The $^{45}$Ca turnover in the membranous labyrinth
of chick embryos during development

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INTRODUCTION

As is well known, otoliths are composed in most vertebrates of calcium salts which are in the form of carbonates. Yet, Hastings (1935) in Amblystoma tigrinum and Carlström & Engström (1955) in Petromyzon found otoliths in these species to be made of calcium phosphate. From a crystallographic point of view the researches of these two authors have provided evidence that the otoliths of birds, of cartilaginous fishes and even those of mammals (contrary to the generally accepted opinion) are made of calcite, while those of amphibians and of bony fishes are made of aragonite.

In foetuses of fishes (Acanthias vulgaris) Vilstrup (1951) records the presence of 'vacuoles' containing calcite crystals in the epithelium of the endolymphatic duct and on the surface of the sacculus. He thinks he can show a triple origin of otoliths in Elasmobranchs: (1) exogenous otoliths made of quartz crystals coming from sand grains, which, according to him, reach the labyrinth through the endolymphatic duct which, in the Elasmobranchs, opens freely on the surface of the skin over the dorsum cranii; (2) otoliths produced in situ at the level of maculae; and (3) otoliths coming from the endolymphatic duct which on reaching the level of the maculae become embedded within a gelatinous substance. This last process takes place only after birth of the animal.

In embryos and new-born mammals (mice, cats) Lyon (1955) found a gelatinous layer with an organic matrix and some inorganic salts in the otolithic membrane. In the early stages of development she found a PAS-positive precipitate (organic matrix) within the sacculus and the utriculus. This precipitate shows a birefringence due to the presence of calcium salts. According to this author, the first appearance of calcium salts would be simultaneous with that of the matrix. Yet Lyon (1955) did not determine exactly the ways in which the calcification of otoliths takes place. As for the place of origin of the material concerned with the formation of otoliths, part of it is thought to be produced

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by that region of the membranous labyrinth which is called ‘secretory’ by Iwata (1924) and Hazama (1929), while most of it appears to be secreted by the maculae.

In chick embryos Vasquez (1955) has noticed the presence of calcareous formations of aragonite and, in a smaller quantity, of calcite from the earliest stages of otolith development (5–5½ days). They occur in the endolymphatic duct and the author thinks that they are otoliths.

Such formations have the morphological, physical and chemical characteristics of the otoliths of the utriculus, of the sacculus and of those contained in the lagena; they are, however, of a smaller size and are placed in transparent ‘vesicles’ of a globoid or amoeboid shape which may contain one or several crystals. The author thinks that the endolymphatic sac is very important in the production of otoliths, yet their production would not be limited to this organ as, according to Vasquez, ‘almost all the epithelium originating from the otic placode has the power of forming crystals in the epithelial cells or within the endolymph’.

Thus the region of the maculae and of the endolymphatic sac are involved in the production of otoliths. However, opinions concerning the ways in which otoliths arise are still contradictory. This may reflect differences between the classes of vertebrates.

In order to contribute to the problem of the genesis of such formations we thought it interesting to study the turnover of ⁴⁵Ca in the membranous labyrinth in chick embryos of different stages.

In an autoradiographic study of the mineralization of teeth and bones Bélanger (1956) had incidentally noticed the presence of radioactive calcium after injections of ⁴⁵CaCl₂ at the level of the otoconiae of the otolithic membrane of rats and hamsters of various ages. This author did not find any activity after injections of ³²P (as phosphoric acid) at the same regions. This observation is a further confirmation of what we know about the chemical constitution of the inorganic salts of otoliths. On the other hand Guardabassi (1960) finds the highest content of ⁴⁵Ca in the inside of the endolymphatic sac and a small quantity in the membranous labyrinth in larvae of *Bufo bufo bufo*, treated with ⁴⁶Ca before the mineralization of the skeleton. At the end of metamorphosis the inside of the endolymphatic sac was only partially occupied by crystals of ⁴⁵CaCO₃, while the localization of ⁴⁵Ca in the epithelium of the sac and in the developing bones was quite clear. The author thinks that this might mean that the endolymphatic sac is involved in processes of ossification.

**MATERIALS AND METHODS**

Our experiments have been carried out on chick embryos of 4, 5, 6, 7 and 12 days of incubation (stages according to Lillie, 1952).

25 µc of ⁴⁵CaCl₂ dissolved in distilled water at pH 7 were injected into the
\textbf{RESULTS}

Autoradiographic observations have shown the following results.

\textit{Stages 22–26 (ca. 4 days of incubation)}

The otocystic bud does not show any particular localization of radioactive material.

\textit{Stages 26–28 (ca. 5 days of incubation)}

At this stage radioactive material is present at the level of the sacculus and at the base of the cochlear duct (mainly extracellular) at the level of the endolymphatic duct, and in the bud of endolymphatic sac (Plate 1, figs. 1, 3, 4). In the same places PAS-positive material is found which does not always have the morphological features of the otoliths in the endolymphatic duct (Plate 1, fig. 2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Plate1.jpg}
\caption{Plate 1}
\end{figure}

\begin{itemize}
\item Fig. 1. Five-day chick embryo: bud of the sacculus. Autoradiography shows accumulation of $^{45}$Ca within the bud. $\times$ 213.
\item Fig. 2. The same section as fig. 1: PAS reaction. PAS-positive material found at the same places which incorporated $^{45}$Ca. The PAS-positive material is formed both by small otoliths and by material not having the morphological features of otoliths. $\times$ 213.
\item Fig. 3. Five-day chick embryo: section of the membranous labyrinth. Autoradiography shows $^{45}$Ca at the level of the central part of the sacculus just outlined and within endolymphatic duct (pointed out by the arrows). $\times$ 69.
\item Fig. 4. Five-day chick embryo: endolymphatic duct. Autoradiography shows presence of $^{45}$Ca at the level of the interior part and of the epithelium. $\times$ 316.
\item Fig. 5. Seven-day chick embryo: region of the macula utriculi. Autoradiography. A large accumulation of $^{45}$Ca at the level of the otolithic membrane. $\times$ 185.
\item Fig. 6. Twelve-day chick embryo: autoradiography. Otoliths in which is seen the scanty incorporation of $^{45}$Ca. $\times$ 708.
\end{itemize}
Stages 28–30 (ca. 6 days of incubation)

A large quantity of radioactive material is found at the inferior wall of the utriculus, in the bud of saccusculus and at the upper part of the cochlearis ductus by the epithelial wall. We can notice also radioactive material in some parts of the epithelium of the endolymphatic sac and inside it as well as in the endolymphatic duct. In these last parts it is often located in small circumscribed areas.

Stages 30–33 (ca. 7 days of incubation)

Radioactivity is found on the lower wall of the bud of the utriculus, on the middle wall of the saccusculus, and on the epithelium at the top of the lagena (Plate 1, fig. 5). Radioactivity is also found on the wall of the endolymphatic sac and within its central part. Both in this stage and in the previous one the presence of radioactive material is associated with PAS-positive material, almost always in the form of otoliths.

Stage 38 (ca. 12 days of incubation)

A little radioactivity is found in the otoliths of the utriculus, saccusculus and lagena (Plate 1, fig. 6).

DISCUSSION

We think that these observations make a contribution to the problem of the site and the mode of formation of otoliths.

The places where the first otoliths appear in the chick are regions of the endolymphatic sac and the maculae. Therefore Vasquez's hypothesis (1955), by which all the epithelium deriving from the otic placode is able to form crystals, does not appear very likely.

Besides, it is important to notice the presence of activity in part of the endolymphatic sac in the early stages of development; this finding differs from what Vilstrup (1951) observed in Acanthias vulgaris where it is suggested that the endolymphatic sac is a source of otoliths only after birth.

As to the different forms of otolith morphogenesis, these observations have shown that the appearance of $^{45}$Ca in the above-mentioned zones of the membranous labyrinth always comes along with the appearance in such places of an organic matrix chiefly made of acid mucopolysaccharides (de Vincentiis, Marmo & Materazzi, 1964).

Probably in analogy to what has been described in the regeneration of the shell of Helix pomatia (AbolinS-Krogis, 1958), in the morphogenesis of otoliths in the chick we must also distinguish two stages; a first stage in which the organic matrix is secreted, and a second one in which a process of mineralization takes place and calcium salts are deposited. Such a hypothesis is supported by our experiments in which we notice that $^{45}$Ca is always associated with the mucopolysaccharide which constitutes the otolithic matrix when the turnover of $^{45}$Ca
is very high in embryos of 5, 6 and 7 days incubation (periods in which the morphogenesis of otoliths is very active). Turnover is very low in embryos of 12 days incubation in which otoliths are already formed and mineralized to a great extent.

To such observations we must add also the presence of alkaline phosphatase in the gelatinous substance of the otolithic membrane (de Vincentiis & Marmo, 1964) and of an acid phosphatase in the very gelatinous substance and in the macular epithelium, besides more prominently in that of the endolymphatic sac (Marmo, 1965). Such enzymes, of course, must have a part in the processes of morphogenesis and mineralization of otoliths. However, it is difficult to determine exactly the ways in which the union of calcium salts and the otolithic organic matrix takes place. We are studying this problem at present and also that of the origin of the calcium salts.

As to this last point we think that both the calcium which is present in the endolymph (calcium which could join the otolithic organic matrix), and that secreted at certain zones in the endolymphatic sac, together with the organic matrix, take part in the mineralization of otoliths.

SUMMARY

An autoradiographic study of the $^{45}$Ca turnover in the membranous labyrinth of chick embryos has shown the following.

1. An early presence of $^{45}$Ca in the surface of the endolymphatic duct and of the lower part of the otocyst from which the saccusculus and the ductus cochlearis will originate.
2. $^{45}$Ca is always connected to an organic matrix of mucopolysaccharide.
3. The turnover of $^{45}$Ca in these zones is very active in embryos of 5, 6, 7 days of incubation (a period in which otolithic morphogenesis is occurring), while it is very low in embryos of 12 days of incubation (in which the otoliths are already formed and mineralized in large amount).

These results are discussed, together with the problem of the origin of otoliths.

RIASSUNTO

_Il ricambio del $^{45}$Ca nel labirinto membranoso dell'embrione di pollo durante lo sviluppo_

Viene condotto uno studio autoradiografico sul ricambio del $^{45}$Ca nel labirinto membranoso di embrione di pollo nel corso dello sviluppo. I risultati hanno mostrato:

1. Una precoce presenza di $^{45}$Ca in corrispondenza della parete del dotto endolinfatico e della porzione inferiore dell'abbozzo otocistico da cui si origineranno sacculo e canale cocleare.
2. Il $^{45}$Ca è sempre associato ad una matrice organica composta, in gran parte, da mucopolisaccaridi.
3. Il ricambio del $^{45}$Ca, in corrispondenza delle zone suddestrate, è molto vivace in embrioni di 5, 6, 7 giorni di incubazione (periodi nei quali è attiva la morfogenesi otolitica) mentre è molto lento in embrioni di 12 giorni di incubazione (nei quali gli otoliti già si sono, in gran parte, formati e mineralizzati).

L’insieme dei risultati ottenuti viene discusso nell’ambito del problema concernente la genesi degli otoliti.

REFERENCES


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