of 100 mg per kg body weight, 24 hours before sacrifice.

In a second series, mice of 17 and 19 days of pregnancy were also injected with the same dose of oxytetracycline. After birth the young ones were decapitated at the age of 2, 8 and 16 days post partum. The specimens of both series were prepared in the manner described above. The histological sections were examined unstained under the fluorescence microscope.

C. Demonstration of alkaline phosphatase

Alkaline phosphatase was localized with the technique described by RUTENBURG (1966). This technique was modified in such a way, that the counterstaining was made by a Feulgen method with cold hydrolysis, according to JAMES (1965).

D. Demonstration of carbohydrates and related substances

1. *PAS staining*

Carbohydrates and related substances were demonstrated by oxidizing the sections with $\frac{1}{2}$% periodic acid for 30 minutes, after which the aldehyde groups formed, were stained by means of the Schiff reagent, prepared according to LONGLEY (1952). To differentiate clearly between glycogen and other PAS (Periodic-Acid-Schiff) positive substances, every third section in this staining series was treated with 5% diastase (BDH) before staining by the PAS method or the section was only treated by the buffer in which the diastase was dissolved.

2. *Staining with alcian blue*

This staining material that mainly reveals protein-polysaccharide complexes was applied in a 1% aqueous solution for 30 minutes. The staining was applied both separately and in connection with the PAS technique.

3. *Staining with toluidin blue*

The acid protein-polysaccharide complexes were also stained with toluidin blue in a aqueous solution for 60 minutes.

Electron microscopy

Beside the light-microscopical investigations, the ultrastructural aspects of the statoconial membrane were studied on electron micrographs made by means of a Siemens Elmiskop 1A.

The skulls of the mice were fixed in 4% glutardialdehyde in cacodylate buffer (pH 7,1) for 3 hours and stored in the same buffer to which sucrose was added in order to obtain the same osmolarity as the fixing solution. After postfixation in 1% osmiumtetroxide in phosphate buffer (pH 7,3) the specimens were dehydrated in an alcohol series followed by propylene oxide, and finally embedded in a standardized position in Epon 812. Sections were made with an LKB Ultrotome and stained with uranyl-acetate and lead tartrate.

Chapter IV

MICRO-ANATOMICAL ASPECTS OF THE MOUSE LABYRINTH

Introduction

The membranous labyrinth is a complex system of cavities and canals that in general shows the same morphology in all mammals. However, the data concerning the stereoscopic anatomy of the labyrinth of the mouse to be found in the literature were too restricted to allow a correct interpretation of the two-dimensional histological sections. It became clear that some extension of the anatomical knowledge of the labyrinth of the mouse was necessary. Therefore, before passing on to the description of the results of the histological investigations we will devote this chapter to the border region between microscopic and macroscopic anatomy.

Material and methods

The material of this part of the investigation consisted of petrous bones of adult mice of the same strain (RQ) as used for the histological part.

After sacrifice, the skin was removed from the skull, the cranial cavity was opened and its contents were removed. Immediately afterwards, the skull was immersed in alcohol 80% at 4 °C. In this fixative the specimens were kept in the refrigerator for 24 hours. The microdissection was performed under a Zeiss operating microscope with ENT middle ear instruments and home-made fine needles and rasparatories which were made to meet specific demands. All manipulations were always performed below the surface of the alcohol as specimens can be preserved indefinitely in this fluid if they are protected from drying.

The inner ear was approached from the medial towards the lateral aspect. The orientation within the cranial cavity of the mouse is not difficult, because the bony superior and posterior semicircular canals protrude from the petrous bone with a slight bulging. The bony surrounding of the vestibulum was carefully scraped away dorsally by means of a fine rasparatory until a thin shell remained. After that the thin shell of bone was gradually removed with fine needles, beginning over the posterior part of the utriculus (fig. 3). The first perforation of the bony capsule will show the brown to dark brown spotted membranous labyrinth. Over the medio-anterior surface of the utriculus the spots are not seen because of lack of pigment in this area. The utriculus and cristae are easily exposed in this way without severe damage (plate 2). The macular side of the sacculus, however, is firmly attached to the medial wall of the bony otic capsule and is seldom freed from the surroundings without damaging the sagitta.

The names given to statoliths derive from the original morphological description of the statoliths of the carp. The urticular stone was called *lapillus* or little stone, the saccular stone *sagitta* or arrow, and the stone of the lagena *asteriscus* or little star. Later on these names were applied to all fishes. QUIX and WERNDLY (1924) were the first to apply these terms to the macular covering membranes of mammals. In the official P.N.A. anatomical terminology, as mentioned in the "Nomina Anatomica" (1966) the macular covering membrane is called "*Membrana statoconiorum*". No special name is given to the "*membrana statoconiorum maculae utriculi*", nor to the "*membrana statoconiorum maculae sacculi*". Following the proposal of Quix and Werndly, in this thesis the names lapillus and sagitta will be used respectively for these structures in order to avoid a too complicated terminology.

Finally the preparations were measured under the operating microscope for morphological reconstruction. The lapillus and sagitta were removed in toto and studied with a polarizing microscope. The isolated macular membranes were then divided into three groups for different purposes, i.e.:

a. X-ray contact microradiography.
b. X-ray projection microradiography.
c. X-ray diffraction.

a. *X-ray contact microradiography*

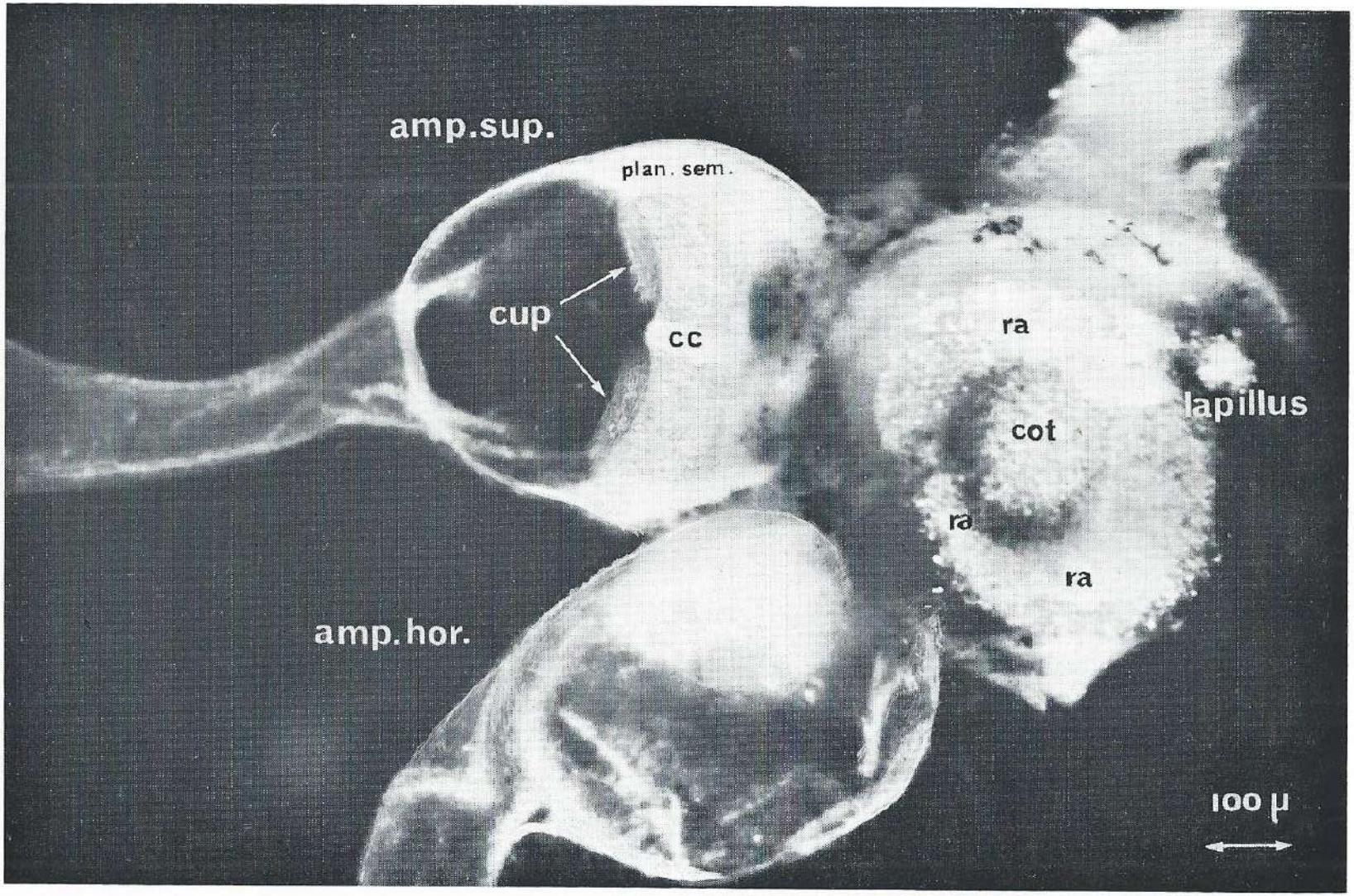
In order to make x-ray contact microradiographs of the total lapillus, the prepared covering membranes were placed into distilled water via an alcohol series. After this, the membranes were mounted with albumin-glycerin on stretched Mylar film, as described in chapter III. Subsequently, the dried sensularia were photographed once more under the polarizing microscope to study any effect of drying on the localization of the statoconia in the macular membrane. Finally, an x-ray contact microradiograph (plate 3B) was made in the same way as described in chapter III, however, this time with an exposure of 10 minutes.

b. *X-ray projection microradiography*

The x-ray projection microradiography was carried out with the modified Delft microscope (see chapter III). The macular covering membranes were prepared in the same way as for x-ray contact microradiography and, mounted on stretched Mylar film, brought into the vacuum chamber of the microscope.

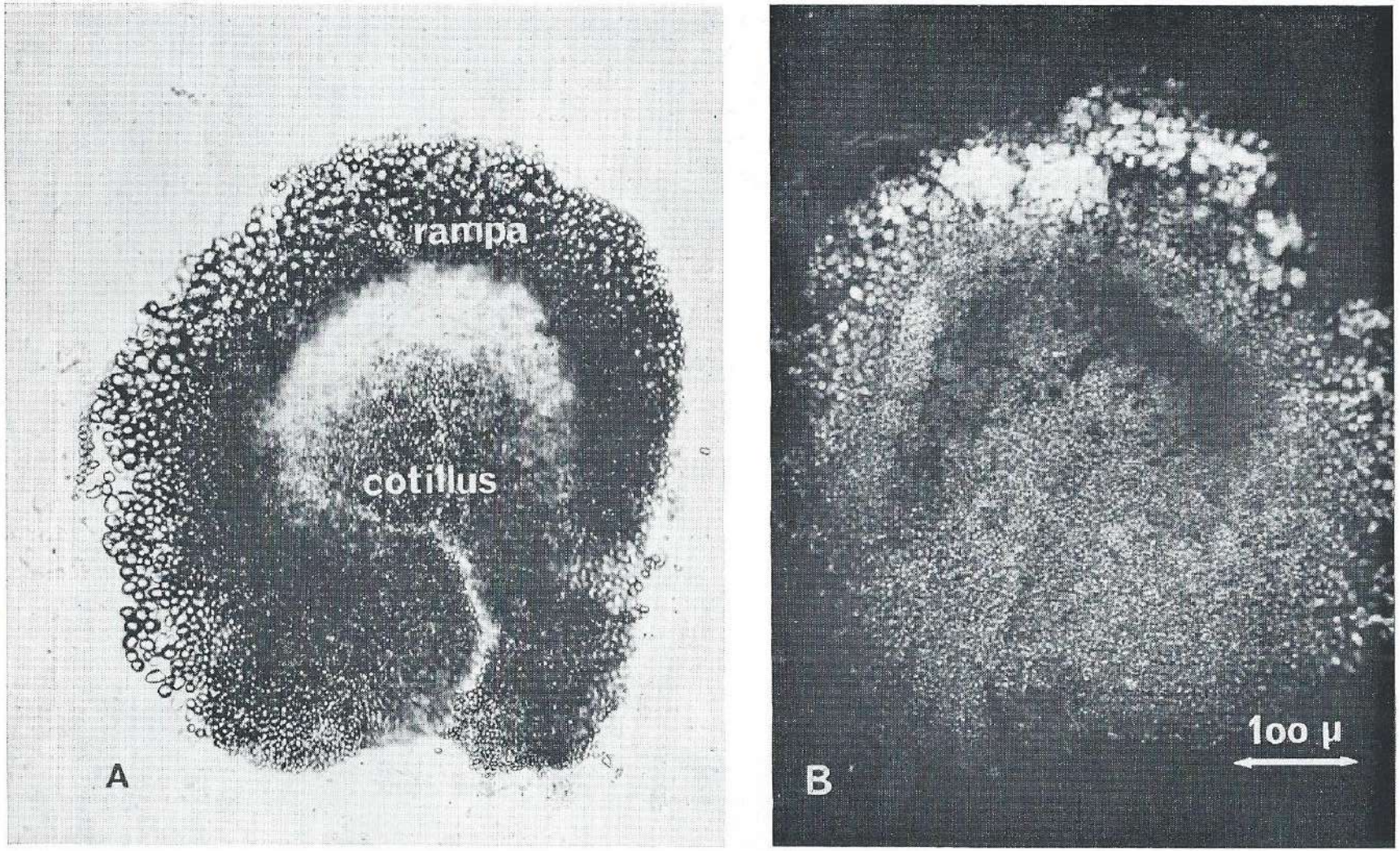
Exposures of the specimens were made under the conditions specified in Chapter III. The primary magnification ranged from 5 to 10 times, with a subsequent magnification of 25 times (plate 4).

X-ray projection microradiographs of the total petrous bone were made as well; as there is a large depth of focus with this instrument, due to the point-projection principle (COSSLETT and NIXON 1960, COSSLETT 1966, BOERSMA 1969), we were able to make sharp stereoscopic photomicrographs of the complicated system of cavities and canals in the labyrinth of the adult mouse.



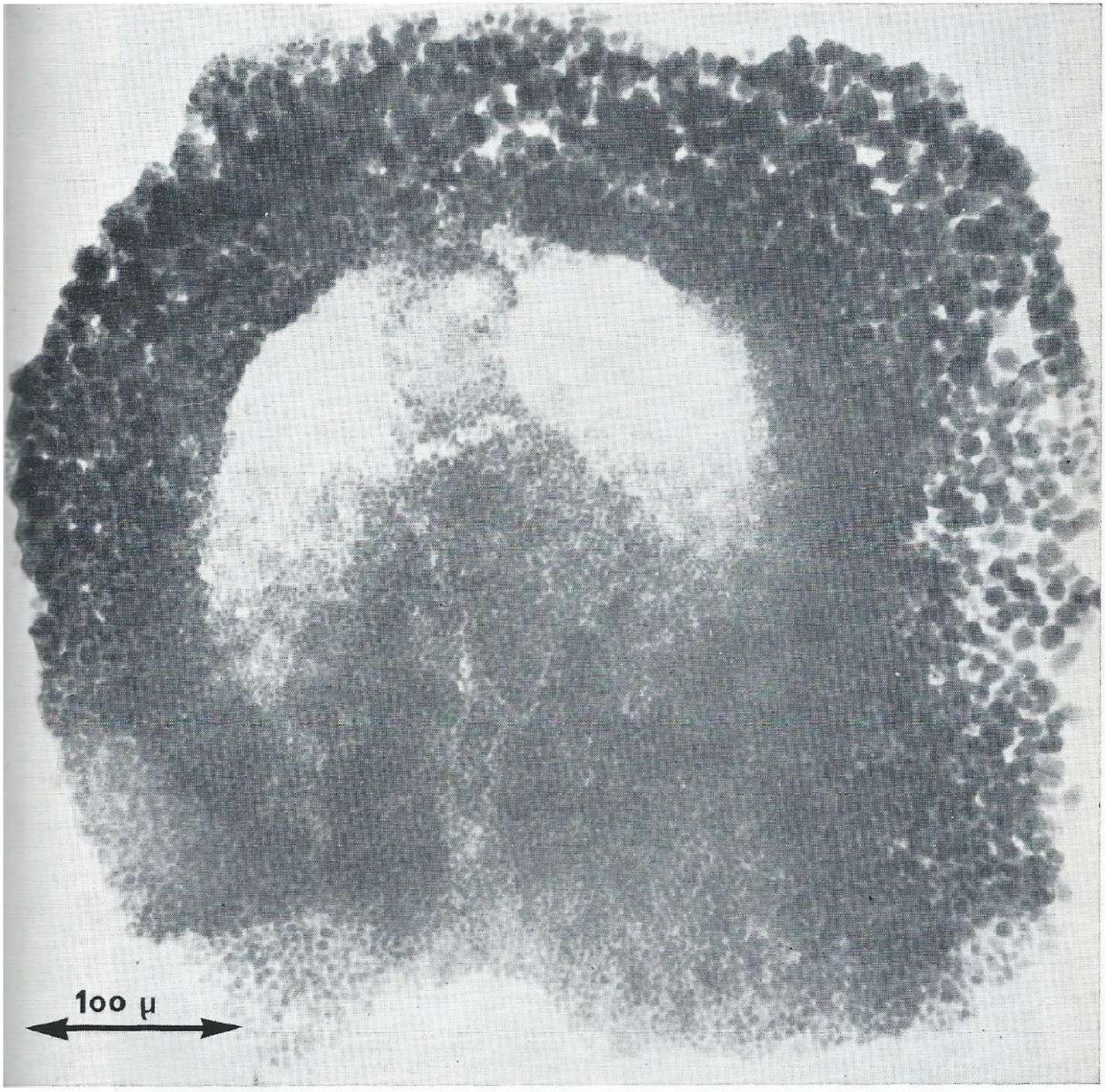
Microdissection preparation of the labyrinth of an adult mouse. The utricular statoconial membrane (lapillus) is prepared free together with the horizontal and superior ampulla. cup, cupula; plan. sem., planum semilunatum; cc, colliculus cristae; ra, rampa; cot, cotillus.

PLATE 2

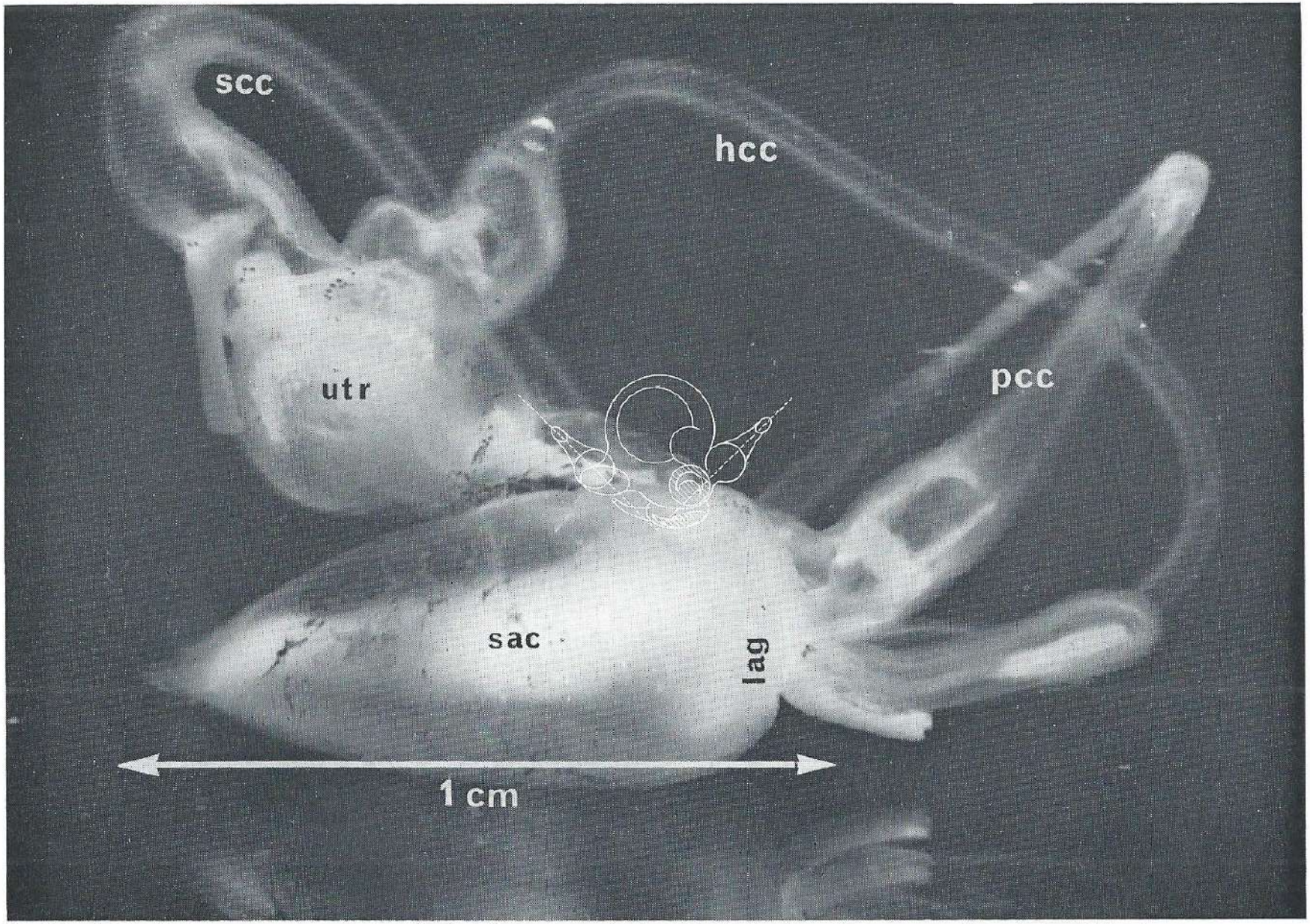


Utricular statoconial membrane of an adult mouse. **A**, light-microscopic micrograph showing the highly refractile stones with polarization only; **B**, x-ray contact microradiograph.

PLATE 4



X-ray projection microradiograph of a total utricular statoconial membrane of an adult mouse. The photograph clearly shows the differences in size of the stones in the various parts of the statoconial membrane.



The labyrinth of the mouse compared with the dimensions of the labyrinth of a pike, as seen from below. The inset represents a schematic drawing of the mouse labyrinth, as seen from above. hcc, horizontal semicircular canal; scc, superior semicircular canal; pcc, posterior semicircular canal; utr., utriculus with lapillus; sac., sacculus with sagitta; lag, lagena with asteriscus. Note the absence of a clear border between sacculus and lagena in the labyrinth of the pike; the sagitta and asteriscus are lying in the same sac.

c. *X-ray diffraction*

Some microdissection preparations of the macular covering membranes were used for investigation of the crystallographic structure of the statoconia of the mouse. The crystalline structure of the sagitta of a pike was also analyzed in order to compare the various structures which were to be expected.

These techniques were carried out by Mr. A. Kreuger at the Crystallographic Laboratory of the University of Amsterdam.

The results of these investigations will be dealt with at the end of chapter V.

Some anatomical considerations on the labyrinth of the mouse

The mammalian inner ear has two major subdivisions, i.e.: the superior part, consisting of the canal system with the utriculus, and the inferior part, consisting of the sacculus and the cochlea. The semicircular canals, utriculus and sacculus form the vestibular portion of the inner ear. This thesis is mainly concerned with the statoconia of the utriculus and sacculus. Certain micro-anatomical features of the lapillus and the sagitta, however, are better understood in relation to the entire membranous labyrinth. In the drawings and photographs most other parts of the membranous labyrinth will be represented as well; the text however, will be restricted to the description of features of the utriculus and the sacculus (fig. 3).

The *utriculus* is an elongated, flat, thin-walled tube of about 1300–1400 μ length, lying lengthwise in a naso-occipital direction. The cross section has an oval form with the greatest diameter medio-laterally. The tube contains in its antero-medial part a patch of sensory epithelium, the *macula utriculi*, which lies almost in one plane, making an angle with the base of the skull of approximately 30° and with an almost perpendicular position to the median plane. The form of the macula is flat and nearly round, it has a diameter of 450–500 μ. The macula utriculi is covered by a jelly-like structure, the *lapillus*, containing the glittering statoconia in large numbers. The crystals of the lapillus are different in size, and form a special pattern over the sensory epithelium. Over the periphery of the macula big stones are found, and over the mid-portion and the median margin of the macula, stones of smaller size are localized (plate 3, 4).

The epithelium of the utricular macula is thickened over a horseshoe-like area, the *eminentia striolaris*, at a certain distance from the macular border. This special area is represented in the macular membrane, the lapillus, as a sickle-like area in which statoconia are lacking (plate 3, 4). The striola divides the utricular macula in an outer zone, the *rampa*, and an inner zone, the *cotillus*. The rampa surrounds the cotillus except on the medial side. The lack of statoconia over the striola is particularly pronounced in the lateral part.

The wall of the utriculus is heavily pigmented by chromatophores in the perilymphatic space directly surrounding the epithelium. This pigmentation is missing below the macula and over the macula in the roof of the utriculus.

The second cavity containing statoconia is the *sacculus*. This epithelial sac lies opposite the oval window, and is firmly connected by connective tissue at the macular side to the medial wall of the bony vestibulum.

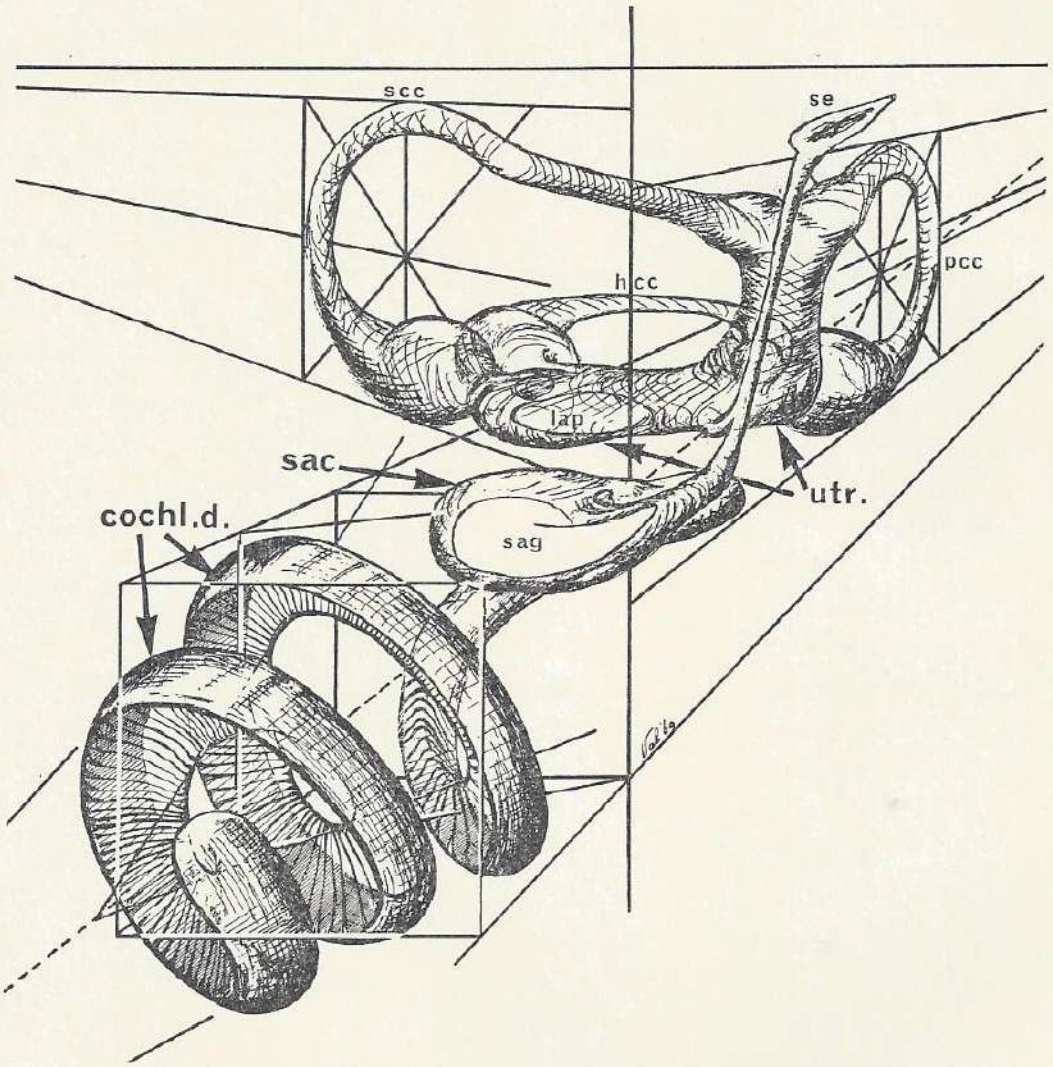


Fig. 3. Drawing of the right membranous labyrinth of the mouse as seen from the medial side, based on micro-anatomical preparations. utr., utriculus; sac., sacculus; cochl.d., cochlear duct; sag, sagitta; lap, lapillus; hcc, horizontal semicircular canal; scc, superior semicircular canal; pcc, posterior semicircular canal; se, endolymphatic sac.

The upper wall of the sacculus is attached to the under side of the anteromedial part of the utriculus. The lateral wall of the sacculus lies free between endo- and perilymph and has no anchorage by strands of connective tissue, as in the case of the utriculus and the semicircular canals.

The form of the saccular macula is quite different from the form of the utricular macula (plate 1, 5). Roughly, this macula is oval and lies in a sagittal plane with its longest diameter (650 μ) in a naso-occipital direction. From its antero-superior border a small oval patch protrudes in an occipital direction. The plane in which this small oval protrusion is found makes a rounded angle of 120° with the big oval patch.

In the microdissections the macular membrane of the sacculus is less clearly visible than is the case in the utriculus. The statoconia of the sacculus are smaller in size than the statoconia of the utriculus. With the magnification of the dissecting microscope used by us (up to 40 times), no local size differences in statoconia could be observed; the entire macular membrane of the sacculus has a homogeneous aspect.

Chapter V

RESULTS

In this chapter we shall describe our histological material in the same sequence as the techniques employed are dealt with in chapter III. Before a detailed description is given, a brief general outline of the histogenesis of the statoconia as seen in our material may be useful.

The first trace of crystalline calcium salts in the statolith sacs in mice was found in 14 days old embryos as minute birefringent particles. The crystalline particles then increased in number and in size in a few days, until a sensularium was formed of definite size and structure. This period of calcium deposition in the sensularium of the maculae lasted about 6 days. The greatest activity took place on the 15th and 16th days of embryonic life, whereafter the activity gradually diminished, until finally growth of the statoconia had finished some days after birth.

Sections stained according to von Kossa

The macular sensularia in all specimens of our material showed a negative reaction with the von Kossa staining method. Apparently, this cannot be attributed to a technical defect in our handling of the von Kossa slides, as the developing bone in the cap and base of the skull showed a highly positive reaction. Embryos of 15 days gestation showed yellow-brown staining in places of calcifying bone, while the staining intensity of the skeleton of the skull progressed with age into dark-brown to black. All this was to be expected with a well balanced von Kossa technique.

The von Kossa stained slides were also studied under the polarizing microscope. The statoconial layers showed anisotropy, in which each statoconium passed from darkness to brightness and back again in every quadrant when the section was rotated through 360° degrees between crossed polarization filters. This polarization phenomenon was observed for the first time in sections of 15 days old embryos. Under the conventional microscope the statoconia could be distinguished in this stage as highly refractile particles. The calcified skeleton showed no birefringent properties *with this method*.

Alizarin red-S staining

From 16 days onwards, the statoconia stained red, as did the calcifying parts of the skeleton of the skull. Under the fluorescence microscope, however, the stones did not show the characteristic yellow-orange fluorescence with blue-violet excitation light, which was observed clearly in the growing bone tissue of the skull. It thus seems that the red staining

of the statoconia is aspecific and bears no relation to the calcium localizing effect of alizarin red-S; in fact it is as negative as the von Kossa staining.

The alizarin red-S sections showed birefringent properties of the statoconia for the first time in 16 days old specimens. As compared with sections treated with other techniques, the statoconia seemed to be diminished in size by this staining method. This difference became less clear in sections from older embryos or from specimens after birth; the negative fluorescence of the red stained sensularium remained the same, however.

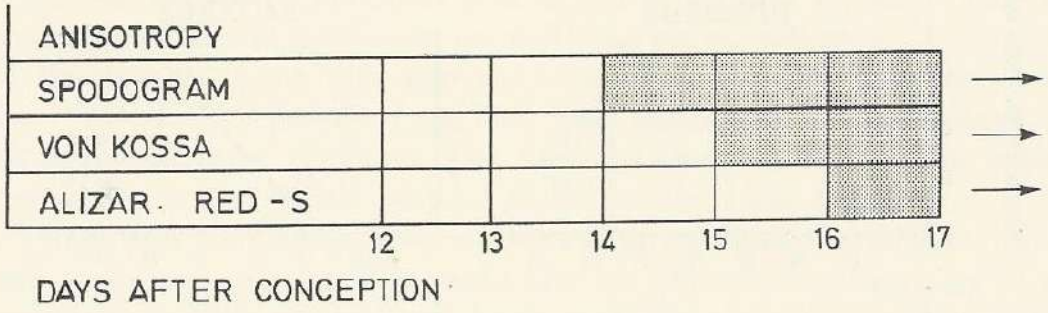


Fig. 4. Explanation see text.

Microincineration

Spodograms from sections of the earliest available embryos, i.e. from 12 and 13 days gestation, did not show any birefringence in the macular sensularia.

Specimens one day older showed minute bright spots between crossed polarization filters. The statoconia in 15 days embryos showed typical anisotropy (fig. 4); at this stage the stones were spread already over the entire macula of utriculus and sacculus. The forms of the statoconia were different from those in older animals. They had a dumb-bell-like shape, consisting of two identical spheres connected by a short rod; the spheres were also formed separatedly. There was little variation in the size of the spheres and in the size of the dumb-bells as a whole (plate 8).

The 16 days old embryos were the first to show the same forms of the maculae and their covering membranes as adult mice; the sizes, however, were smaller. In the same way the statoconia showed adult forms, i.e. more or less spindle-shaped forms (plate 9).

Especially in the utriculus, there was a great variation in size of the statoconia at the older ages. On the contrary, the stones of the sacculus seemed to be of more constant size, and remained smaller in size in comparison with those of the utriculus. The dimensions of the utricular stones thus showed a definite correlation with their localization over the macula. This is clearly demonstrated in plate 3 and 4, showing a low power view of an undecalcified lapillus of an eight month old mouse.

The stones of the cotillus are small, like the stones on the striolar side of the rampa. The biggest stones lie on the peripheral side of the rampa.

In specimens of different stages a clear increase in size and number of the statoconia could be observed with age. In order to follow the growth quantitatively, the following data were collected.

The contours of the statoconia were drawn with a magnification of 500 times by means of a camera lucida; subsequently, the surface area of each statoconium contour was determined by means of an OTT-planimeter. In this way data were collected from embryos of 16, 19 and

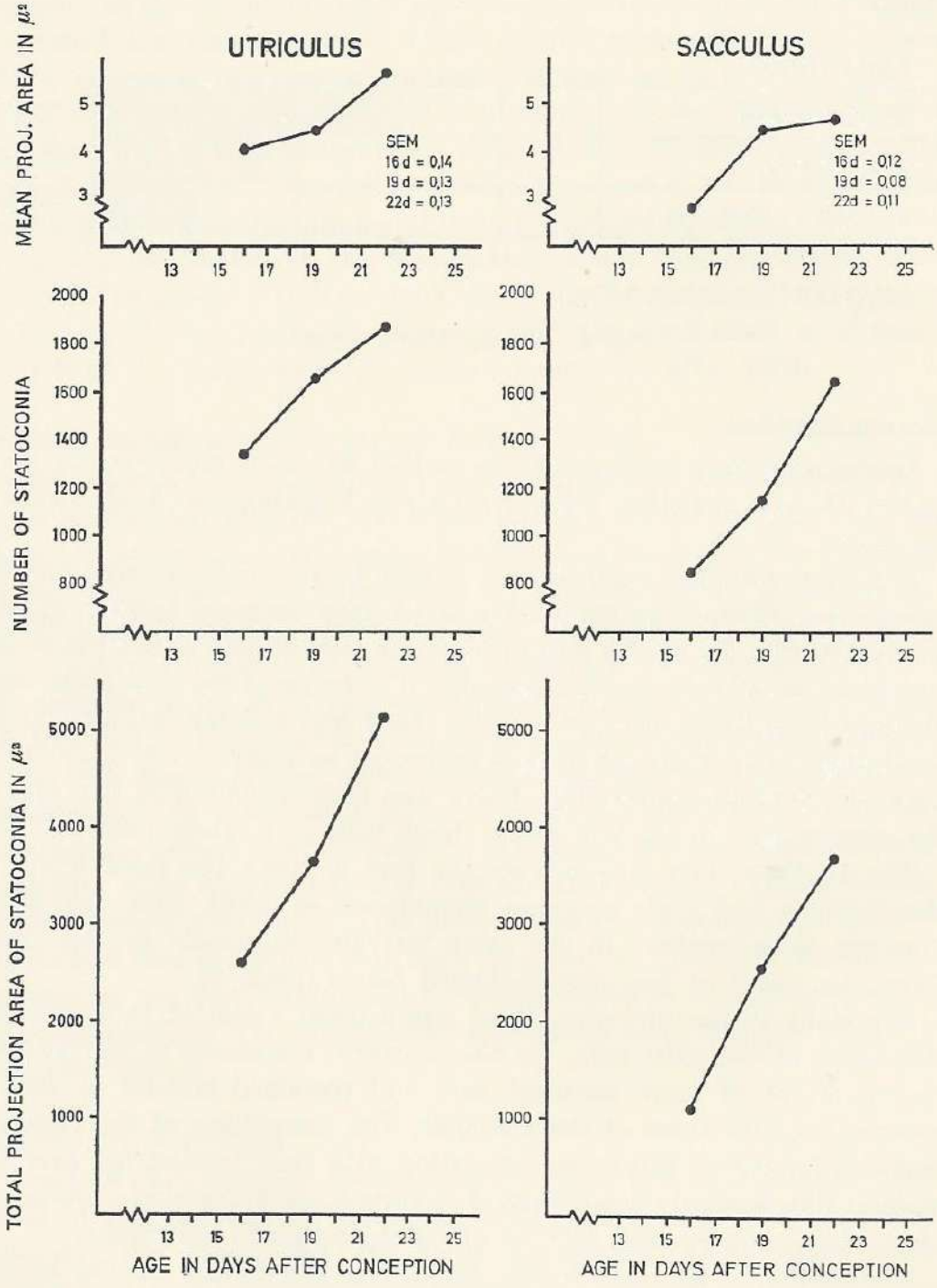


Fig. 5. Explanation see text.

22 days, which gave a rough idea of the size of the stones at these different stages. From two different animals of each age one section was used. Sections going just through the middle of the lapillus were selected for this investigation.

The drawing of the contours and the measuring of the areas of hundreds of statoconia per macula is an exceedingly time-consuming and laborious technique, which makes a more extensive investigation virtually impossible. However, with the limited samples taken, a few things emerged clearly enough (fig. 5).

The calculation of the mean area of the statoconial silhouette and of the total number of statoconia per section gives an indication of increase of the weight of the utricular and saccular statoconial membranes in two ways, i.e. by *growth of the individual stones* and by *enlargement of the total number of statoconia*. The utricular stones show a larger mean projection area than the saccular stones at the same stages.

As no real volumetric determinations can be done, these data have only a limited value, but they do give an objective confirmation of a general impression we got from microscopic investigations, namely, that a continuous growth of utricular and saccular stones takes place until shortly after birth. This period of growth will be called the *crystallization period*.

X-ray contact microradiography

In contact microradiographs statoconia could be clearly distinguished from 15 days gestation onwards.

In 14 days embryos, the region over the maculae was only slightly denser than in sections of 13 days embryos, so that the presence of calcium salts in solid form in this stage could not be ascertained. In sections of embryos of over 15 days gestation, the images of the statoconia acquired the characteristic pattern as described in the previous paragraph.

In contact microradiographs the sharpness of the images of statoconia is limited by the unavoidable penumbra effect, due to the large focus, short focus-film distance and insufficient contact between film and specimen. Consequently the smallest statoconia (1 μ and smaller) could not be visualized with this technique. In addition small x-ray dense images resembling statoconia were occasionally difficult to distinguish from images due to small bone fragments displaced during the cutting procedure.

X-ray projection microradiography

In the x-ray projection microradiography, the penumbra effect is less marked than in the contact method. This is due to the fact that in the TPD Delft microscope the diameter of the x-ray source is extremely small, about 1000 times smaller than the diameter of the focus of the Philips LMR 5 apparatus, used for x-ray contact microradiography. According to Ong Sing Poen (1959) the resolving power in the x-ray

projection method is proportional to the diameter of the source, which is for the Delft microscope 0,1 μ.

On the projection microradiographs of the histological sections some very interesting aspects of the statoconia of the mouse were revealed. In the first place, most statoconia did not show the regular angular forms as seen under the conventional microscope. Only the very large stones presented angular faces, resembling crystals. The smallest stones showed spindle-shaped or ellipsoid forms and had approximately the same size. Medium-size statoconia appeared in the projection microradiographs as consisting of two spherical forms, connected by an x-ray transparent mass and surrounded by a thin, x-ray opaque, shell. These forms will henceforth be refered to as *bipolar forms*. The large statoconia with the angular faces did not have uniform x-ray density; the x-ray cores with low x-ray density in these large forms, however, were not as clearly visible as in the bipolar forms. The faces and angles of all statoconia were rounded rather than sharp as may be expected from the preceding paragraphs. This is not caused by the techniques used, as demonstrated in plate 10, 11 and 12.

The correlation between the size of the statoconia and their position over the utricular macula in these sections was the same as described above in other paragraphs. The bipolar forms are mainly found in the cotillus and in the striolar side of the rampa. The large statoconia were found mainly on the periphery of the rampa (plate 12).

Autoradiography

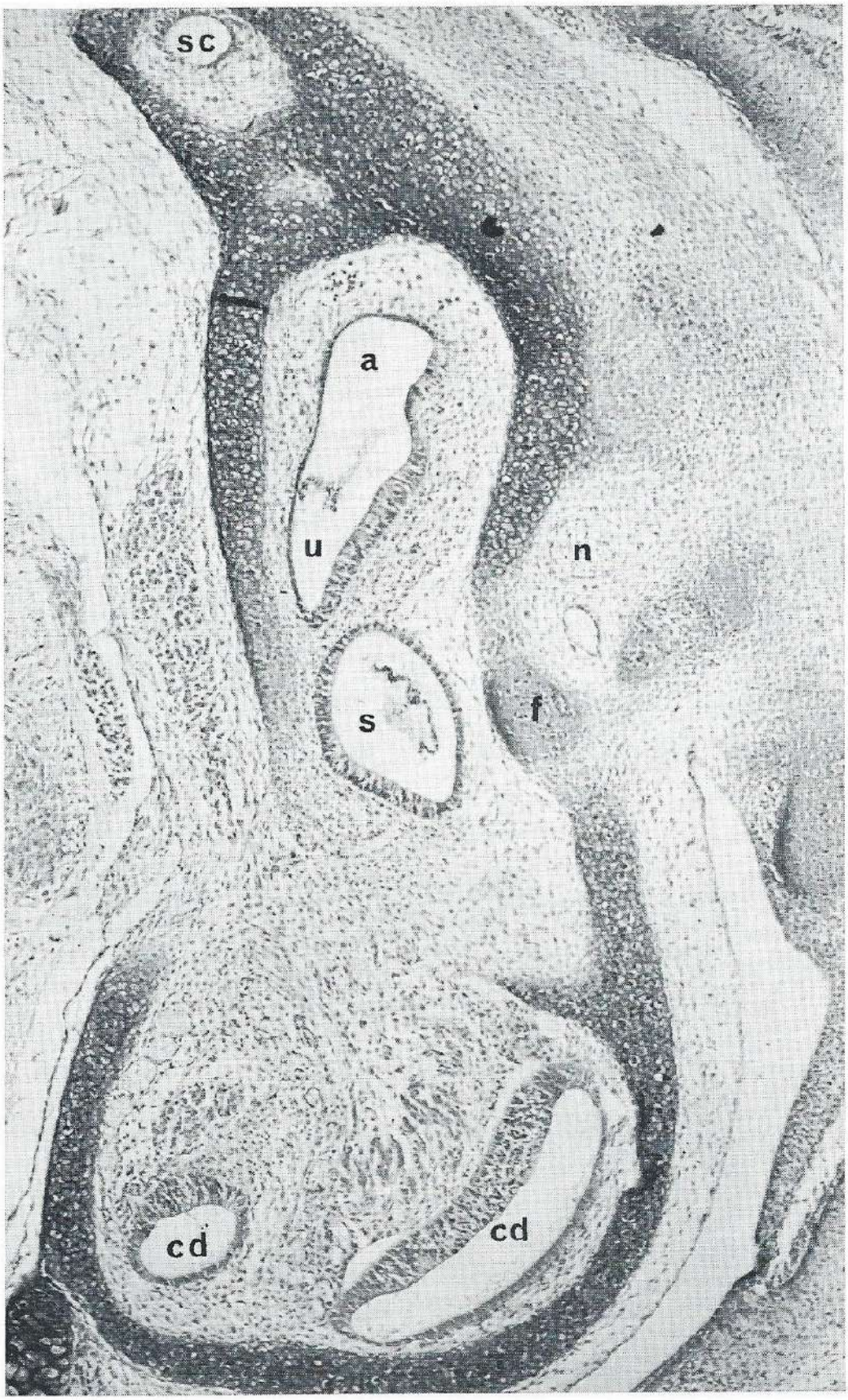
The youngest embryos in which incorporation of radioactive calcium could be observed were 15 days old. The skeleton of the skull showed clear patches of incorporation of radioactive Ca^{45}. The statoconial membranes, however, showed only a faint concentration of grains, as compared with the background. The incorporation in the statoconial area was increased in older embryos; the most intense incorporation was found in 16 and 17 days old embryos (plate 13 and 14).

In 19 days embryos the Ca^{45} activity in the autoradiographs was clearly diminished as compared with animals two days younger. Two days after birth, the audioradiographs barely showed any differences between maculae and background.

In none of our preparations could a concentration of grains over the background density be observed in the macular *epithelium* throughout the whole period investigated. As was to be expected, the growing bone showed an intense incorporation of Ca^{45}.

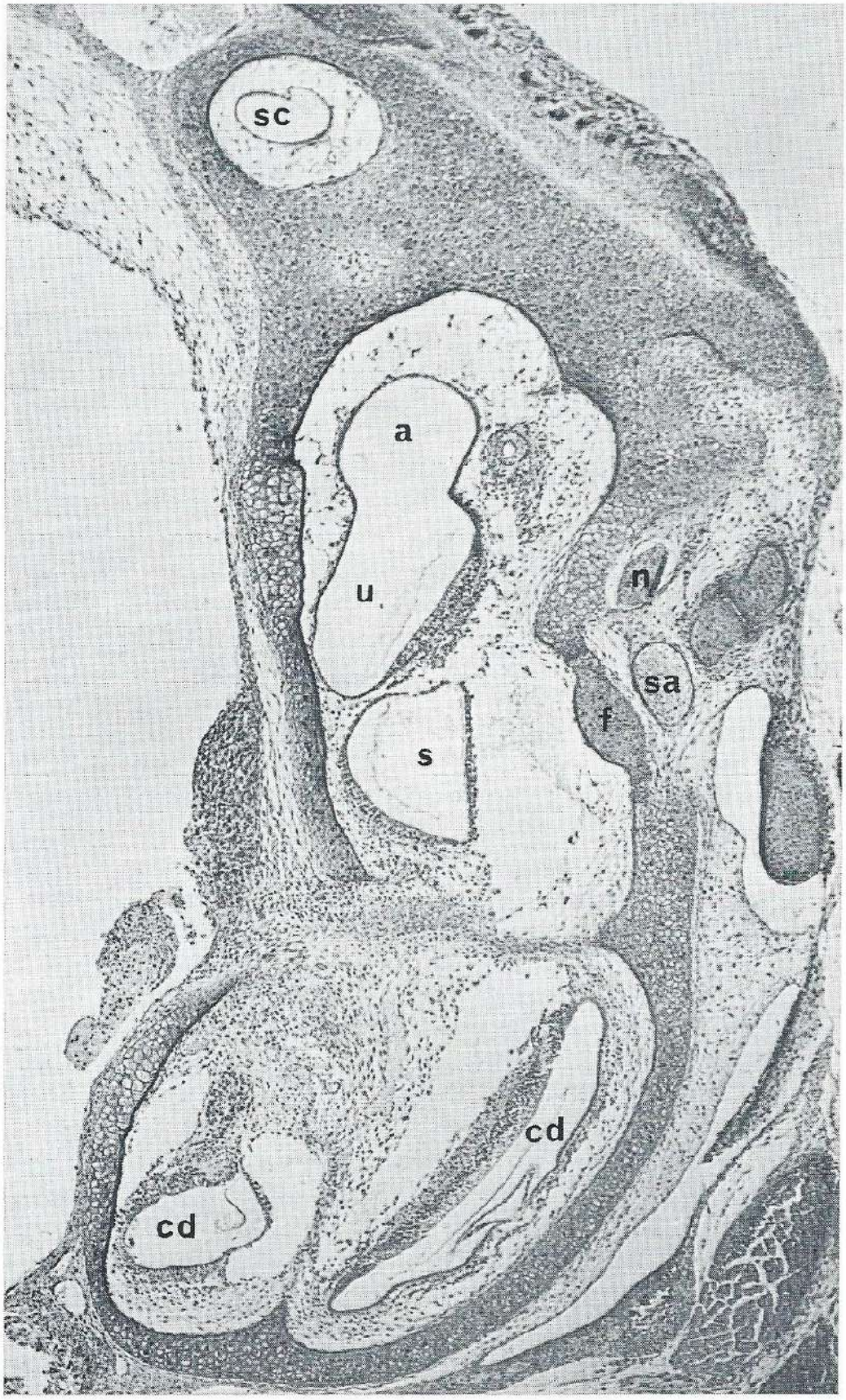
Special care must be taken with the interpretation of these findings. The incorporation of radioactive material takes place during about six hours following the injection. As the injection is given to the mother 24 hours before sacrifice, the results from the autoradiographs refer to the calcium metabolism from 24 to 18 hours before the moment of fixation.

PLATE 6



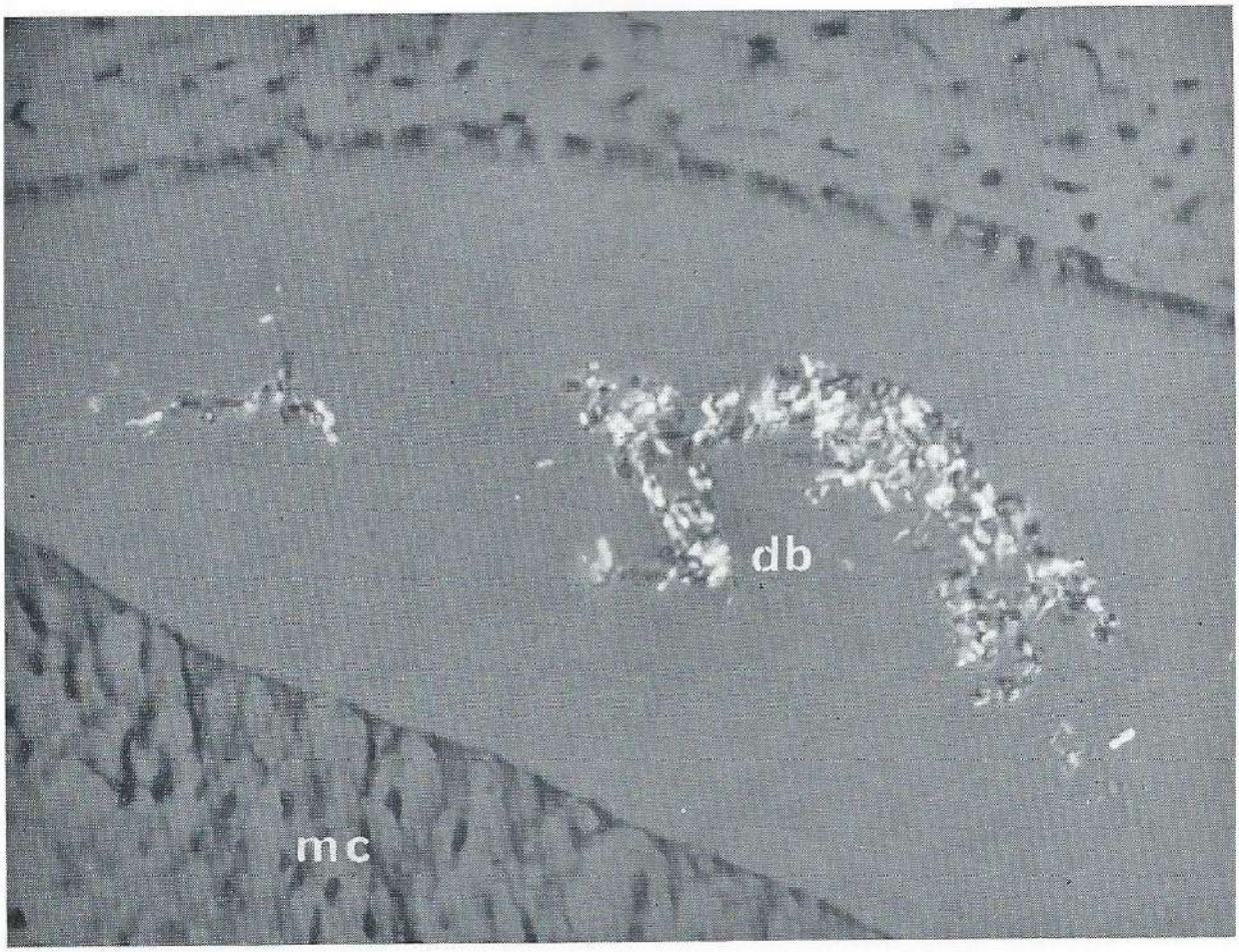
Section of a labyrinth of an embryo of 15 days gestation; alcian blue-PAS, 95×. u, utriculus; s, sacculus; cd, cochlear duct; a, ampulla; sc, semicircular canal; f, footplate of stapes; n, facial nerf.

PLATE 7



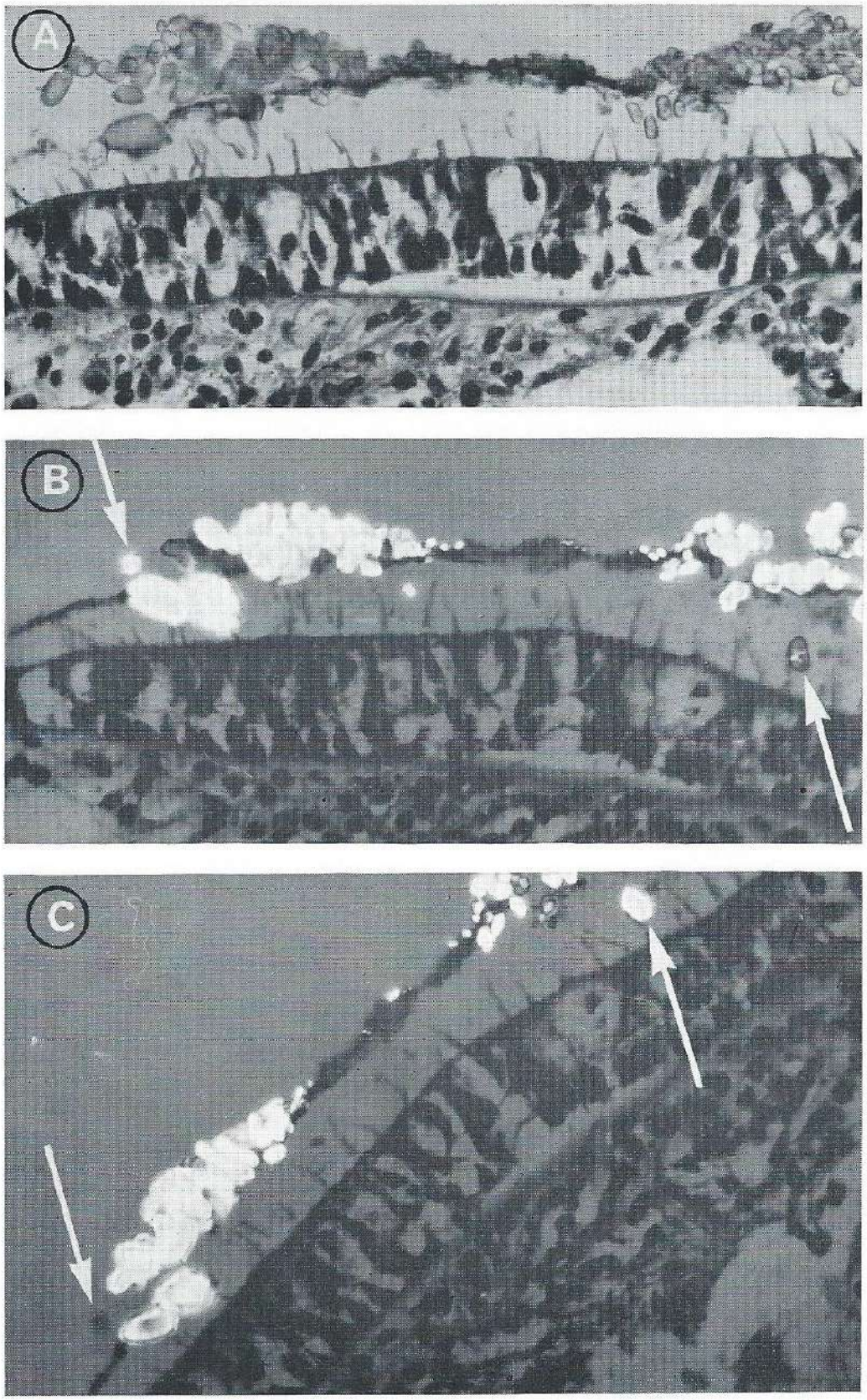
Section of the labyrinth of an embryo of 19 days; 65×. Legends as in plate 6.

PLATE 8



Spodograph of the utricular statoconial membrane of an embryo of 15 days gestation. mc, macula; db, dumb-bell forms. The microphotograph is taken with uncompletely crossed polarizing filters.

PLATE 9



A, photomicrograph with ordinary light of the utricular stones adjacent to the pars lucida lapilli; **B**, adjacent section photographed with incompletely crossed polarizing filters; **C**, the same section with the same light conditions; the specimen, however, is rotated over 45° with regard to the position in B. Note the extinction of the stones as indicated by arrows.

With this in mind, it can be concluded that the absence of grains over the macular areas of 14 days old embryos means in fact that no incorporation of radioactive calcium has taken place in the first six hours of the 13th day of gestation. A second conclusion is that the statoconia are formed and grow after the 13th day of embryonic life and the first postnatal days. Investigation of the macular sensularia of the mother animals, injected with radioactive Ca^{45}, showed a total absence of Ca^{45} incorporation. This points to a complete standstill of the growth in statoconia of mice.

Tetracycline incorporation

Statoconia in sections from temporal bone of mice of all ages in both series of experiments did not show any uptake of oxytetracycline under the fluorescence microscope. On the contrary the growing bone in the skull showed clear incorporation of this antibiotic in both series of animals with the well-known bright yellow fluorescence, when excited with proximal UV-light.

Alkaline phosphatase

The alkaline phosphatase activity, as localized with the Rutenburg method, was found as scattered blue grains in the lumen of the membranous labyrinth and as a faint blue staining of the apical parts of some epithelial cells of the maculae. In general the blue granular mass was found in the vicinity of the blue stained cells. The overall intensity of this blue staining was much less than that found in osteoblastic borders in growing bone of the skull, indicating a low level of activity. The greatest dye-binding occurred in embryos of 13 days gestation. The older embryos showed a gradual decrease of the staining intensity and a sharply localized binding of the blue stain. Finally, only a bluish haze was left over the cells of the eminentia striolaris utriculi and over a few isolated cells of the saccular macula in the neighbourhood of the striola saccularis.

A more detailed description of the localization of alkaline phosphatase in the membranous labyrinth can be given as follows. The embryos of 12 days had blue grains in all lumina of the membranous labyrinth with the greatest concentration in the ampullae, otolith sacs and cochlear duct. At this stage the granular mass was lying mainly against the wall opposite the sensory epithelium. The localization was the same in the 13 days old embryos with a more intense staining affinity, however. Sections of embryos of 14 days showed the first diminishing in the distribution of the blue grains, especially in the semicircular canals. Embryos of 15 days gestation no longer showed any alkaline phosphatase activity in the canals, the ampullae and the cochlear duct. The localization of the blue dye in the otolith sacs was restricted to the by now clearly recognisable utriculus and sacculus. At this age sensularia in the sections were still lying against the wall opposite the macular epithelia.

Carbohydrates and related substances

The organic matrix in which the statoconia were embedded, consisted of a variety of substances of which we demonstrated two groups of carbohydrates by means of histological methods. The Periodic-Acid-Schiff (PAS) reaction stains a heterogeneous group of so-called "neutral sugars". With the basic stains, alcian blue and toluidin blue, acid protein-polysaccharides were demonstrated.

The staining affinity of the macular sensularia was compared with that of the cupula, membrana tectoria cochleae and with the organic matrix of growing bone. These comparative studies were done in each individual histological section.

The PAS positive staining of the macular sensularia appeared in two forms, namely, as a diffuse magenta staining and as more intensely stained granules. In general, both appearances of PAS positive material showed the same localization, although the granules were found in larger numbers in the non-macular area of the sensularium. If the PAS technique was performed after treatment of the sections with a diastase-buffer solution almost no PAS positive granules were found. Treatment of the sections with the buffer solution only gave a clear, but less pronounced decrease of the number of PAS positive granules in the sensularia. It can be concluded, therefore, that these granules are, at least for a large part, composed of glycogen.

We evaluated carefully the intensity of staining of the covering membrane with Periodic-Acid-Schiff and alcian blue.

With respect to the cupulae and tectorial membrane of the cochlea, we observed an opposite affinity for these stains as compared with the statolithic membranes. The results are illustrated in fig. 6 from which it can be clearly seen that the statolithic membranes react PAS positively only in the crystallization period. Another striking feature is the temporary decrease of the affinity for the basic stains in the beginning of the crystallization period.

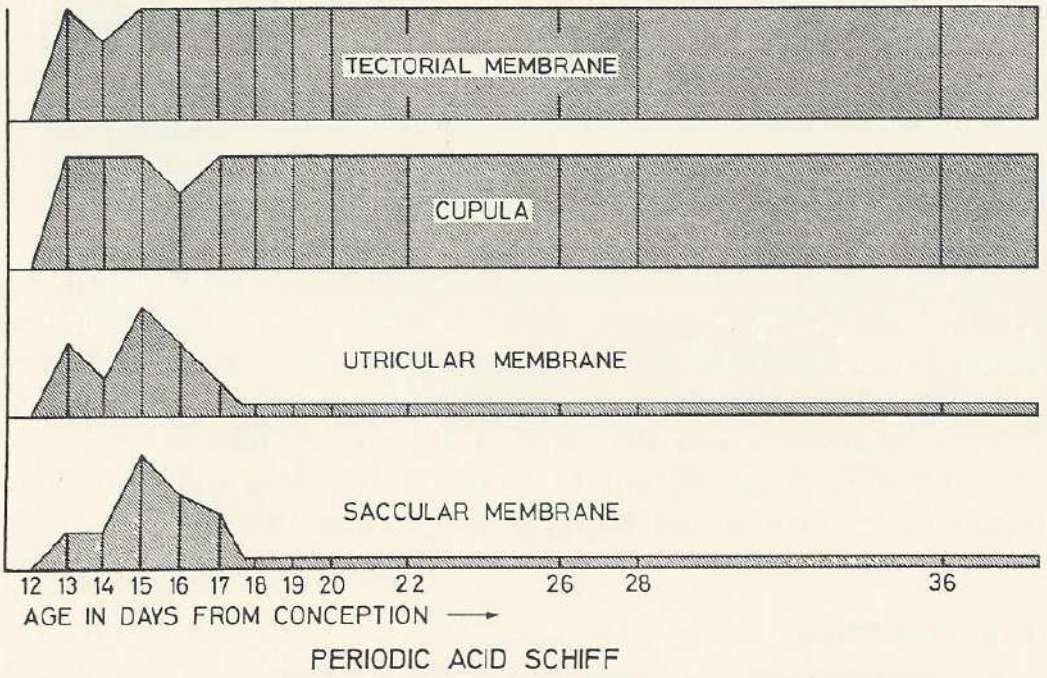
After the crystallization period we found a stabilized dye affinity in the covering membranes. The macular covering membranes showed hardly any reaction with the Periodic-Acid-Schiff staining method and a strong reaction with alcian blue. On the contrary, the cupulae and the tectorial membrane of the cochlea reacted strongly with PAS and showed hardly any reaction with alcian blue.

In sections of older specimens the glycogen containing granules were also found in the sensularia, however, the number was greatly decreased. Toluidin metachromasia was found virtually at the same places in adjacent sections as those in which there was alcian blue positivity.

The staining properties of the carbohydrates and related substances in the macular sensularia are quite different from the staining properties

of these substances in growing bone. In the macular sensularia, there was strong PAS positive reaction solely in the crystallization period, after which mainly affinity for alcian blue and toluidine blue was observed.

In growing bone we first observed a positive reaction with alcian blue and toluidine blue; later on the affinity for the basic stains decreased with increasing degree of calcification, and at the same time the staining intensity of the PAS reaction increased.



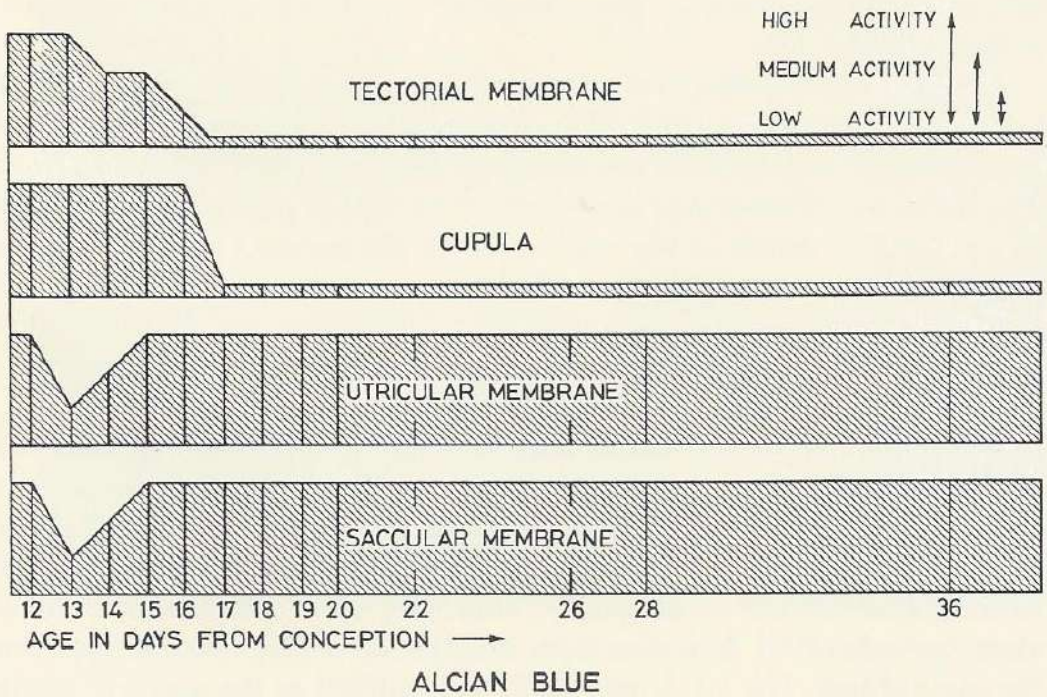


Fig. 6. Explanation see text.

Electron microscopy

For technical organisational reasons our microscopical data had to be limited to material from embryos of 15 days gestation; the 1 μ serial sectioning in order to keep the topography in view is extremely time-consuming.

Nevertheless the information obtained from these specimens was of great use when taken in connection with our other investigations. On the electron micrographs somewhat round, granular masses of low irregular electron density were lying over the macular cells (plate 15). The diameter of these granular blobs varied at this stage from 0,5 to 2 micron. On close inspection the blobs contained isolated round spots of very high electron density with diameters of approximately 300 Å (plate 16 and 17). The blobs were here and there connected to each other by a very thin thready texture.

At some places membrane-like annular structures with diameters of approximately 2,5 micron were seen. The annular structures contained material of the same apparent electron density as the surrounding material (plate 16 and 17). The electron micrograph even at this stage showed the presence of kinocilia and stereocilia of the sensory cells as well as the microvilli of the supporting cells in the same way as seen in adult mammals (WERSÄLL, ENGSTRÖM and HJORTH 1954, FLOCK 1964).

X-ray diffraction

In accordance with the description in the literature, we found that the sagitta of the *pike* is composed of calcium carbonate in the lattice of aragonite. This structure was identified by x-ray powder diffraction patterns (plate 18).

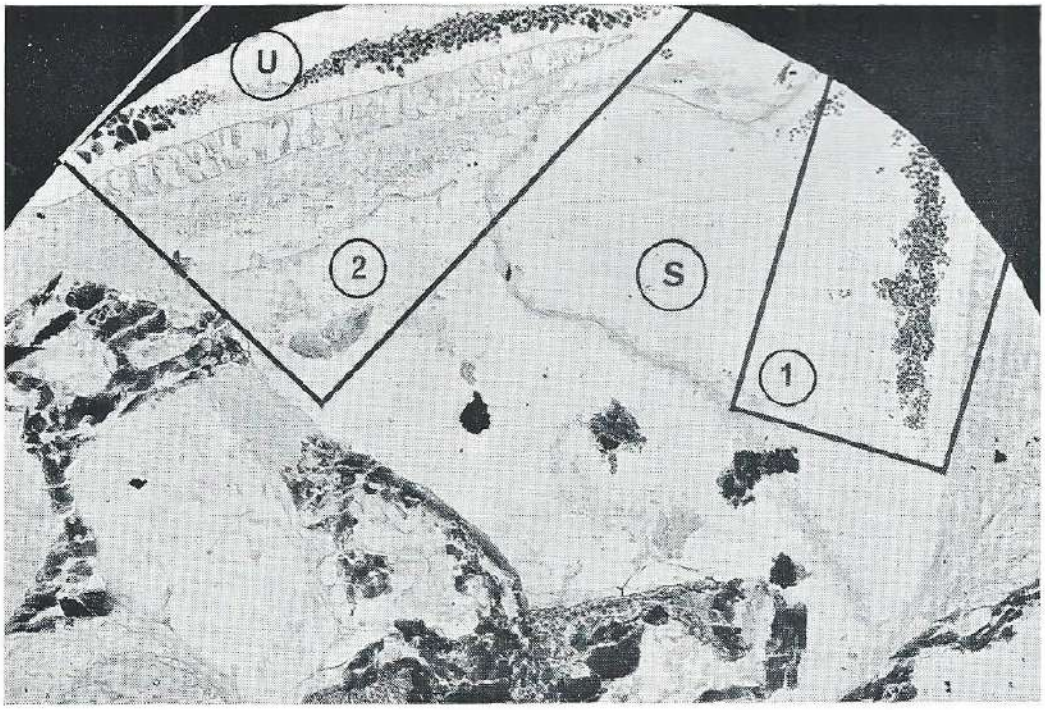
The aragonite crystals in a splinter of the sagitta did not show a random texture on the x-ray diffraction pattern, but did show a spherulitic arrangement (radially built up, going out from one point) of the crystals. The spherulitic texture was also observed under the polarizing microscope. The spherulitic center of the sagitta lies on the macular side of the stone.

Also, as was to be expected from the literature, we found a calcite structure in the x-ray powder diffraction pattern of the statoconia of the *mouse* (plate 18).

A single big statoconium was investigated more thoroughly. Oscillation about the axis of the x-ray camera gave a diagram which appeared to be that of a single crystal. A setting about a crystal axis of 9,7 Å was found to give a zero layer Weissenberg diagram which could not be identified as calcite or aragonite. However, in a different orientation, about an axis of 5,1 Å, a zero layer diagram of calcite was obtained from the same object. The 5,1 Å axis could be identified as the a-axis of calcite.

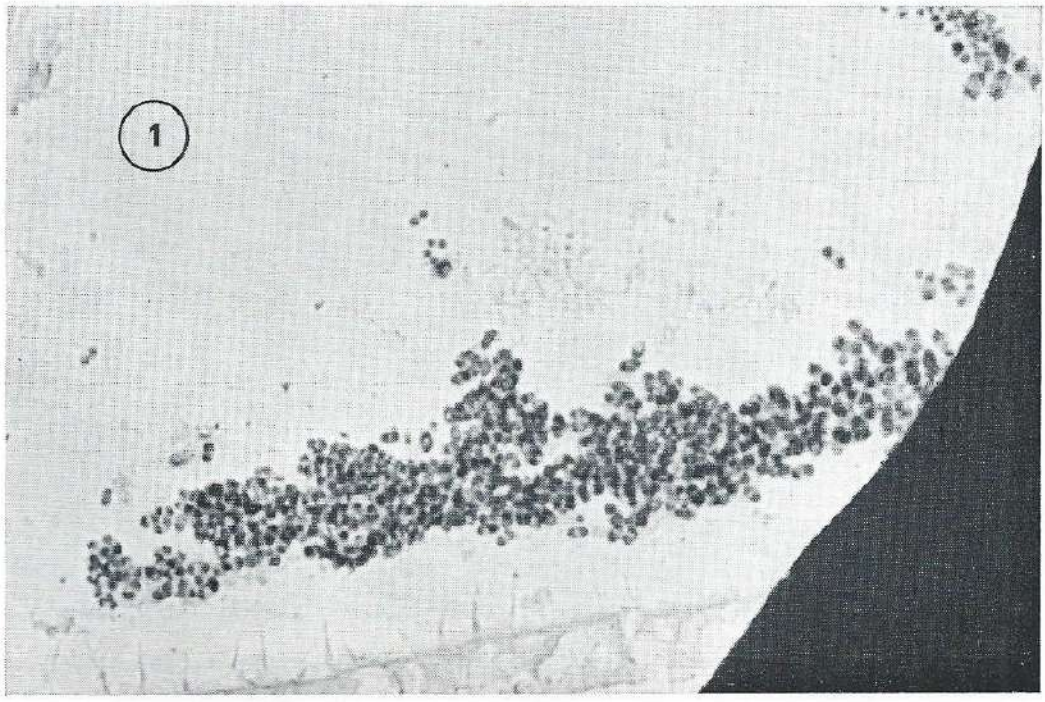
Apparently the object consisted of two components, both crystallized,

PLATE 10



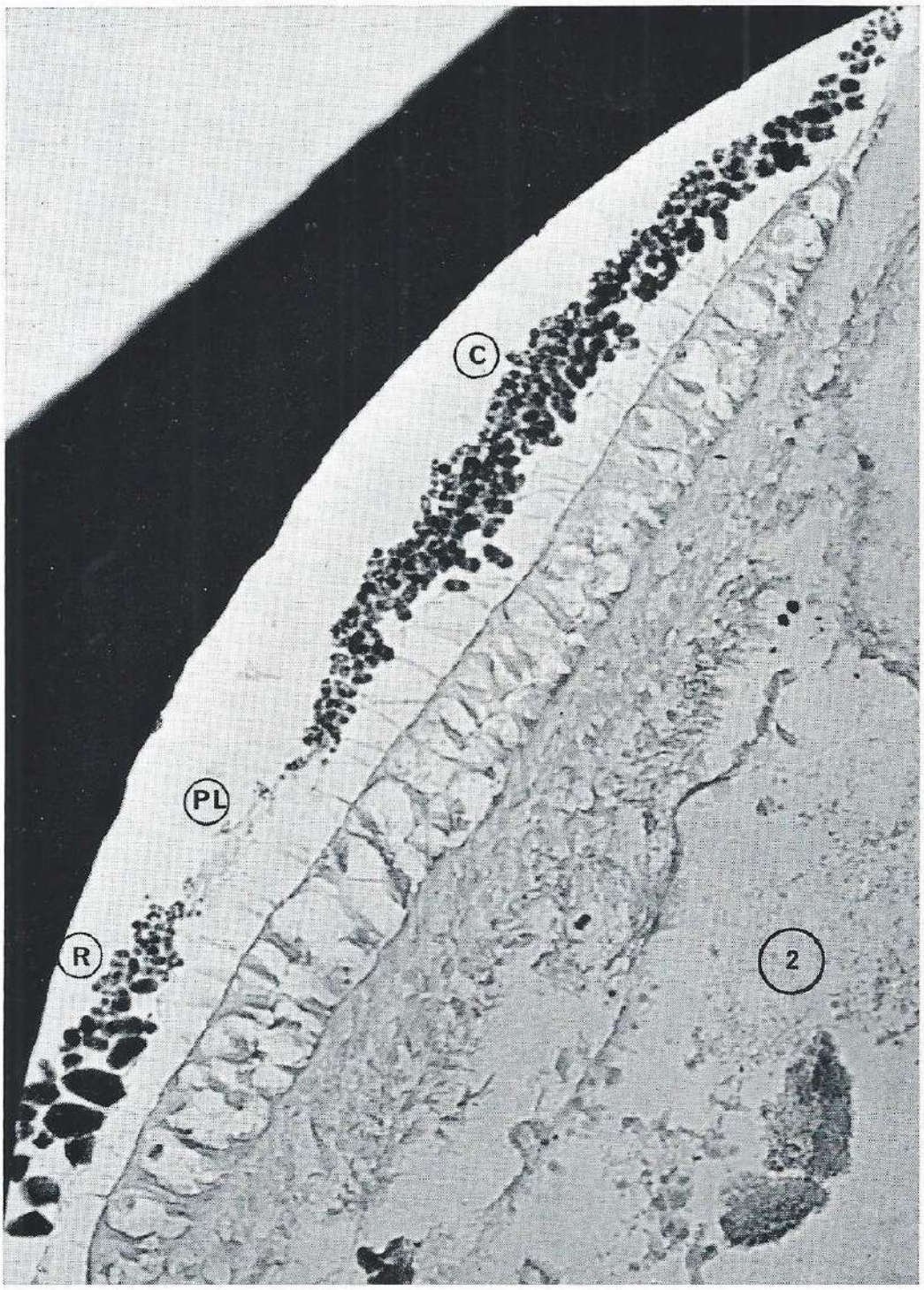
General view of an x-ray projection microradiograph of an unstained section of the labyrinth of a 2 days old mouse. u, utriculus; s, sacculus. For higher magnifications see plate 11 and 12.

PLATE 11



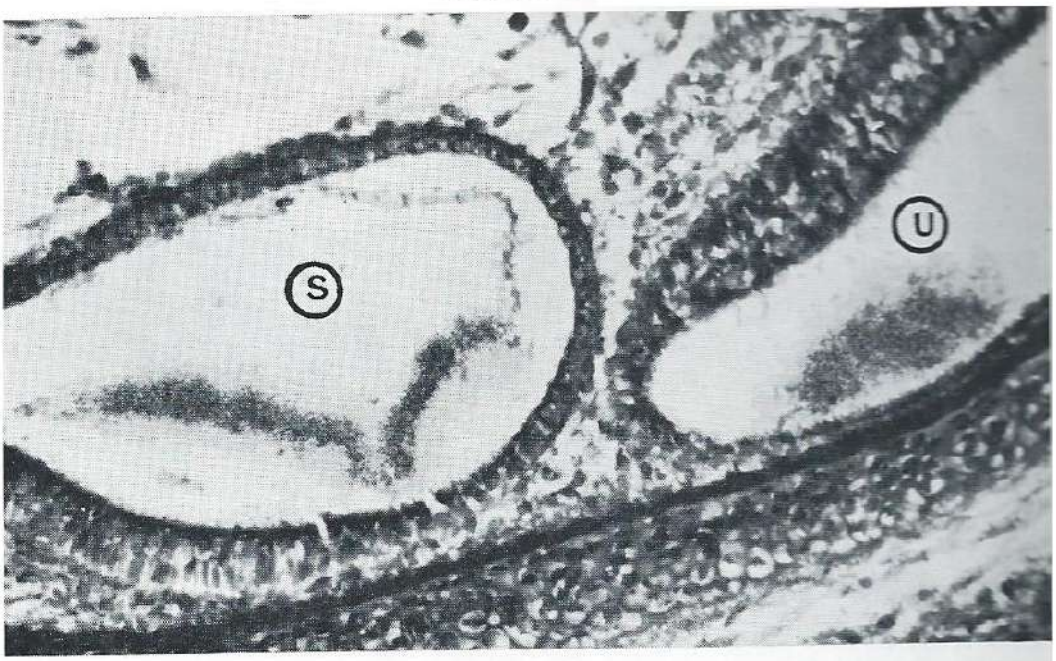
Detail of plate 10. The bipolar form of the saccular statoconia is clearly distinguishable.

PLATE 12



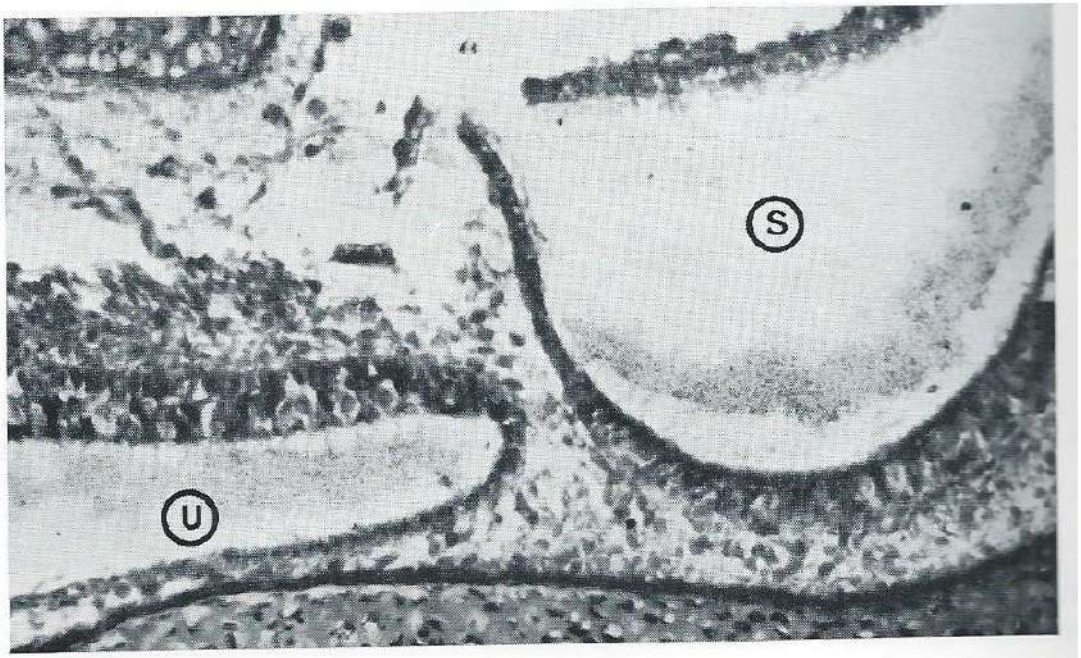
Detail of plate 10. R, rampa; pl. pars lucida lapilli; c, cotillus.

PLATE 13



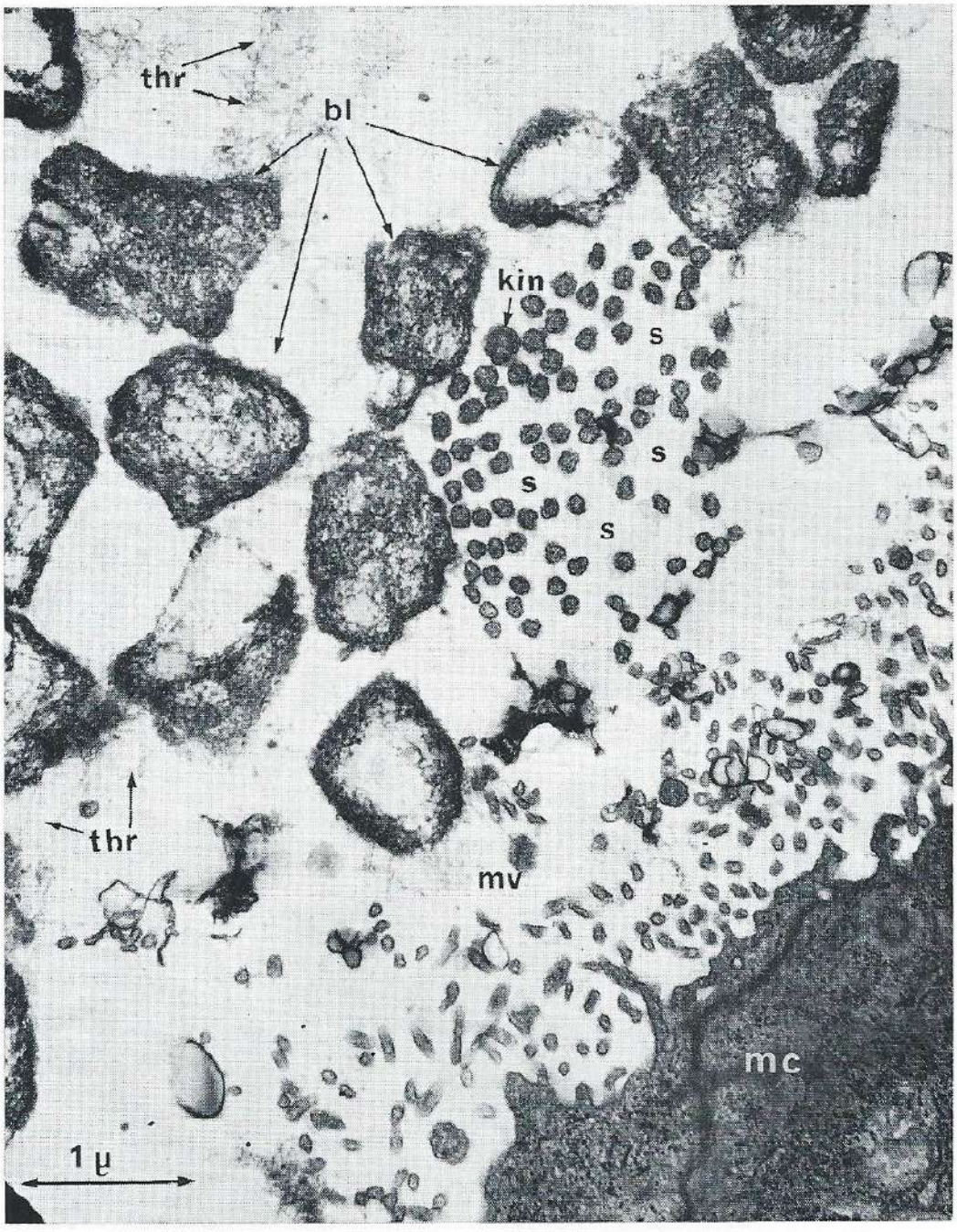
Autoradiograph of an embryo of 17 days gestation, 24 hours after injection of Ca^{45}; 270×. Strong incorporation of radioactive calcium in both statoconial membranes. u, utriculus; s, sacculus.

PLATE 14



Autoradiograph of an embryo of 19 days gestation, likewise 24 hours after injection of Ca^{45}; 270×. A clear decrease of incorporation as compared to plate 13 can be observed.

PLATE 15



Electron micrograph of the supra-macular region of a 15 days old embryo. mc, macular cells; mv, microvilli; bl, blobs; thr, thready texture; kin, kinocilium; s, sterocilia.

and each presented either as a single crystal or as a set of more than one parallelly oriented crystals. Moreover, the direction of the axis of 9,7 Å of the unknown component does not coincide with any lattice row of calcite, and vice versa, no axis of the unknown component coincides with the direction of the a-axis of calcite. On closer inspection, especially on the first oscillation diagram about the 9,7 axis, a number of reflections were seen which did not conform with the expected layer line type of this diagram and which can not tentatively be ascribed to calcite.

From the general aspect of the x-ray diagram of the unknown component, this appeared to be an organic compound of medium molecular weight.

The powder diagram of the statoconia of the mouse showed almost exclusively calcite reflection without clear indication of other crystalline structures. However, the strong x-ray powder diffraction pattern of calcite can easily mask the presence of other components of the statoconia.

Chapter VI

DISCUSSION

The crystallization period

In the previous chapter the results of various methods for the detection of calcium salts in the macular covering membranes were described. Microincineration in combination with polarisation microscopy appeared to be the most sensitive test for detecting crystalline salts in the sensularia. With this method it was possible to demonstrate minute birefringent particles in the statoconial membranes of embryos of 14 days.

From this fact and from the absence of incorporation of Ca^{45} in 14 days old embryos the conclusion can be drawn that deposition of calcium salts in the pre-otolithic material in the lumen of the statolith sacs starts somewhere in the last two-thirds of the thirteenth day of embryonic life. This is partly in agreement with the findings of Lyon (1955), who also found the first trace of calcium crystallization in 14 days embryos. In other specimens, however, she found no birefringence in the statolith sacs of embryos of 14 and 15 days gestation.

Autoradiographs from Ca^{45} treated animals showed that no incorporation of radioactive calcium takes place in animals injected later than about 7 days after birth. These findings are in agreement with the data found by Bélanger (1960). He found slight, but clear incorporation of radioactive calcium in the statoconia of juvenile rats of 3 and 9 days old.

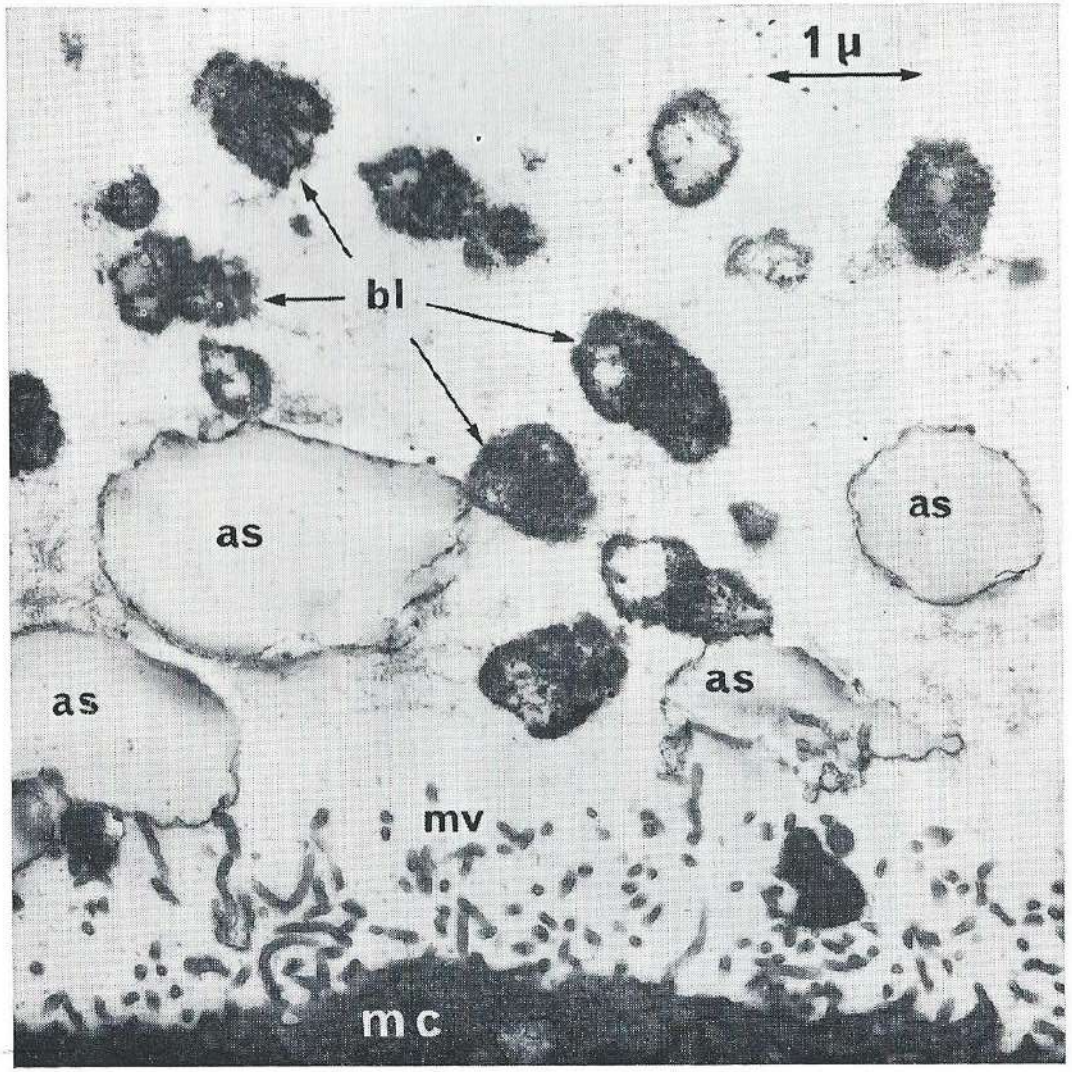
From this the conclusion can be reached that the crystallization period in the statoconial membranes of the mouse takes about 10 days, from the 13th day of gestation until 3 to 7 days after birth.

The formation of statoconia

In the problem of formation of statoconia there are two main questions, i.e. where and what is the origin of the statoconium, and where and how does the statoconium grow?

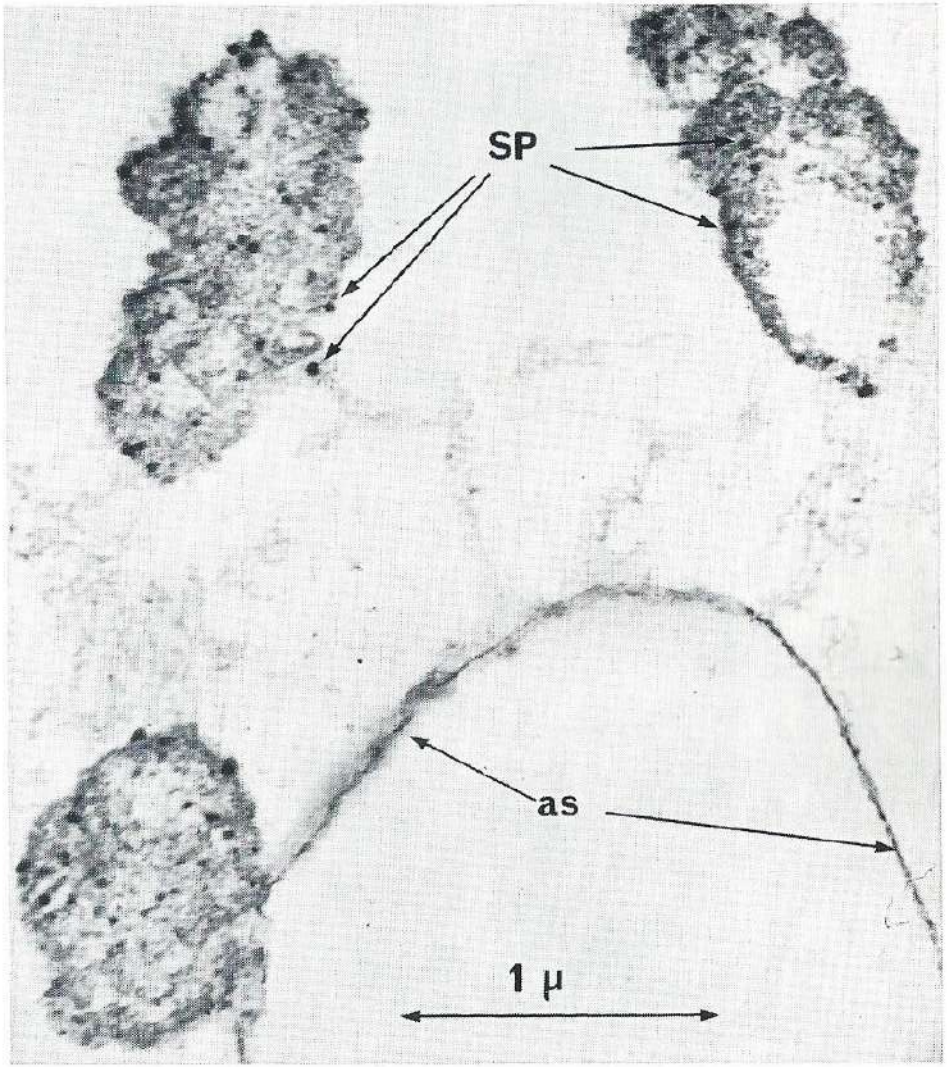
The results of our investigation demonstrated that the crystallization of calcium carbonate apparently takes place in the *lumen* of the statoconial sacs. It is also shown that as well as calcite another crystalline substance can be located in the statoconium. It is, however, far from clear where the organic substance of the statoconia comes from. Are they condensation products of organic material inside the lumen of the statoconial sacs, or are they cell products formed in the way described by Geuze (1968) in the statocysts of water snails? The findings of Geuze were made on invertebrates and thus can hardly be compared with the findings in

PLATE 16



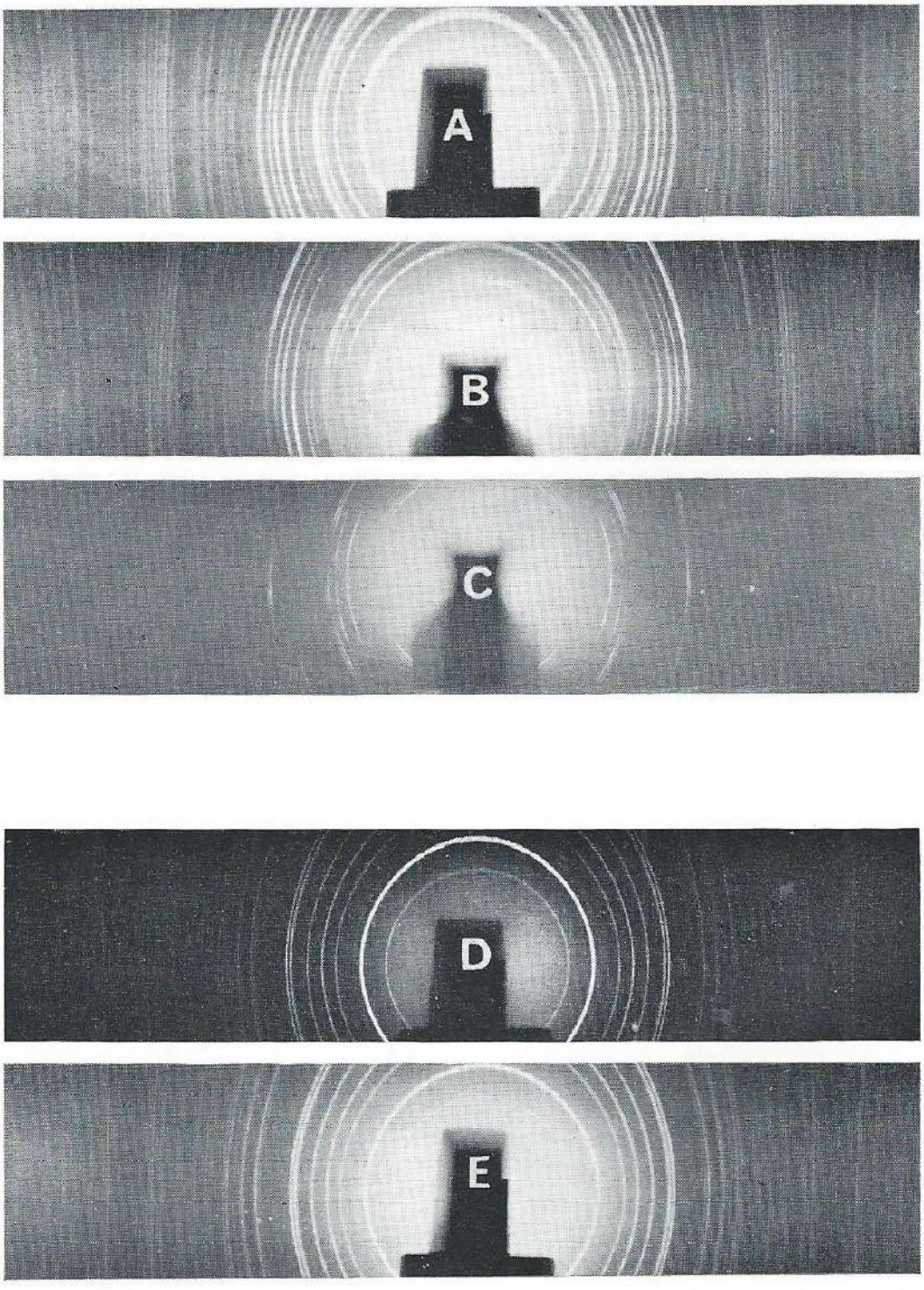
Electron micrograph of the supra-macular structures of a 15 days old embryo. mc, macular cells; mv, microvilli; bl, blobs; as, annular structure. Note the thready texture stretched in between the blobs and the annular structures.

PLATE 17



Electron micrograph of 15 days old embryo. The organic masses with high electron dense spots (sp) are clearly seen, as well as the thread-like texture between the less dense masses and the annular structure (as).

PLATE 18



X-ray diffraction patterns. **A**, aragonite standard; **B**, fragment of the statolith of the pike; **C**, small splinter of the statolith of the pike; **D**, calcite standard; **E**, statoconia of the mouse.

vertebrates. On the other hand, it is a fact that the statoconial sacs are phylogenetically the oldest parts of the membranous labyrinth and thus the parts best comparable with the primitive statocyst. Taking this into account, we consider it not impossible – although there is no direct proof – that the rounded masses observed with the electron microscope over the macular surface are products of macular cells.

One might speculate, however, that the irregular masses lying over the maculae on the electron micrographs correspond with the PAS positive granules observed over the maculae at this stage. The gradual diminishing of the PAS positivity of the statoconial layer might be explained, then, by a gradual apposition of calcite around a nucleus, composed of protein-polysaccharide material.

It is not proven in our work that the irregular masses over the macular cells in the 15 days embryos are identical with the nuclei of low x-ray density as found in the statoconia by means of x-ray projection microradiography. However, there are strong arguments that this might be the case. The moderately dense structures over the macular epithelium are very similar to the organic pre-otolithic material described by GEUZE in 1968, and confirmed by GEUZE (1969). Secondly, the x-ray diffraction patterns of the statoconia of the mouse showed two different crystalline structures in the very same statoconium, i.e. calcite and an unidentified organic substance of low x-ray density.

The precipitation of crystalline calcium in biological systems takes place under the influence of various factors which are only partly known at present and which give rise to rather controversial hypotheses. (NEUMAN and NEUMAN 1953, 1958, GLIMCHER 1959, 1960, SOBEL 1955, SOBEL et al. 1960).

BACHRA et al. (1963, 1964, 1966) presented evidence for the so-called nucleation theory. This theory starts from the principle that spontaneous nucleation in an unstable calcium phosphate or calcium carbonate solution is possible. A metastable solution, however, needs nucleation centers in order to start crystallization.

The results in our investigation point to a mechanism in the deposition of calcite, which might well fit into this nucleation theory. The electron-microscopic findings of small particles lying over the macular surface in the labyrinth (JAMES, SCHELLENS, VEENHOF 1969) might be a nucleation center formed intracellularly that initiates the crystallization of calcium carbonate. These small PAS positive particles (see speculation above) were not found in the tectorial membrane of the cochlea by IURATO (1956, 1960), BAIRATI jr. and IURATO (1957, 1958) in electron-microscopical investigations.

Each granule is then mounted in calcite and looks like an x-ray transparent core on the projection microradiographs, and gives an x-ray diffraction pattern of a crystalline organic substance.

The supply of calcium salts

From our experiments with radioactive labelled Calcium (Ca^{45}) it is evident that the embryos do not show a local increase in concentration of bound calcium in the statoconial sacs in the pre-otolithic stage. The calcium ions which are in *solution* are of course not traced by this method because they are washed out from the sections during processing.

In the crystallization period, no local increase of radioactive calcium concentrations could be traced except in the macular covering membrane. These facts do not point to the presence of any special pathway for the calcium supply, nor the existence of intracellular precursors.

In fact it is not necessary for special pathways to exist. It may be possible that calcium salts are laid down on a nucleus from a metastable calcium ion solution under the influence of certain physical and chemical balance mechanisms, as is the case in bone formation. In bone formation there is evidence that indicates that some physical attribute of collagen influences the initiating of the crystallization of calcium salts in bone without a supersaturated solution of calcium ions being attained. Alkaline phosphatase is one of the factors that plays a role somewhere in this complex mechanism of calcium *phosphate* precipitation.

Bachra et al. (1963, 1964, 1966) demonstrated in their experiments that in most biological fluids deposition of calcium carbonate cannot take place unless simultaneously the level of carbonate ions is raised and the level of phosphate ions is markedly lowered. They explained the inhibition of calcium carbonate precipitation by phosphate ions by the assumption that phosphate and carbonate ions compete for a binding with the calcium ions. They found inhibition of the calcium carbonate precipitation when the PO_4^{3+}/CO_3^{2+} ratio was higher than about 1/300. This occurred at PO_4^{3+} concentrations which were considerably below the initial crystallization point. The results of our histological investigations on alkaline phosphatase activity suggest a similar mechanism. At places in the statoconial membrane where alkaline phosphatase is found in the crystallization period, no statoconia are formed.

Thus nucleation centers enhance calcite deposition whereas alkaline phosphatase might counteract calcite formation.

Another possible balance system in the mechanism of calcium carbonate crystallization in the labyrinth is suggested by the remarkable differences in the polysaccharide content of the macular sensularia in regard to the non-calcifying sensularia (fig. 6).

Before the crystallization period, all sensularia reacted equally to the PAS reaction as well as to alcian blue and toluidin blue. In the last stage of the crystallization period and later, the non-calcifying sensularia reacted mainly with the PAS technique, whilst the calcified sensularia reacted mainly with alcian blue and showed toluidin blue metachromasia. Thus a clear distinction in the content of sugar complexes in the sensularia has been created.

However, in the first days of the crystallization period an alternating staining pattern was observed, i.e. PAS positivity in the calcifying sensularia with a temporary decrease of the affinity for alcian blue, whilst the non-calcifying sensularia at the same time gradually lose their affinity for this stain.

It may be possible that the PAS positive substances initiate the crystallization in the macular covering membranes, whereas, in later periods, further calcification is held back by acid polysaccharides. Now, logically, the question arises, why do not the cupula and tectorial membrane calcify, in spite of their strong PAS positive reactions? Reasoning in the same way as in the case of the macular covering membranes, we must point to the fact that the acid polysaccharides by their presence may protect the non-calcifying sensularia from deposition of calcium crystals in the very beginning of the crystallization period. Besides we refer to the work of IURATO (1960) who did not find anything resembling nucleation centers on electron micrographs of the tectorial membrane.

The metabolism of calcium in statoconia

It is a well known fact that statoliths of fishes grow continuously during their whole life (see chapter II). In this process apparently no cells are directly involved. It is most likely that the calcium metabolism in statoliths of bony fishes is a one-way transport of calcium ions to the otolith surface where the crystallization will proceed.

In the literature hardly any data can be found about the growth or the calcium metabolism of statoconia in adult mammals.

In our material we found a clear increase in the size of the average stone in mice from 16 to 22 days after conception (fig. 5). We have not been able to trace incorporation of radioactive calcium in the statoconia later on in postnatal life or in adult mice. From these findings we may safely conclude that no growth (i.e. calcite apposition) of any importance takes place in statoconia of normal mice after the crystallization period of the statoconia.

WERNER (1933) reported new formation of statoconia in rabbits and guinea-pigs after removal of the statoconial membrane from the macula by means of centrifugation. On the other hand, JAMES (1962) did not observe any new formation of statoconia in adult rabbits, 3 months after destruction of the labyrinth. However, he observed regeneration of the epithelial vesicles in those animals. GEUZE (1968) described both processes, i.e. new formation of a sensularium, as well as regeneration of epithelial vesicles, after operative removal of the statoconia in invertebrates. He demonstrated with the electron microscope that the very beginning of the new formation of the statoconia of the Lymnaea stagnalis is an *intracellular* process.

Taking into account that the findings of Geuze were made on an invertebrate, we are of the opinion that the findings in the mammals of Hasegawa and James, together with our (negative) findings of growth in adult mice do not exclude the possibility that, under certain pathological conditions, a tendency may exist in adult mammals to form new statoconia. In the experiments of Hasegawa, as in those of Geuze, regeneration might have been initiated by the lack of interplay between weight of the statoconial mass and the macular sense cells. This is in accordance with the arrest of growth of the statoconia if a certain mass (weight, age) is reached, as recorded in our experiments. From this one might speculate that weightlessness in space flight might be a condition similar to the experimental conditions in the experiments of Hasegawa, James and Geuze. In that case, prolonged weightlessness might induce new formation of statoconia.

Bone formation and formation of statoconia

LYON (1955) drew attention to the similarity between the substances present in bone formation and in formation of statoconia; she suggested that both processes might be similar to a great extent. The substances she mentioned were polysaccharides, glycogen, alkaline phosphatase and calcium salts. Lyon had her doubts about her own suggestion because of the relatively weak alkaline phosphatase activity she found. Furthermore, she drew attention to the fact that the calcium salts of the statoconia are calcium *carbonate* and not calcium *phosphate*, as is the case with the calcium deposits in bone.

We shall now briefly summarize some pros and cons of a possible similarity between those processes:

1. Deposition of calcium salts in bone formation as well as in the formation of statoconia takes place in PAS positive material.
2. Both processes occur in close relation to extracellular fluids. The endolymph, however, has a quite different ion pattern from that in the extracellular fluid in bone, for instance, a high K^+ ion content and a low Na^+ ion content.
3. The calcite deposition occurs in an organic matrix of epithelial origin, whereas the organic apatite is formed in connective tissue.
4. In both processes nucleation centers are supposed to be involved. In bone the initiation of the crystallization occurs in close relation to collagen. The covering membranes, however, do not show a collagen structure, either on electron micrographs, or on x-ray diffraction patterns.
5. The statoconia consist of calcite (or aragonite) whereas the calcium deposits in bone have an apatite lattice.
6. The apatite crystals are invariably of the same shape and size, whereas the statoconial crystals differ markedly in these respects.

7. The activity of alkaline phosphatase in the statoconial membranes is low during the crystallization period as compared to places of growing bone in the same histological section.
8. If treated in vivo during the crystallization period with tetracycline, no tetracycline incorporation is found in the statoconia, whereas in the same sections incorporation is found at places of growing bone.
9. The statoconia stained in vitro with alizarin red-S, do not show fluorescence as does the growing bone in the same histological section.

Our conclusion should therefore be that the formation of statoconia and bone are different processes with a strong parallelism.

SUMMARY

The origin, development and maintenance of statoconia in mice have been studied in embryonic, juvenile and adult specimens, using the micro-dissection technique, various histological techniques, autoradiography, x-ray microradiography and x-ray diffraction.

The following facts have been established:

1. By means of x-ray diffractometry it was found that each statoconium contains two crystalline substances, namely, calcite and a crystalline organic substance of unidentified chemical structure.
2. In the early stages PAS positive granular material has been found in the primitive statoconial membrane, whereas the electron microscope showed small irregular masses with dark spots over the macular epithelium.
3. The process of calcite deposition takes place in the lumen of the otolith sacs.
4. The formation of statoconia in the mouse takes place from the 13th day of embryonic life until some days after birth. Before and after this period no radioactive calcium incorporation is found. The weight of the macular membrane increases in this period by growth of the statoconia in number and in size.
5. A decrease of PAS reactivity of the macular membranes in the last part of embryonic life and the presence of x-ray transparent cores in the stones is tentatively interpreted as an appositional growth around a nucleation center.
6. Some pecularities concerning topography and sizes of various parts of the labyrinth of the mouse are described.

SAMENVATTING

In dit onderzoek werd het ontstaan, de groei en de instandhouding van de statoconia bestudeerd bij muizenembryos, jonge en volwassen muizen. Hierbij werd gebruik gemaakt van microdissectie, verschillende histologische technieken, autoradiografie, röntgen micrografie en röntgen diffractometrie.

De volgende feiten konden worden vastgesteld:

1. Door middel van röntgen diffractometrie vonden we dat ieder statoconium twee kristallijne stoffen bevat, namelijk, calciet en een kristallijne organische substantie.
2. In de jongste stadia werd boven de maculae PAS positief korrelig materiaal aangetoond in de primitieve membrana statoconiorum, terwijl op de microfoto's van de electronen microscoop boven de macula klonten met een onregelmatige structuur gevonden werden.
3. De vorming van calciet geschiedt in het lumen van de otolithenzakjes.
4. Het calciet wordt vanaf de 13e embryonale dag aangemaakt tot enige dagen na de geboorte, waarna dit proces definitief stilstaat.
5. De PAS positieve korreling verdwijnt in de loop der kristallisatie periode en tegelijkertijd neemt de hoeveelheid afgezet calciet toe. Deze feiten en het vinden van de röntgen transparante kernen in de statoconia hebben wij als mogelijke aanwijzing aangevoerd voor groei van de statoconia door appositie van calciet rond een nucleatie centrum van organisch materiaal.
6. Enige bijzonderheden betreffende de topografie en maten van verschillende onderdelen van het vliezig labyrinth van de muis werden beschreven.

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STELLINGEN

I

De cupula is behalve aan de crista tevens bevestigd aan het epitheel tegenover de crista.

II

Voor de clinicus is de bepaling van het fibrinogeen-gehalte van het bloed van veel minder belang dan de bepaling van de protrombinetijd.

III

Wanneer in het serum van patienten met myastenia gravis geen antilichamen aantoonbaar zijn tegen dwarsgestreept spierweefsel, kan men de aanwezigheid van een thymoom uitgesloten achten.

IV

Het is van belang bij prematuur geboren kinderen, die enkele maanden na de geboorte een anaemie blijken te hebben, een onderzoek te verrichten naar het bestaan van een foliumzuur-deficientie.

STRELLING, M. K. e.a., Lancet I, 898, 1966.

V

Tijdens de groeiperiode dienen fracturen niet operatief behandeld te worden.

VI

Bij labyrintitis geve men bij voorkeur geen tetracyclines.

VII

De anticrista in het labyrint van de Octopus vulgaris heeft geen hydrostatische functie, maar dient ter aanhechting van de supra neuro-epitheliale structuren.

YOUNG, J. Z., Proc. Roy. Soc. B. vol. 152, 1–77 (1960).

VIII

Fracturen van het zygomatico-maxillaire blok dienen zo snel mogelijk na het ontstaan gereponeerd en gefixeerd te worden.

IX

Bij arthrosis van de articulatio mandibularis dient extirpatie van de discus slechts overwogen te worden indien tandheelkundige prothetische correctie niet tot verbetering van de arthrosis heeft geleid.

X

Door de medische faculteiten in Nederland wordt het onderwijs aan afgestudeerde medici veronachtzaamd.