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ELECTRON PROBE MICROANALYSIS OF THE OTOLITHIC MEMBRANE. A METHODOLOGICAL AND QUANTITATIVE STUDY

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Abstract

The effect of tissue preparation on calcium and potassium weight percents in the otoconial layer in the utricle and saccule was studied in four groups of OF1 mice with electron probe X-ray microanalysis. Glutaraldehyde and freeze-drying, glutaraldehyde and air-drying, air-drying, and cryo-fixation and freeze-drying were compared. Ca and K changed significantly in the utricle depending on the method used ($P < 0.001$), and K changed significantly in the saccule ($P < 0.001$). We chose cryo-fixation with freeze-drying for the quantitative analysis of the otolithic membrane because this method provided the highest values of Ca and K with minimum loss of Ca and K. Microcrystalline salt standards mounted on scanning electron microscopy holders were used for the quantification of Ca and K by the peak-to-local-background (P/B) ratio method. The P/B ratio in standards with reproducible results, when plotted against weight percent, gave a straight line for Ca ($r = 0.99$, $P < 0.001$) and K ($r = 0.98$, $P < 0.001$). The Ca and K weight percents in otoconia showed similar frequency distributions in the utricle and saccule.

Key Words: Electron probe X-ray microanalysis, otolithic membrane, otoconia, scanning electron microscopy, calcium, potassium, biomineralization.

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Introduction

The vestibular maculae in the inner ear are made up of a sensory epithelium formed of two different types of cells: sensory cells and cells supporting an overlying structure, the otolithic membrane. This membrane consists of two layers: an underlying gelatinous layer in contact with neurosensory cell cilia, and a superficial layer of otoconia, crystals of calcium carbonate, in the form of calcite which contain a small amount of organic material (Lim, 1973; Campos *et al.*, 1990). The otoconia act as weight-lending structures, making the membranes more responsive to linear acceleration-induced forces.

Electron probe X-ray microanalysis (EPMA) is a analytical tool which has been used to investigate biomineralization in calcified tissues, mainly in two systems: bone (Ali *et al.*, 1977; Krefting *et al.*, 1981) and teeth (Engel and Hilding, 1984; Sánchez-Quevedo *et al.*, 1989, 1991; Höhling *et al.*, 1991).

In the inner ear, EPMA has been used to study the ionic environment (Ryan *et al.*, 1979; Anniko and Wroblewski, 1981) with special attention to otoconial biomineralization under normal and pathological conditions (Anniko *et al.*, 1984, 1985; Cañizares *et al.*, 1990; López-Escámez *et al.*, 1993). Although calcium, the most abundant element in otoconia, has been widely studied (Anniko *et al.*, 1984, 1987; Ciges *et al.*, 1985; Cañizares *et al.*, 1990), potassium has seldom been examined in mineralized systems (Höhling *et al.*, 1991) despite the fact that the otolithic membrane is located in a highly K-enriched endolymphatic environment.

A quantitative approach to EPMA, initially developed by Hall *et al.* (1973) for thin specimens, has been widely revised (Gupta and Hall, 1982; Hook *et al.*, 1986; Roomans, 1988; Hall, 1989) and applied to bulk specimens (Zs.-Nagy *et al.*, 1977, Zs.-Nagy and Casoli, 1990; Boekestein *et al.*, 1980, 1983, 1984; Roomans, 1981). In addition, different types of standards have been used for EPMA of biological specimens (Janossy and Neumann, 1976; Roomans, 1979; Russ, 1980; Krefting *et al.*, 1981; Warley, 1990). However, despite the many studies based on quantification by EPMA in biological specimens, no attempt has been made to

undertake a fully quantitative analysis of the otolithic membrane. Quantitative studies have been hampered by problems associated with specimens preparation for microanalysis, and the lack of suitable standards. Although procedures for specimen preparation have been widely discussed elsewhere (Morgan, 1985; Gupta, 1991), we were interested in comparing air-drying versus freeze-drying of the otolithic membrane as a model for evaluating mass loss during specimen preparation in calcified tissues.

Therefore, the purpose of the present study was to establish the most suitable method for the determination of Ca and K and to provide a quantitative analysis of the otoconial layer using the chosen method.

Materials and Methods

Animal and tissue collection

OF1 adult mice of both sexes (body weight 25-30 grams) were studied. The mice were anesthetized with ether before killing by decapitation according to the European Community guidelines regarding experimental animal protection (86/609). The temporal bones were excised bilaterally and placed in a petri dish with saline solution for microdissection. The bullae were then opened rapidly, the stapes was removed, and the round window perforated to expose the utricle and the saccule.

EPMA Sample Preparation

Experiment 1. Sixteen otolithic membranes were used for this study. Four different experimental groups were established to study the effect of tissue preparation on the elemental analysis in the otoconial layer in the utricle and saccule:

Group A. Four otolithic membranes (2 saccules and 2 utricles) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4) at 4 °C for 4 hours, and freeze-dried at -80 °C, for 48 hours in a Polaron E5300 freeze-drying apparatus. Samples were left in the freeze-drying chamber to return slowly to room temperature.

Group B. Another four otolithic membranes were fixed in glutaraldehyde as described for group A, but samples were air-dried at room temperature for 48 hours on the specimen holders.

Group C. Otoconia from four otolithic membranes were isolated during micro-dissection, mounted on scanning electron microscope (SEM) holders and air-dried for 48 hours.

Group D. Four otolithic membranes were plunge-frozen in liquid nitrogen-cooled Freon 22. Samples were transferred to the freeze-drying apparatus and dried as in group A.

All specimens were sputter-coated with a thin layer of carbon in an argon atmosphere (at 0.1 Torr) for 30 seconds.

Experiment 2. Twenty-eight otolithic membranes (14 saccules and 14 utricles) were processed, on the basis of the results of experiment 1, as in group D above, to determine Ca and K concentrations.

Electron probe X-ray Microanalysis

Sample. Otoconia 5-7 μm long with regular morphology were studied in a Philips 505 SEM (operating voltage = 18.5 kV; spot size = 50 nm; tilt angle = 35°; take-off angle= 50°).

Detector. An energy dispersive spectrometer (EDAX PV9900) was used for quantitative analysis (count rate = 2000 cps; live time = 50 seconds).
We used the peak-to-local-background (P/B) ratio method, a well established technique for measuring the elemental concentration of biological bulk samples (Zs.-Nagy, *et al.*, 1977; Hook *et al.*, 1986; Zs.-Nagy and Casoli, 1990). Spectra were collected by pin-point electron beam at 40,000 x. The number of analyses was 160 in experiment 1 (20 for the saccule and 20 for the utricle, in each group) and 560 in experiment 2. Elemental peaks in the X-ray emission spectra were considered to be significant when $P\text{-}b > 2\sqrt{(P+b)}$, where P is the total peak integral and b the estimated continuum component of peak integral according to Chandler (1977).

Ca and K quantification. Microcrystalline salt standards used for Ca and K quantification (Campos *et al.*, 1992) were: $CaCO_3$, $Ca(H_2PO_4)_2 \cdot H_2O$, $CaHPO_4$, $Ca_2P_2O_7$, $Ca_3P_2O_8$, $Ca(OH)_2$, $Ca(H_2PO_4)_2$, $(CH_3\text{-}COO)_2Ca$, $CaHPO_4 \cdot 2H_2O$, $CaO_4S \cdot 2H_2O$, $Ca(NO)_3 \cdot 4H_2O$, $CaCl_2$, $C_{24}H_{48}CaO_{24} \cdot 2H_2O$, and $Ca_{10}(PO_4)_6(OH)_2$ for Ca, and $KB[C_6H_3(CF_3)_2]_4$, K_2HPO_4, KH_2PO_4, $Cr_2K_2O_7$, $AlKSO_4$, KI, K_2CO_3, KCl, $K_3Fe(CN)_6$, $K_2PO_4 \cdot 3H_2O$, $CH_3\text{-}COOK$, $KCr(SO_4)_2 \cdot 12H_2O$ for K.

Microcrystalline standards were mounted on 200 mesh nickel grids fixed to adhesive graphite lamina in SEM holders. The standards were analyzed in the SEM immediately after preparation to avoid contamination or chemical modification. The element weight percent (WP) of each salt standard was directly proportional to peak to local background, i.e.,

$$Cs = k \cdot (Ps/Bs) \qquad (1)$$

where Cs is the element WP of standard (known), (Ps/Bs) is the element peak-to-local continuum X-ray intensity ratio from analysis of salt standards (determined during the analyses), and k is the characteristic calibration constant for each element, calculated from equation (1).

The element WP in individual otoconia was determined by the direct proportion method based on the following relationship between the otoconia and the salt standard:

$$Co/Cs = (Po/Bo) / (Ps/Bs) \qquad (2)$$

where Co is the element WP of a given otoconia (unknown), Po/Bo is the element peak-to-local continuum

Table 1. P/B ratio for calcium and potassium detected in the saccule and utricle in the otolithic membrane under different procedures (n = 20 for each group in saccule and utricle). All data are expressed as mean ± standard error of mean (S.E.M.).

	SACCULE		UTRICLE	
Group	Ca	K	Ca	K
A	46.2±2.2	0.51±0.08	41.0±1.2	0.21±0.03
B	48.8±1.8	0.30±0.05	51.5±1.4	0.31±0.04
C	47.8±1.8	0.37±0.06	51.4±1.6	0.32±0.03
D	52.6±1.8	0.32±0.03	53.3±1.7	0.82±0.09
F-test	N.S.	$P < 0.001$	$P < 0.001$	$P < 0.001$

Table 2. Comparison between P/B at the saccule and utricle for Ca and K (with different procedures used) by the student t-test.

Group	Calcium (S/U)	Potassium (S/U)
A	N.S.	$P < 0.001$
B	N.S.	N.S.
C	N.S.	N.S.
D	N.S.	N.S.

Table 3. Changes in the P/B ratio for Ca and K depending on the method used for specimen preparation.

	Calcium		Potassium	
	saccule	utricle	saccule	utricle
A/B	N.S.	$P < 0.001$	$P < 0.5$	N.S.
A/C	N.S.	$P < 0.001$	N.S.	N.S.
A/D	N.S.	$P < 0.001$	$P < 0.01$	$P < 0.001$
B/C	N.S.	N.S.	N.S.	N.S.
B/D	N.S.	N.S.	$P < 0.001$	$P < 0.001$
C/D	N.S.	N.S.	$P < 0.001$	$P < 0.001$

X-ray intensity ratio from analysis of an otoconia, and Cs and Ps/Bs are as in eq. [1]. Co may be calculated applying equation [1] to each otoconia.

For experiment 1, the Po/Bo ratio for Ca and K were determined to compare the different procedures. In the second experiment, Ca, K, Cl, P and S were detected. The percentages of different elements were compared with one-way analysis of variance (ANOVA 1) and Student's t-test. The WPs of Ca and K were calculated and drawn as a bar chart to compare the elemental distribution in the otoconial layer.

Results

Experiment 1

The P/B ratios for Ca and K in the otoconial layer are shown in Table 1. Analysis of variance showed statistically significant differences between the P/B ratio for Ca in the utricle, and for K in both the saccule and utricle, in material processed with different methods (Groups A-D). In Table 2, Student's t-test showed significantly different amounts of K only in group A. Table 3 summarizes differences found using Student's t-test in the P/B ratio for Ca and K determined with the different methods used to prepare the specimens for EPMA.

Experiment 2

P/B ratios for the significant peaks detected in the otoconia from cryo-fixed and freeze-dried saccules and utricles were obtained. Ca and K were the most abundant elements, but significant peaks for Cl, S and P were also detected.

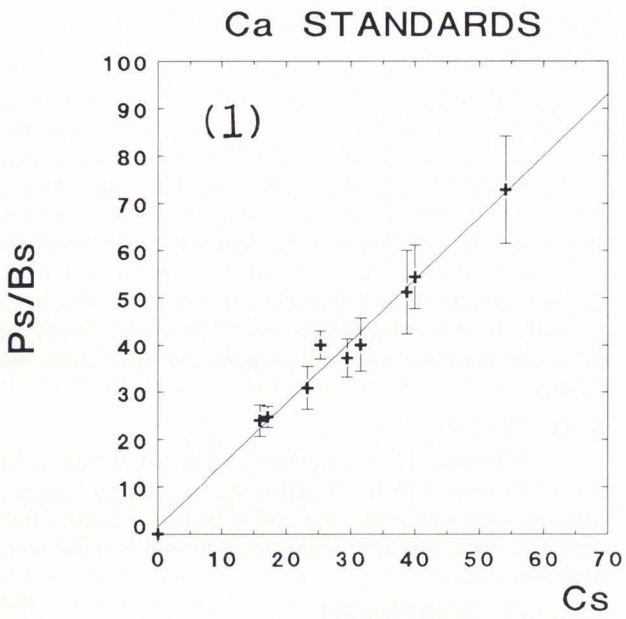
Ca and K quantification

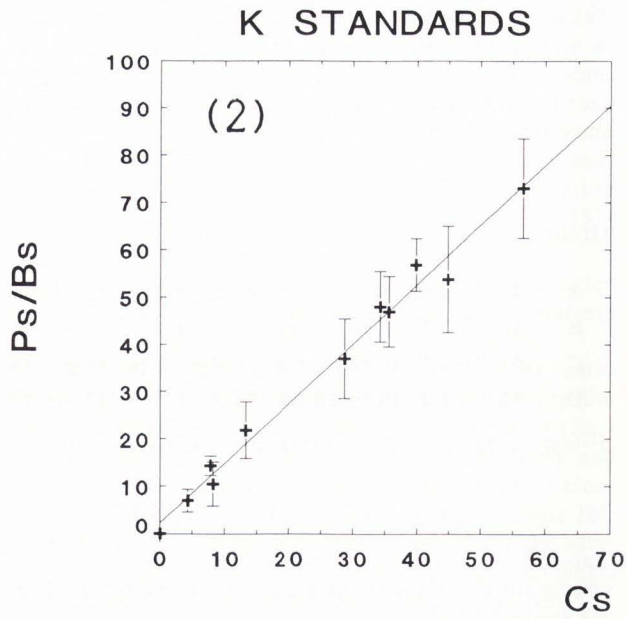
The following Ca and K standards were stable under the beam and yielded reproducible results: $CaCO_3$, $Ca(H_2PO_4)_2 \cdot H_2O$, $CaHPO_4$, $Ca_2P_2O_7$, $Ca_3P_2O_8$, $Ca(OH)_2$, $Ca(H_2PO_4)_2$, $(CH_3\text{-}COO)_2Ca$, and $CaHPO_4 \cdot 2H_2O$, for Ca; and $KB[C_6H_3(CF_3)_2]_4$, K_2HPO_4, KH_2PO_4, $Cr_2K_2O_7$, $AlKSO_4$, $K_3Fe(CN)_6$, $K_2PO_4 \cdot 3H_2O$, and $KCr(SO_4)_2 \cdot 12H_2O$ for K. Plotting Ps/Bs against Cs (Figs. 1 and 2) resulted in a straight line plots for both elements ($P > 0.05$). The regression equations through the origin were $y = 1.35\ x$ ($r = 0.99$, $P < 0.001$) for Ca, and $y = 1.31\ x$ ($r = 0.98$, $P < 0.001$) for K. Co was 38.97 ± 1.27 (WP) for Ca (mean ± standard error of mean, S.E.M.) and 1.01 ± 0.14 (WP) for K in the saccule and 39.61 ± 0.89 (WP) for Ca and 1.32 ± 0.16 (WP) for K in the utricle. The Ca and K frequency distributions in the saccule and utricle are plotted in Figs. 3 (a,b) and 4 (a,b).

Discussion

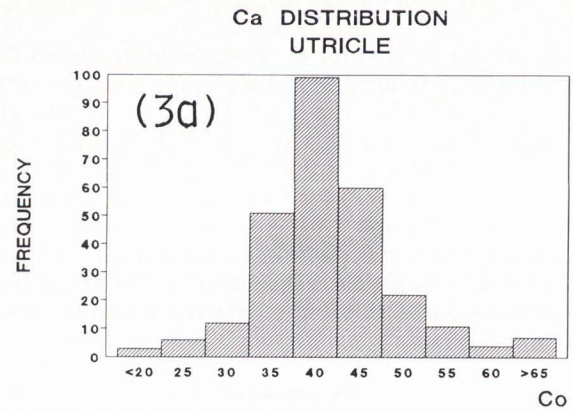
Sample preparation methods for scanning electron microscopy and EPMA for the study of the inner ear have been widely discussed (Anniko *et al.*, 1985; Garcia *et al.*, 1990; Osborne and Comis, 1991). Technical difficulties in obtaining suitable specimens add to the problems generated by measures taken to avoid redistribution of the elements during EPMA sample preparation (Anniko and Wroblewski, 1981). This is especially important because, generally, losses can occur during all stages of processing (Morgan, 1979).

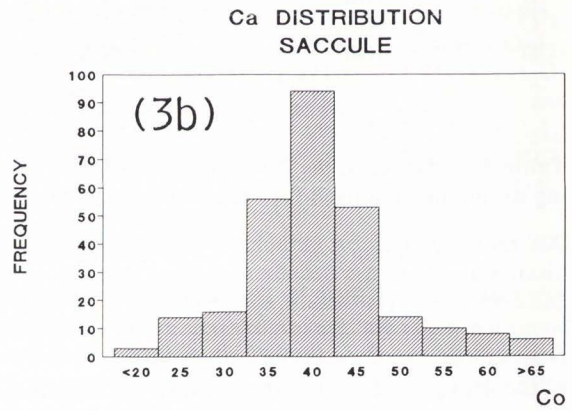
Furthermore, the rate of element loss from a specimen during exposure to histological fluids can be influenced by specimen size (Harvey *et al.*, 1976), the processing temperature (Penttila *et al.*, 1974), and the fixative used (Penttila *et al.*, 1974). We designed a protocol to evaluate the effects of specimen preparation methods on mass loss in the otolithic membrane, a model for the study of biomineralization with EPMA.

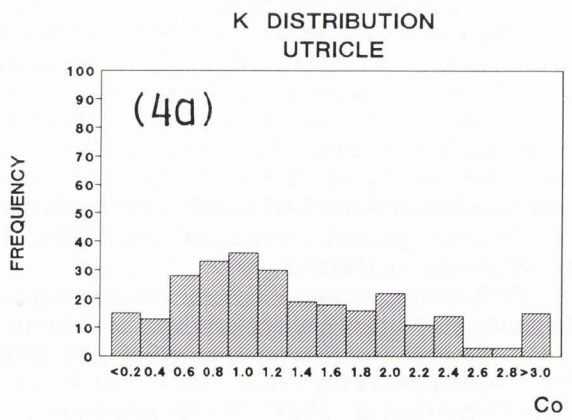


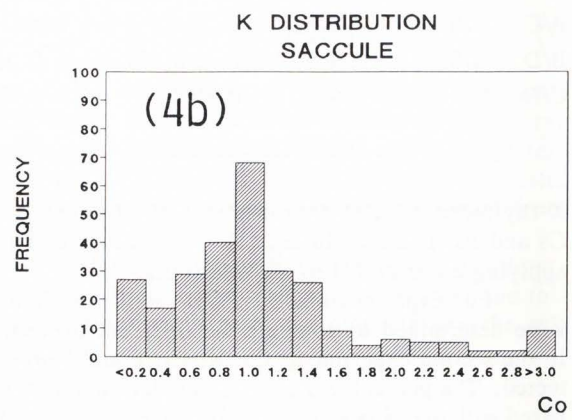


Figures 1 (at left) and 2 (at right). Plots of peak-to-local-continuum X-ray intensity ratio in Ca (**Fig. 1**) and K (**Fig. 2**) standards (Ps/Bs) against Cs. In Figure 1, the equation was $y = 1.35\ x$ ($r = 0.99$, $P < 0.001$); in Figure 2 the equation was $y = 1.31\ x$ ($r = 0.98$, $P < 0.001$).









Figures 3 (above) and 4 (below). Frequency bar charts for WP of Ca (in **Fig. 3**) and K (in **Fig. 4**) in 5-7 μm-long otoconia in the utricle (**Figs. 3a, 4a**) and saccule (**Figs. 3b, 4b**).

In the utricle, both Ca and K changed significantly depending on the method used; the P/B for K changed significantly only in the saccule. Glutaraldehyde fixation followed by freeze-drying (group A) decreased the P/B for Ca in comparison with all other groups. However, in the saccule, the P/B for Ca was not affected, regardless of the procedure used for sample preparation.

Neither the air-drying (group C) nor the freeze-drying (group D) methods yielded different P/B values for Ca. The simplicity of air-drying method for specimen preparation makes this method the most suitable for Ca determinations.

The P/B for K showed significantly lower values in groups A, B and C in comparison with cryo-processed samples (group D). These results may be explained in two ways. The otoconia lost Ca and K during sample preparation, especially in the groups A, B, and C, which consequently decreased the P/B values for K and Ca. This loss was smaller when cryo-procedures and freeze-drying (group D) was used. However, the larger amounts of K in cryo-fixed samples may reflect the fact that this procedure avoids the loss of K ions from the endolymph.

Based on the results of Experiment 1, we chose cryo-fixation with freeze-drying for the quantitative analysis of the otoconia. Quantitative EPMA in biological samples has been performed by the P/B ratio method using standards made with 20-25% gelatin or dextran, to which salts containing elements of interest have been added (Roomans, 1979; Warley, 1990). However, this type of standard is unsuitable for the analysis of mineralized tissues because the standards differ so markedly from the specimens with respect to matrix composition. For this reason, we chose salt micro-crystals containing Ca or K as our standards to estimate Ca and K WPs in the otoconia. Crystal salt standards offer several advantages: they are quick and easy to prepare, their composition is known and their crystalline structure guarantees a constant relationship between the elements, regardless of the area of analysis.

The P/B ratio method (Statham and Pawley, 1978; Small *et al.*, 1979) differs from Hall's continuum normalization method (Hall *et al.*, 1973), which is generally used to quantify thin biological specimens. The P/B method takes into account the background at the same energy as the characteristic peak, in order to have the same absorption by the sample, instead of the background from a selected region of the continuum (usually 4.2-6.2 kV). The principle of this method is that the concentration of an element in the area of analysis in the specimen is proportional to the net counts in the characteristic peak of that element divided by the continuum under the peak. The proportionality constant, k, is characteristic for each element, and can be determined by analysis of standards where the concentration is accurately known. The value of the P/B method is that it is independent of absorption and specimen surface effect, and is, therefore, suitable for rough surfaces and variations in the beam current intensity (Armstrong, 1991).

The most abundant elements in otoconia were Ca and K, but significant peaks for Cl, S and P in the saccule and utricle were also detected. Ca in the otoconia was crystallized in the form of calcite, and showed similar frequency distributions in the saccule and utricle. The WP of Ca we calculated in cryo-processed samples (38.97 ± 1.27 in the saccule and 39.61 ± 0.89 in the utricle), was close to the 40.04 WP of pure crystalline calcite.

Significant amounts of K were detected in 90 % of otoconia in the saccule ($Co = 1.32 \pm 0.16$) and 95 % in the utricle ($Co = 1.01 \pm 0.14$). The K/Ca ratio was 1/39 in the saccule and 1/30 in the utricle; this ratio may be of relevance for the process of biomineralization. The Ca and K ions may be used to balance the charges of the sulfated anionic groups in carbohydrate-protein complexes and in glycosaminoglycans, as well as in acidic amino acids and phosphate groups in matrix protein, as described for dentine mineralization (Sánchez-Quevedo *et al.*, 1989; Höhling *et al.*, 1991). López-Escámez *et al.* (1992) found a linear relationship for both Ca and K concentrations, in the utricle and saccule. However, no relationship was found between Ca and K in the saccule or the utricle. This study supports the idea that most of the K detected in the otolithic membrane is deposited from the endolymph upon the otoconia during freeze-drying.

The presence of sulfur can be explained either as an extra otoconial element incorporated into the spectra because of over-penetration, or as unmineralized residue of the protein matrix. This element is thought to be related, as Engel and Hilding (1984) have suggested, with sulfated glycosaminoglycans of the matrix. Because sulfur decreases as mineralization progresses (Engel and Hilding, 1984), it seems reasonable to assume the absence of sulfur in mature otoconia.

The biomineralization process in the otolithic membrane is still poorly understood, and further studies of the underlying gelatinous layer and epithelial cell surface are needed.

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Discussion with Reviewers

M. Anniko: You constantly describe the presence of a potassium peak when analyzing otoconia and the otolithic gelatinous mass. Can this finding represent a contamination from endolymph? If not, why should there be potassium in otoconia also in cases when analysis is made from the ends of otoconia where there is no organic matrix?

G.M. Roomans: It seems possible that the otoconia could be contaminated at different stages of the preparation, e.g., by the saline used during dissection, the phosphate buffer, the endolymph. If contaminating crystal cover the otoconia, this could also (by absorption of Ca X-ray) affect the Ca signal. Maybe washing with water or with a volatile buffer (ammonium acetate) would remove the contaminating fluid.

Authors: All the analyses were performed on the central area of a "clean" otoconia. If an amorphous mass covered the surface of an otoconia in the SEM image, that otoconia was not analyzed. The amorphous masses were usually identified as KCl micro-crystals, probably formed *in situ* during specimen isolation. However, although endolymphatic contamination cannot be excluded, the absence of a Cl peak might also indicate that K is actually an element that exists in otoconia, and not a preparation artifact.

The use of a washing solution to remove the contaminating fluid is an interesting suggestion, but we have not tried it yet.

G.M. Roomans: How were the microcrystal prepared? Were they recrystallized from commercially available crystals? Why were the micro-crystals mounted on nickel grids and not on carbon planchet? How were they mounted? Glue?

Authors: The micro-crystals were not prepared in any special way. A pre-glued carbon disc was mounted on a conventional pin type stub, a 200 mesh nickel grid for TEM was fixed to the disc, and powdered micro-crystals were mounted on the grid. The nickel grid was used to avoid over-penetration of the crystal.

M. Anniko: How have the micro-crystalline standards been checked for their exact mineral content?

Authors: Micro-crystalline standards have a known, stoichiometrically defined composition and their crystalline structure guarantees a constant relationship between the atoms. We were especially careful to avoid radiation damage in the standards, using a low accelerating voltage, but enough to produce Ca Kα emission, and a short acquisition time (i.e., 50 seconds). However, if spots or bubbling appeared in the standard during irradiation, the standard was considered unstable and was not used for the calibration procedure.

M. Anniko: Why does a difference exist for Ca changes between the utricle and the saccule depending on the methods used?

D.J. Lim: Differential mineral loss following fixation and tissue preparation is of interest, particularly difference among different organs, e.g., saccule, utricle, etc. What could be the reasons?

Authors: Table 2 shows that the P/B ratio was different only for K when the saccule and utricle were compared in group A, with lower values in the utricle. This seems to indicate that the rate of K loss during glutaraldehyde fixation is higher in the utricle. For the rest of the experimental groups, otoconia from the saccule or the utricle did not behave differently.

D.J. Lim: What kind of SEM holders have you used? If made of metal, would it interfere with the elemental analysis?

Authors: We used carbon SEM holders to reduce elec-

trostatic charging problems. It also avoided the elemental interference from the holders in microanalysis.

A. Boekestein: The statistics of your measurements in Figure 1 and 2 show a relative error of 20% or more for the specific concentration of Ca and K. Have you compared these relative errors with calibration measurements using net peaks only?
Authors: No. The use of net X-ray intensities to calibrate the concentration in bulk specimens involves several correction factors. The emitted X-rays are affected by local variations of the take-off angle and electron beam current fluctuations.

A. Boekestein: In principle, the peak-to-local-background method is optimal if the background radiation is measured at the same energy as the peak intensity and is generated in the same analyzed micro-volume. As there will also be some extraneous background generated elsewhere in the specimen environment, I would like to know whether you have some ideas on how to take this contribution into account.
Authors: Nil do not know of any procedure to evaluate the extraneous background contribution to the spectrum in a SEM. The use of specimen holders made of low atomic number materials (Be or C) or coated with C, the coating of metallic parts near specimen with C, operation at a low tilt angle and optimization of the detector-to-specimen distance and accelerating voltage can help to reduce extraneous background sources.

A. Boekestein: As nb are merely composed of calcite as solids, I wonder why you have not processed these specimens using, for instance, (cryo)-polishing methods which eventually would yield a flat surface?
Authors: These samples are difficult to manipulate because of their small size. The surface of the otolithic membrane is rough and cannot be polished easily.

G.M. Roomans: In group A, were the specimens frozen before freeze-drying, how?
Authors: No. The specimens were transferred to the freeze-drying apparatus after chemical fixation.

H.J. Höhling: Why have the authors given only the P/B ratios for Ca and K in Table 1, not the quantitative elements contents, which would have shown directly the amount of element loss during tissue preparation in relation to the shock freezing method ?
Authors: The WP is a linear function of the P/B ratio in the standard and in the specimen (equation 1), so there are no differences in using P/B or WP to evaluate the rate of mass loss.

H.J. Höhling: Why do the authors think that the decreased P/B ratios for K are due to mass loss during preparation also for the air-dried specimens, in relation to the shock-frozen ones?
Authors: We think that the lower values in the air-dried specimens are due to specific loss of K induced by the air-dried method, rather than K contamination in the shock-frozen ones.

G.M. Roomans: Although method A gives the lowest Ca values, this is not proof that glutaraldehyde removes Ca from the otoconia, since method B does not differ from methods C and D. Are we to believe that air-drying is significantly better than freeze-drying? (comparison A and B). Although I *a priori* would prefer freezing followed by freeze-drying, the data show that the Ca in the otoconia is very firmly bound.
H.J. Höhling: Why do the authors recommend the air drying method for Ca-analysis, considering that during this process a redistribution of ions will also take place?
Authors: Our results show that Ca is not affected differentially by air-drying or shock-freezing followed by freeze-drying. Ca is very strongly bound in otoconia and a more aggressive procedure is required (e.g., glutaraldehyde) to remove Ca.

G.M. Roomans: It is known that the continuum intensity is dependent on the square of the atomic number. Therefore the Hall equation contains a correction factor Z^2/A. This dependency does not change if one takes the background under the peak instead of a peak-free region. It is remarkable that the authors obtain a straight line without using the Z^2/A correction factor; can you please discuss this further.
Authors: We have studied the influence of Z^2/A on the calibration accuracy at different voltages (10-25 kV) by simple linear regression, plotting the coefficient of correlation (r), calculated for each curve, as a function of voltage to determine the optimal voltage which reduces the error in the calibration procedure. We found that the accuracy of the calibration was not dependent of the voltage, in the range studied for the set of Ca standards. K was also independent when the P/B was corrected for Z^2/A. The correlation coefficient did not differ regardless of whether Z^2/A was or was not used to correct the P/B for Ca or K standards and was always > 0.98.

H.J. Höhling: Assuming that the measured phosphorus represents at least partly organic phosphorus, e.g., in the form of phosphoproteins, and sulphur, at least partly, sulfated proteoglycans, did you measure or consider a correlation between the K content and that P and S?
Authors: The presence of P or S in mature otoconia is probably due to contamination or over-penetration to the gelatinous layer. We are studying P, S and K in the gelatinous membrane to determine the relationships between these elements and their possible role in the biomineralization process.

A. Boekestein: Have you noticed any Ca concentration profile on a cross-section of an otolith?
Authors: We are presently studying Ca and K concentrations in shock-frozen, freeze-dried and resin-embedded otoconia.