Chemical composition of saccular endolymph and otolith in fish inner ear: lack of spatial uniformity

PATRICK PAYAN, ANAICK EDEYER, HÉLÈNE DE PONTUAL, GIL BORELLI, GILLES BOEUF, AND NICOLE MAYER-GOSTAN

Laboratoire de Physiologie et Toxiquologie Environnementales, EA 2138; Laboratoire de Physiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, UMR 6548; Université de Nice-Sophia Antipolis, Faculté des Sciences, 06108 Nice Cedex; and Laboratoire de Scérochronologie des Animaux Aquatiques et Laboratoire de Physiologie des Poissons, IFREMER, DRH, 29280 Plouzané, France

Payan, Patrick, Anaick Edeyer, Hélène De Pontual, Gil Borelli, Gilles Boeuf, and Nicole Mayer-Gostan. Chemical composition of saccular endolymph and otolith in fish inner ear: lack of spatial uniformity. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol., 46): R123–R131, 1999.—Fish otoliths provide a record of age, growth, and environmental influences. In both trout and turbot, spatial chemical investigation of the endolymph surrounding the otolith (sagitta) showed a lack of uniformity. Proteins, PO₄³⁻, and Mg²⁺ were significantly more concentrated in the proximal (facing the macula) than distal zone, whereas the opposite was observed for K⁺ and total CO₂ (totCO₂). Na⁺ concentration ([Na⁺]) was 20% higher in the proximal zone in trout but not in turbot. Total Ca and Cl⁻ contents were uniformly distributed in both species. We propose that the endolymphatic gradients of protein and totCO₂ concentration within the endolymph are involved in the otolithic biocalcification process. Microchemical analyses of otolith sections by wavelength dispersive spectrometry showed a lack of spatial uniformity in the K/Ca and Na/Ca ratios, whereas the Sr/Ca ratio was uniform. There is a clear relationship between endolymph and otolith [K⁺], but the interpretation of the results for [Na⁺] needs further investigation. Thus the lack of uniformity in the otolith composition must be taken into account when investigating otolith microchemistry.

endolymph chemistry; otolith mineralization; otolith microchemistry

IT IS THE CONCERN of all countries with fishery industries that their fishing practices should be efficient within the limits imposed by the maintenance of an ecological equilibrium in the exploited waters. Estimation of age and growth of fish is essential for fisheries management. The standard method of obtaining such information for teleosts (which are the predominant marketed fish) is by reading and interpreting calcified structures and most often otoliths. Otoliths are calcareous structures located in the inner ear and bathed in endolymph. They are involved in mechanoreception (6). Fish have three pairs of otoliths, the largest one being called sagittae. Otoliths grow throughout the life of the fish, without resorption (4), in a series of concentric layers of alternating high and low optical density zones that are assumed to record temporal events of seasonal (macroscopic) and daily (microscopic) periodicities (22) (Fig. 1A). In addition to age estimation, recent research has indicated that the chemical composition of otoliths could also provide information concerning fish movement and population dynamics (2, 14, 29, 33), because it is influenced by environmental parameters (temperature, salinity, pollution, etc.) and metabolic events in the life cycle (1, 15, 17, 27, 34, 35).

Considerable progress has been made in improving the accuracy of age estimation, but the mechanisms of otolith formation are still poorly understood. During ontogenesis, variously directed growth gradients result in an elliptical, laterally compressed, distally concave otolith. Unlike most calcifying systems (vertebrate bones, enamel, mollusk shells, corals, etc.; Ref. 31), mineralization takes place in an acellular medium, the endolymph, which contains all the precursors of otolith formation. By comparison with plasma, the composition of fish endolymph is characterized by high K⁺, relatively low Na⁺, a total Ca²⁺ (totCa²⁺) content of ~1–2 mM, and a low protein concentration (2 g/l) (20, 23, 24). Unlike that of higher vertebrates, fish endolymph is more alkaline (pH 8) than plasma (pH 7.20–7.40), mainly due to a high total CO₂ (totCO₂) concentration (20, 23, 24).

The ionic composition probably depends on the activity of ion-transporting cells called ionocytes or mitochondrial rich cells (18, 25, 32). In both trout and turbot, the distribution of ionocytes in the saccular epithelium is in two zones (18): the first consists of a ring of large ionocytes around the macula and the second is of smaller cubital ionocytes unevenly grouped at the opposite side of the macula. We thus hypothesized that the location of these two types of cell in relation to the orientation of the sagitta could result in a nonuniform composition of the endolymph. The microtechnique recently developed in trout and turbot (24) permitted the various chemical concentrations to be determined on single 4- to 5-μl samples of endolymph. We therefore selected two sampling sites (Fig. 1B): one distal, facing the smaller ionocytes (sites 1 and 2), and the other proximal, near the ionocytes around the macula (sites 4 and 5). In the present study we improved the technique to enable endolymph samples to be taken at various sites around the otolith and found a lack of uniformity in its composition. Therefore, we carried out microchemical analyses of otolith sections by wavelength dispersive spectrometry (WD-EM) to study whether the lack of uniformity of the endolymph...
induced a similar nonuniformity in the composition of the otolith.

MATERIAL AND METHODS

Fish handling. Trout (Oncorhynchus mykiss) of ~200 or 600 g body wt (depending on the experiment) were supplied by a local fish farmer near Nice, maintained in running tap water at ~14°C in circular tanks, and used for experiments at least 3 wk after transfer from the fish farm. Turbot (Psetta maxima) of 150 g or 2 kg body wt were reared at IFREMER Brest and kept in a running seawater temperature-controlled circuit (14°C). Both groups were fed every morning.

Collection and analysis of blood and endolymph samples. After blood sampling by puncturing the caudal artery, the sample was centrifuged and the separated plasma was kept on ice until analysis. Then endolymph was sampled as already described by Payan et al. (24). Briefly, after the fish were decapitated, the operative field was washed and dried, the saccular wall was incised, and the endolymph was sampled under a stereomicroscope with a capillary tube connected to a micromanipulator and withdrawal pump. In fish (trout or turbot) weighing ~150–200 g, distal samples were withdrawn from sites 1 and 2 and proximal samples from 4 and 5 (see Fig. 1B). In turbot of ~2 kg, six sampling sites (1-6) could be exploited per saccule.

The volume of sampled endolymph was calculated from the length of the fluid in the calibrated capillary as measured with a slide caliper. Protein, Na\(^+\), Cl\(^-\), and K\(^+\) concentrations were determined on individual samples diluted in 0.5 ml of distilled water. totCa, totCO\(_2\), PO\(_4\)\(^{3-}\), and Mg\(^{2+}\) content measurements required the whole sample to be directly introduced into the measurement vial containing the reagent.

For both plasma and endolymph, Na\(^+\) and K\(^+\) were determined by flame photometry (Eppendorf); totCa, totCO\(_2\), Cl\(^-\), PO\(_4\)\(^{3-}\), and Mg\(^{2+}\) were measured by spectrophotometry using Sigma kits; and protein content was determined using Coomassie blue with BSA as a standard.

Preparation and analysis of section of otolith turbot for microchemistry assay. After extraction, the otolith was cleaned in millipore deionized water (MilliQ). Otoliths were then dried at 40°C. After drying, the entire otoliths (sagittae) were embedded in Araldite epoxy resin and polymerized at 25°C for 24 h. Transverse and frontal sections (200 µm thickness), containing the nucleus, were obtained from the otolith with a low-speed saw. Sections were then embedded on glass slides with epoxy resin. After polymerization, they were ground

![Fig. 1. A: photograph of transverse section of turbot otolith. Arrows, sites of analysis by wavelength dispersive spectrometry (WD-EM) (results in Fig. 5). B: dorsal view of left saccule, showing disposition for endolymph sampling (sites 1-6).](image-url)
down to the nucleus with successive grades of carborundum paper (800–1200 grade) and polished with successive diamond sprays (9, 3, 1, 0.25 µm) and the sections were cleaned in an ultrasonic bath in deionized water (MilliQ) (13). The usual precautions were taken to avoid chemical contamination of the otoliths and to obtain perfect surface conditions as required in microprobe analyses. These analyses were carried out on a Camebax SX 50 WD-EM microprobe fitted out with five spectrometers used to assay calcium (Ca), potassium (K), sodium (Na), strontium (Sr), and phosphorus (P). Operating conditions were as follows: spot size 5 µm, voltage 10 kV, beam current 15 nA, and peak counting time 40 s for Ca and P and 120 s for Na, K, and Sr.

Five transverse sections from five different otoliths (left sagittae sampled from 5 different turbots) were prepared, and eight sampling sites of analysis zones by WD-EM were defined on each section as indicated in Fig. 1A: proximodorsal, dorsal, distodorsal, distal, distoventral, ventral, proximoventral, and sulcus. Three analyses were carried out at each otolith site. The limits of detection (LD) were 158 parts/million (ppm) for K, 109 ppm for Na, 328 ppm for Sr, and 520 ppm for Ca. The LD of K/Ca, Na/Ca, and Sr/Ca ratios were estimated using the ratio between the K, Na, and Sr LD and the composition of the endolymph surrounding the otolith site. The limits of detection (LD) were 158 parts/million (ppm) for K, 109 ppm for Na, 328 ppm for Sr, and 520 ppm for Ca. The LD of ratios were estimated as mentioned above.

Phosphorus was undetected in all the analyses and thus rejected from the subsequent data analysis. Data analysis was performed using the mean value of the three assays of each otolith site.

RESULTS

Ionic, totCO2, and protein concentrations of plasma and saccular endolymph in trout and turbot are given in Table 1.

In the same way, 5 frontal sections from five different otoliths (left sagittae sampled from 5 different turbots) were prepared, and eight sampling sites of analysis zones by WD-EM were defined on each section: proximoposterior, posterior, distoposterior, distal, distoanterior, anterior, proximoanterior, and sulcus. Three analyses were carried out at each otolith site. The limits of detection (LD) were 158 parts/million (ppm) for K, 109 ppm for Na, 328 ppm for Sr, and 520 ppm for Ca. The LD of ratios were estimated as mentioned above.

In trout, the negative K+–protein relationship was largely counterbalanced by a positive Na+–protein and Cl– showed no correlation. Thus the estimated osmolarity (sum of the 3 main monovalent ions) was constant. In the turbot, the protein range was about half that of trout, there was no relationship between Na+ and protein, but there was a negative relationship between Cl− and protein. Consequently, the sum of the three monovalent ions was negatively correlated to protein. The origin of these differences between trout and turbot needs further investigation.

Lack of spatial uniformity of ion and protein in endolymph. In both trout and turbot, proteins, PO4 3−, and Mg2+ were significantly more concentrated in the proximal than in the distal zone, whereas the opposite was the case for K+ and totCO2. [Na+] was 20% higher in the proximal zone than in distal in trout, but in the turbot there was no difference. Total Ca2+ and Cl− contents were similar whatever the sampling zone in the two species. These results clearly demonstrate that the composition of the endolymph surrounding the otolith is not uniform.

Thanks to our microtechnique, Na+, K+, Cl−, and protein concentrations were measured in each sample and thus their interrelationships could be studied. In one series of experiments, different samples of endolymph were withdrawn at different sites inside the saccule. Results are presented in Fig. 2. A (trout) and B (turbot), in which ion concentrations are plotted as functions of protein concentrations. In both species, similar significant negative relationships exist between the concentrations of K+ and protein: a high protein content (proximal zone) corresponds to a low K+ content. In some high K+ samples, proteins were not even detectable by the Coomassie blue method used. There were certain differences between the trout and turbot values. In the turbot, the negative K+-to-protein relationship was largely counterbalanced by a positive Na+–protein and Cl− showed no correlation. Thus the estimated osmolarity (sum of the 3 main monovalent ions) was constant. In the turbot, the protein range was about half that of trout, there was no relationship between Na+ and protein, but there was a negative relationship between Cl− and protein. Consequently, the sum of the three monovalent ions was negatively correlated to protein. The origin of these differences between trout and turbot needs further investigation.
phatic CO₂ concentrations. Two series of samplings were made, alternating the initial sacculus (left or right) to be sampled. In each series, two orders of sampling were used inside the sacculus: 1) a clockwise order in which the first sample was taken in area 1 and the last in area 6 and 2) a counterclockwise order in which the first sample was taken in area 3 and the last in area 4 (see Fig. 1B). ANOVA for repeated measurements indicated that totCO₂ concentrations were significantly different with the otolith sites (P < 0.001) but
that sampling order and sacculus position (right or left) made no difference.

Chemical composition of otolith sections by WD-EM microprobe. On frontal and transverse sections, a variation in K/Ca and Na/Ca ratios was observed from zone to zone, whereas Sr/Ca ratios remain roughly uniform. Multivariate ANOVA tests for repeated measurements showed significant differences (P < 0.001) between otolith sites for both K/Ca and Na/Ca ratios (Fig. 4). On the transverse sections, the average K/Ca ratio of the distal zone was twice that of the proximal, whereas the Na/Ca ratio was only 20% higher. On the frontal sections, the K/Ca and Na/Ca ratio differences between the two zones were less marked, being 27 and 12%, respectively. In fact, data from frontal sections showed greater variability both within and between samples, although the chemical patterns are very similar in the two types of sections. The electron microprobe technique is considered to be one of the best for measuring Na and K ions (5). Actually, in this experiment, all K⁺ concentrations measured in the proximal zone in transverse and frontal sections are below or very close to the K⁺ limit of detection. This makes it difficult to interpret the slight differences between the two types of sections.

**DISCUSSION**

Lack of uniformity within the endolymph. The present study confirms that the endolymph is a K⁺- and totCO₂-rich fluid (see Table 1) and also brings to light a hitherto unknown and exceptional feature of this fluid in that its main constituents are spatially distributed in a nonuniform way, creating important increasing or decreasing proximodistal (P-D) gradients, a phenomenon that has a physiological relevance to the biomineralization process of the otolith.

In the inner ear of higher vertebrates, Sterkers et al. (30) reported that endolymph was variable in composition. There are small but significant differences in the ionic concentrations between the cochlea and the vestibule and also along the length of the cochlea from base to apex. However, these differences only represented ~8% of the maximum K⁺ and Cl⁻ values, and no associated physiological significance was proposed.

The very high K⁺ concentration of the vertebrate inner ear endolymph is unique among extracellular fluids, and its functional significance in mammals is generally related to electrophysiological events in the macula (21, 30). K⁺, being the ion with the highest concentration in the endolymph around the hair cells, normally carries most of the transduction current (21). However, in the present study, the lowest K⁺ concentration was measured near the macula area of the saccus, which contains the sensory hair cells. However, transduction channels are relatively nonselective cation-passing pores (16) and, because Na⁺ is present at a higher concentration than K⁺ near the macula in fish, this ion would assume a role in the mechanically sensitive transduction mechanism.

Origin of ionic gradients within the endolymph. The presence of these gradient in the endolymph implies separate zones of secretion and reabsorption, and its maintenance depends on several parameters, including the ratio between transepithelial flux and pool, diffusional characteristics in the endolymph, and the presence of chelating agents that have not as yet been studied. In higher vertebrates, it has been clearly established that the secretion of endolymph depends on Na⁺-K⁺-ATPase activity in several areas of the inner ear (30). These are zones of specialized epithelial cells, called dark cells, concentrated in relatively small areas around the sensory hair cells. These cells are osmophilic and produce various enzymes, such as Na⁺-K⁺-ATPase, Ca²⁺-ATPase, and carbonic anhydrase generally involved in ion transport and energy metabolism (30). In teleosts, recent studies (25, 32) have demonstrated that saccular ionocytes are mitochondrial-rich cells (DASPMI probe), exhibiting high Na⁺-K⁺-ATPase (antroylouabain) and carbonic anhydrase II activities. In trout and turbot, Payan and coworkers (24) demonstrated that energy-dependent mechanisms are responsible for the maintenance of the K⁺ and H⁺ levels in endolymph. Therefore, from our results in fish and by analogy with the situation in higher vertebrates, we propose that the presence of chemical gradients of monovalent ions in the endolymph is the result of the
uneven distribution of ionocytes in the saccular epithelium. The chemical gradients, whether positive or negative, are oriented along a P-D axis related to the distribution of the two ionocyte populations. As yet, it is not possible to assign specific roles to each type of ionocyte present in this epithelium, but we suggest that these specialized cells are the sites of active transfers of $\text{K}^+$ and $\text{H}^+$ (or related $\text{HCO}_3^-$) as illustrated schematically in Fig. 5A.

Origin of protein gradient within the endolymph. The sensory area of the vertebrate inner ear contains supporting cells displaying a secretory phenotype that are thought to be involved in the formation of tectoria associated with the macula (16). In fish, the secretory activity of the macula was demonstrated by Gauldie and Nelson (12) with hematoxylin and eosin. More recently, Davis et al. (7, 8), using a molecular biology approach, reported that supporting cells located at the outer perimeter of the saccular macula were the site of collagen secretion. These observations and our results suggest the involvement of the macula as the region of endolymphatic protein production. A limited zone of production followed by dilution within the endolymph could explain the establishment of the protein gradient (Fig. 5, A and B). Furthermore, the shape and location of the sagitta would act as a physical barrier to the diffusion of the locally produced ions and proteins and would thus help to maintain the chemical gradient (see Fig. 5A).

Are the chemical gradients significant to otolith growth? Otolith mineralization consists of the deposition of $\text{CaCO}_3$ on a protein matrix (9), thus the principal substances involved in otolith growth are proteins, ionized calcium ($\text{Ca}^{2+}$), and bicarbonate ions ($\text{HCO}_3^-$). Growth is not continuous, because inorganic deposition must necessarily follow the formation of the organic
tein concentration is only half that of the proximal, trout and flatfish. Because the distal endolymph found 72% of diffusible calcium in the endolymph of proteins are strong chelators of Ca\(^{2+}\). Increasing P-D gradient of protein has a further significance in relation to the fact that in all biological fluids proteins are strong chelators of Ca\(^{2+}\). Mugiya (19) found 72% of diffusible calcium in the endolymph of trout and flatfish. Because the distal endolymph protein concentration is only half that of the proximal, whereas the totCa\(^{2+}\) remains constant (Table 1), a P-D increase of ionized calcium is created (Fig. 5B). We also recorded a twofold increase of totCO\(_2\) from proximal to distal. Inasmuch as CO\(_2\) is a highly diffusible gas, the partial pressure of CO\(_2\) is probably constant throughout the endolymph and, with a pH of ~8.0 (20, 23, 24), most of the totCO\(_2\) would be as bicarbonate (HCO\(_3^-\)). The constants of the Henderson-Hasselbach equation (pK and solubility coefficient) are not known for endolymph, so the absolute pH value cannot be calculated. However, on the assumption that these parameters are constant, a difference of 0.3 U pH between proximal and distal zones can be estimated, and this corresponds to a P-D gradient of ~10 mM of totCO\(_2\) (Table 1).

Relationship between the chemical compositions of endolymph and otolith. The chemical composition of the otolith depends on exogenous factors (chemical constituents of the water, food, pollution, factors causing stress, etc.), and endogenous factors (ontogeny, metabolism) (26). As yet, the mechanisms of incorporation of chemical substances are not well known. A narrow relationship probably exists between the chemical compositions of endolymph and otolith, and it was

![Diagram](image-url)

**Fig. 5.** A: hypothetical model of protein, K\(^{+}\), and H\(^{+}\) transports across saccular epithelium. Representation of a transverse section of a saccule. Note that overall movement of H\(^{+}\) results in net excretion of H\(^{+}\) as measured by Payan et al. (24) using a titration technique with isolated saccules mounted as closed sacs. H\(^{+}\) arrow signifies titratable acidity and corresponds either to influx of H\(^{+}\) or influx of HCO\(_3^-\). Influx and efflux of K\(^{+}\) have to be identical to satisfy ionic equilibrium. Shaded areas represent increasing chemical gradient of H\(^{+}\) (or decreasing gradient of HCO\(_3^-\)) and protein between distal and proximal zones and increasing chemical gradient of K\(^{+}\) between proximal and distal zones. B: schematic representation of chemical gradients within endolymph. Macula (proximal zone) is on left and distal zone on right of graph with otolith in middle. y-Axis gives concentration of protein, totCa\(^{2+}\), and HCO\(_3^-\) concentrations were directly measured, whereas Ca\(^{2+}\) and pH concentrations were deduced (see DISCUSSION).
therefore important to study whether the lack of uniformity of the endolymph induces a similar nonuniformity in the otolith.

Variations of the Sr/Ca, Na/Ca, and K/Ca ratios were highly correlated to [Sr], [Na]⁺, and [K]⁺ variations. The 50% difference of the [K]⁺ value between the proximal and distal zones of the otolith is reflected by a similar difference in the endolymph, which clearly indicates a close relationship between endolymph [K]⁺ and otolith [K]⁺. The relationship between endolymph [Na]⁺ and otolith [Na]⁺ has still to be investigated. The fact that, in the endolymph samples, the photometric measurements give the total concentration of ions (i.e., both free and protein linked) may explain the apparent lack of correlation between the two media. Actually, it is the concentration of free ions that is more likely to have a significant correlation with the otolith ionic composition. Further studies of the distributions of free and protein-linked ions, including trace elements used in microchemical studies (3, 10, 11), must also be investigated.

Perspectives

Analysis of the microstructure and microchemistry of the otolith presents considerable potential for the study of fish populations. However, the application of these methodologies seems to be restricted by our present limited understanding of the intimate mechanisms involved in otolith formation. Of the various calcifying systems, the otolith can provide an original contribution because the endolymph is accessible for sampling and analysis. Our study demonstrates that substantial spatial heterogeneity in the concentrations of major ions, totCO₂, and protein exists within the endolymph, which lead us to propose a hypothesis involving a role of such gradients in the degree of CaCO₃ deposition. The nature of the otolith mineralization process is complex. A nonuniform incorporation of the chemical elements into the otolith as a result of the endolymph heterogeneity was demonstrated for the first time and must be taken into consideration in studies based on otolith microchemistry to avoid invalid interpretation. The new dynamic view of endolymph chemistry should promote further research on these interdisciplinary fields, particularly to evaluate the wider importance of this phenomenon in other fish species.

We are indebted to B. Maetz for correcting the manuscript. This study was partly supported by IFREMER, and A. Edeyer is a recipient of a grant from IFREMER and the British council. Address for reprint requests and other correspondence: P. Payan, Laboratoire de Physiologie et Toxicologie Environnementales, EA 2138, Université de Nice-Sophia Antipolis, Faculté des Sciences, BP 71, 06108 Nice Cedex, France (E-mail: payan@unice.fr). Received 20 October 1998; accepted in final form 3 March 1999.

REFERENCES


