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Quantitative histochemistry of phosphorus in the vestibular gelatinous membrane: an electron probe X-ray microanalytical study

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Summary. Electron probe X-ray microanalysis was used to study the phosphorus concentration in the otolithic gelatinous membrane of the saccule and the utricle with scanning electron microscopy. The otolithic membranes were plunge-frozen in liquid N_2 and freeze-dried. Quantitative analysis was carried out with an energy dispersive detector using the peak-to-background ratio method and different concentrations of KH_2PO_4 salts dissolved in dextran solutions.

The otolithic gelatinous membrane consists of a 25-30 µm-thick layer overlying the cilia of the hair cells. Elements detected in the gelatinous membrane are : Na, P, S, Cl, K and Ca. Although Student's t-test did not show significant differences between saccular and utricular concentrations of phosphorus, the distribution of this element in the two organs was different. Regresssion analysis established that the concentrations of phosphorus in the saccular and utricular gelatinous membrane were dependent. The regression equation was: $y = 18.02x^2 + 133.9$ ($r = 0.83$, $P < 0.05$) where y is the concentration of phosphorus in the utricle, and x^2 the concentration of phosphorus in the saccule. The findings obtained in the present study could be related to structural differences in organic phosphate residues of the phosphoproteins associated to collagen, or to different polyphosphoinositide turnover rates in the cell membrane.

Key words: X-ray microanalysis, Phosphorus, Gelatinous membrane, Inner Ear, Vestibular

Introduction

The otolithic membrane in the vestibular receptor consists of two layers: a superficial layer made up of otoconia, crystals of calcium carbonate in the form of calcite, and an underlying, reticulated gelatinous layer in contact with ciliated neurosensory cells (Lim, 1979).

Electron probe X-ray microanalysis (EPMA) is a histochemical technique able to identify chemical elements in the sample while it is examined in the electron microscope. Microanalytical studies of inner ear tissue have been focused mainly on the elemental composition of the otoconia under normal and pathological conditions (Anniko and Wroblewski, 1983; Anniko et al., 1984a, 1985, 1987; Campos et al., 1984; Cañizares et al., 1990). However, the gelatinous layer has seldom been studied by EPMA because of difficulties in the preparation of inner ear samples (Anniko and Wroblewski, 1981, 1986).

A quantitative approach to microanalytical techniques was initially developed by Hall et al. (1973) for this specimens, and has since been extended to bulk specimens (Zs.-Nagy et al., 1977; Boekestein et al., 1980, 1983; Roomans, 1981; Wyness et al., 1987; Zs.-Nagy and Casoli, 1990). However, quantitative studies in the inner ear are lacking because of technical problems with the immobilization of ion content, differential freezing rates in bone and soft tissues, and preservation of the morphological structures (Anniko et al., 1984b).

Immunohistochemical studies have demonstrated the presence of different constituents in the tectorial membrane, including type II collagen (Tomoda et al., 1984; Slepecky et al., 1992), type IX collagen (Slepecky et al., 1992), fibronectin (Fermin et al., 1990), keratin sulphate (Fermin et al., 1990; Munyer and Shulte, 1991) and chondroitin sulphate (Munyer and Schulte, 1991). Sugiyama et al. (1992) examined the tectorial membrane in the gerbil at light microscopic level with lectins. Fermin et al. (1990) have localized glycoconjugates in the gelatinous membranes and statoconia of the chick. However, the chemical composition of the gelatinous membrane is virtually unknown, an article by Khan and Drescher (1990) being the only report on the electrophoretic characterization of nine major proteins of the trout saccular otolithic membrane.

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Organic phosphate residues of the phosphoproteins satisfy the requirements for participation of a charged group in nucleation in hard tissues (Glimcher, 1989). Phosphate and sulphate groups in the matrix protein have been described as regulators in the biomineralization process of dentine (Sánchez-Quevedo et al., 1989; Höhling et al., 1991), and acidic macromolecules have also been implicated in calcite nucleation in mollusc shells (Mann, 1988). The topographical distribution of phosphate groups in the gelatinous membrane may be implicated in otoconial biomineralization. In the present study, we determined the elemental composition of the otolithic membrane gelatinous layer, and present a quantitative analysis of phosphorus by EPMA in the utricle and saccule.

Materials and methods

Six utricles and six saccules were obtained from five adult OF1 mice. The mice were anaesthetized with ether before killing by decapitation, in accordance with 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 were removed and the round window perforated to expose the utricle and the saccule.

The otolithic membranes were plunge-frozen in liquid N_2-cooled Freon 22, and freeze-dried at -80 °C for 24 h in a Polaron E 5300 freeze-dried apparatus. After freeze-drying, the samples were left in the chamber until they had reached room temperature, and were then sputter-coated with a thin layer of carbon (P=0.1 Torr). Samples were examined in a Philips 505 SEM (voltage = 18.50 kV; spot size = 100-200 nm; tilt angle = 35°; take-off angle = 50°). An energy dispersive spectrometer (EDAX PV9900) was used to analyze the gelatinous layer of the otolithic membrane in the utricle and saccule, with a counting rate of 1000 cps. Spectra were collected by point electron beam at x 10,000 during 50 s live time.

Hall's continuum normalization or the peak-to-background ratio method (P/B) was used to measure the concentration of phosphorus in the gelatinous membrane (Hall et al., 1973). However, the background signal was measured between the same energy levels as the characteristic peak (Small et al., 1979; Statham, 1979), to correct for beam current intensity variations and rough surface. Different concentrations of KH_2PO_4 salts were dissolved in 20% dextran solutions and used to quantify phosphorus. Droplets of the standards were mounted on 200 mesh Ni grids fixed to SEM holders, and spread in a thin film with filter paper. The standards were cryofixed in liquid N_2, freeze dried and sputter-coated as described above, and were analyzed in the microscope immediately after preparation to avoid contamination or chemical modification. The concentration of element x in the specimen ($C_{x,sp}$) was calculated according to the formula:

$$C_{x,sp} = \frac{\left(I_x/W\right)_{sp} \cdot \overline{Z^2/A}_{sp}}{\left(I_x/W\right)_{std} \cdot \overline{Z^2/A}_{std}} \cdot C_{x,std} \qquad (1)$$

where C_x is the concentration of the element x in mmol/kg, I_x/W is the peak to background ratio for the element x, the subscripts sp and std refer to specimen and standard respectively, and the value of Z^2/A is the mean value of the atomic number squared, and divided by atomic weight in the sample.

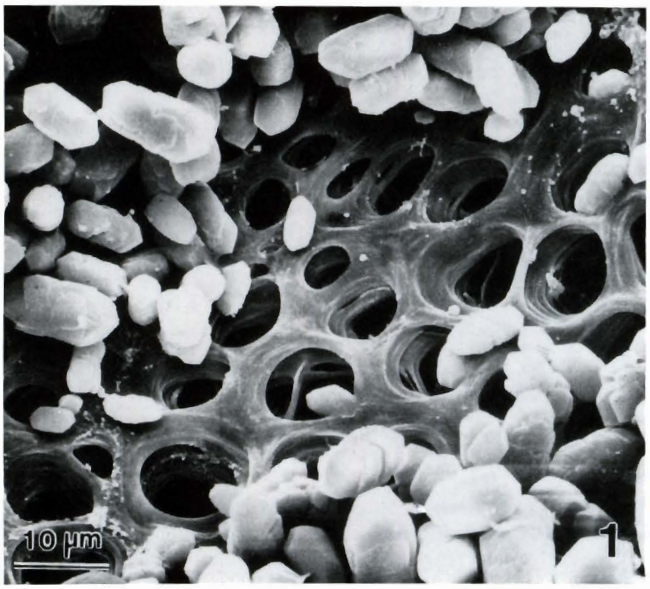
All data were calculated and drawn as frequency histograms to compare phosphorus distributions in the otolithic gelatinous membranes. Final concentrations of phosphorus were compared between saccule and utricle with Student's t-test and least squares fitted to a linear regression model.

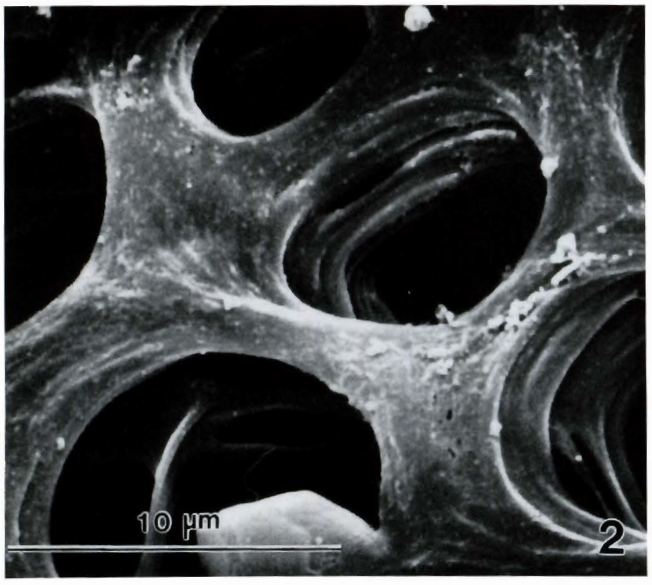
Results

The otolithic gelatinous membrane consists of a 25-30 µm-thick layer matrix forming large cavities at the regions overlying the cilia of the hair cells (Figs. 1, 2). There are no differences in the morphological pattern observed with scanning electron microscopy in the otolithic gelatinous membrane between utricle and saccule in OF1 mice. The elements Na, P, S, Cl, K and Ca are present in the gelatinous layer (Fig. 3). Pottassium and chloride are the most abundant elements, but phosphorus was also consistently detected. Table 1 gives phosphorus concentrations (mmol/kg dry weight) calculated for each sample. Student's t-test did not reveal significant differences between saccular and utricular concentrations of phosphorus. When the mean value of phosphorus concentrations in the utricle were plotted against saccular values, a polynomial curve was obtained (Fig. 4). After transformation of the data in the saccule from x to x^2, the plot became a straight line (Fig. 5). The regression equation was $y = 18.02x + 133.9$ ($r=0.83$, $P<0.05$), where y is the phosphorus concentration in the utricle and x the concentration of phosphorus squared in the saccule. The phosphorus frequency histograms showed different distributions between the saccule and utricle, with an increase in the order of 75 mmol/kg range in the utricle (Figs. 6, 7).

Discussion

Electron probe X-ray microanalysis can be used for the quantitative histochemical analysis of the otolithic gelatinous membrane with scanning electron microscopy. This study presents the first quantitation of phosphorus in the gelatinous layer of the utricle and saccule from OF1 mice, based on the use of dextran solutions containing KH_2PO_4 salts as standards and the





peak-to-background ratio method.

The aim of cryopreparation in EPMA is to produce a specimen whose elemental composition is the same as that observed in the living state. To prevent redistribution of the elements, the specimen is cryofixed as rapidly as possible, and maintained at low temperatures to prevent ice crystal formation (Warley and Gupta, 1991). Regardless of the technique used, ice crystal formation occurs to a varying extent, causing morphological distorsions known as ice crystal damage (Anniko et al., 1984b). However, the gelatinous membrane is a glycosaminoglycan-rich structure which acts as cryoprotective and preserves overall morphology. An essential requirement for the present analysis was the preservation of morphological features, and only well-preserved areas in the membrane were analyzed.

Recently, crystal salt standars have been used to calibrate calcium and pottassium concentrations in the otoconia (Campos et al., 1992). However, this type of standard is unsuitable for the analysis of the gelatinous layer, because the standards differ so markedly from the specimens with respect to matrix composition. Dextran standards may resemble the gelatinous membrane matrix if the standard and the inner ear sample are cryofixed and freze-dried under identical conditions. The differential Z^2/A for dextran and the gelatinous membrane must be taken into account in equation 1 to overcome the dependence of the background on the atomic number (Roomans, 1988).

Although there are no differences between saccule and utricle in the mean value of phosphorus concentration, the distribution of the concentrations was clearly different. The saccule showed a Gaussian distribution with a maximum height at 200 mmol/kg d.w., whereas the utricle was characterized by a bimodal distribution with two peaks, the higher one at the same concentration as that observed in the saccule, and a second peak at 75 mmol/kg d.w. This finding may reflect

Figs. 1 and 2. SEM image of the apical surface of the utricle, showing the gelatinous membrane. Fig. 1. x 1,250. Fig. 2. x 4,580. Scale bar =10 µm

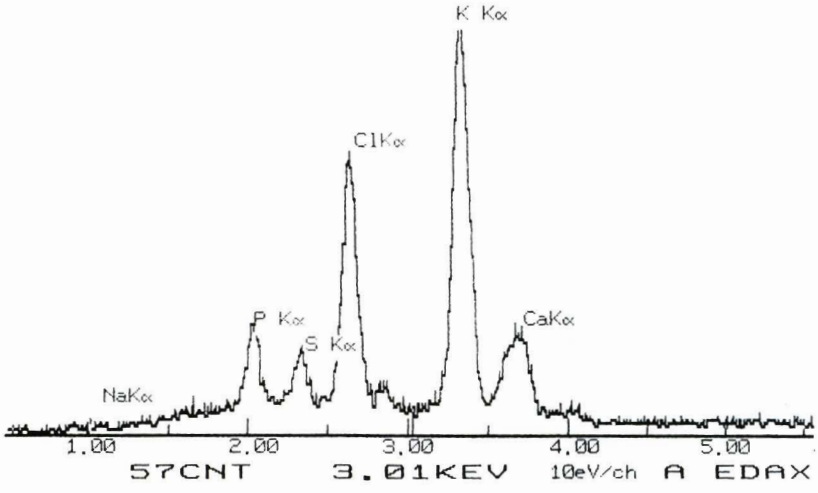


Fig. 3. X-ray emission spectrum obtained from the gelatinous layer.

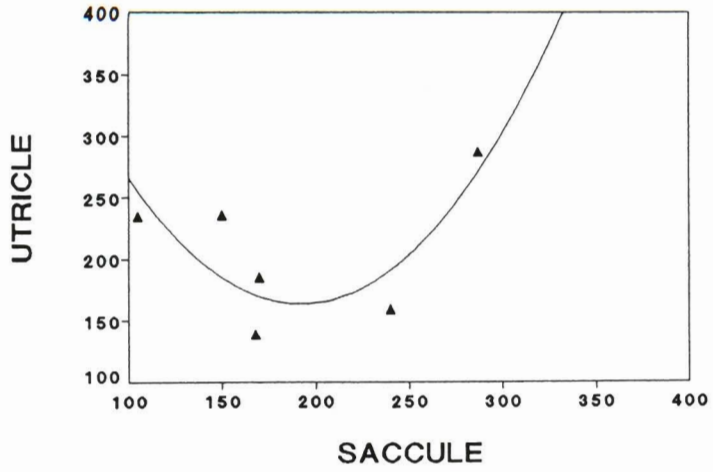


Fig. 4. Plot of the mean value of phosphorus concentrations (mmol/kg d.w.) in the utricle against the saccule.

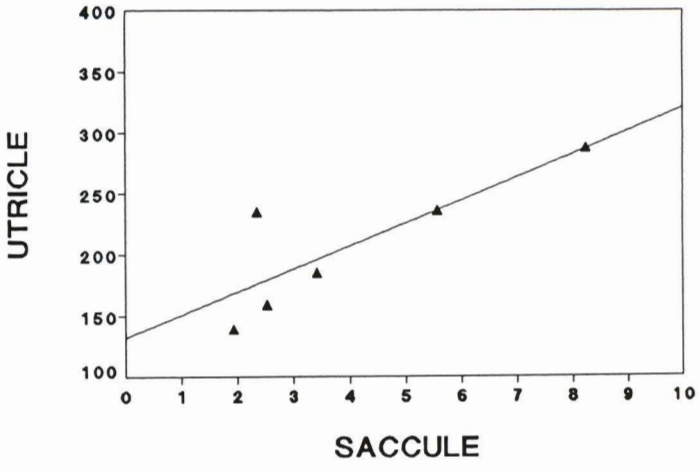
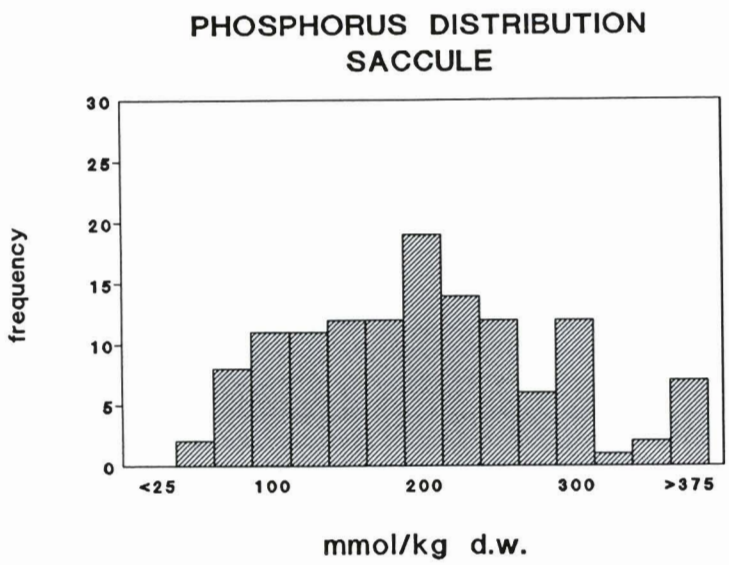
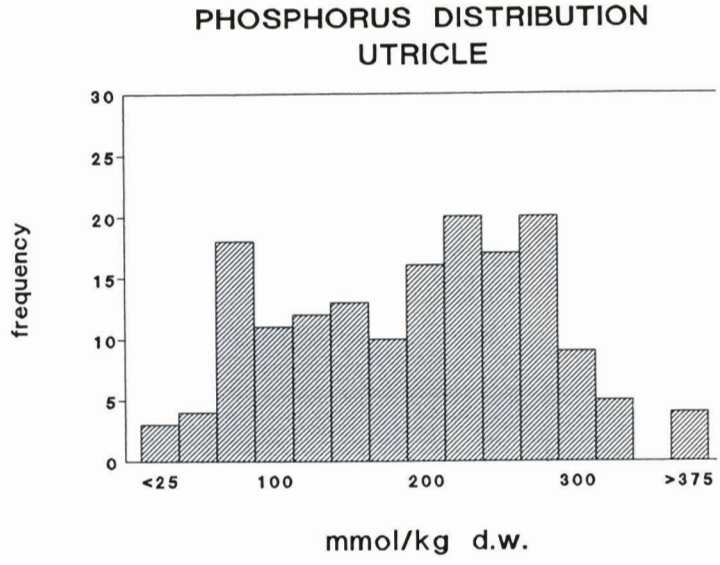


Fig. 5. Plot of phosphorus concentrations (mmol/kg d.w.) after transformation of the data from x to x^2 in the saccule.





Figs. 6 and 7. Frequency histograms for phosphorus concentrations (mmol/kg d.w.) in the saccule (**Fig. 6**) and utricle (**Fig. 7**).

the unequal distribution of phosphorus in the saccule and the utricle. Anniko et al. (1985) found regional differences in the elemental distribution pattern in the adult mouse tectorial membrane, and suggested that these differences can have important physiological implications with regard to ionic permeability of the tectorial membrane and the organ of Corti. This is in agreement with our findings for phosphorus distribution in the gelatinous membrane. The holes in the gelatinous membrane would allow free communication between the fluid in the neurosensory cell epithelium and the endolymph. This implies that the regional differences in phosphorus distribution between the saccule and utricle in the gelatinous membrane are correlated with protein structures of different phosphorus content.

Khan and Drescher (1990) have described the characterization of proteins in the gelatinous layer of the trout saccular membrane by one-dimensional SDS-polyacrylamide gel electrophoresis; they suggested that type II collagen is one of the major proteins. Collagens have the inherent, necessary chemical, stereochemical and electrical charge distributions, which by themselves give rise to sites for the nucleation of calcium crystals. Phosphoproteins may be considered facilitators of nucleation, and when combined with collagen would constitute a highly effective nucleation catalyst (Glimcher, 1989). As far as we know, phosphoproteins have not been studied in the otolithic gelatinous membrane.

Fermin et al. (1990) have identified fibronectin and keratan-sulphate by immunostaining in the otolithic gelatinous layer of the chick, and suggested that the

absence or presence of a particular glycoprotein or glycosaminoglycan in the gelatinous membrane could dictate whether or not the organic template is later mineralized.

Regression analysis is a very useful statistical tool in the study of the relationships among ions and their distribution in biological tissues (Morgan, 1984; Morgan and Winter, 1991). The authors have studied the relationships between calcium and potassium in the saccule and utricle in the otoconia and have shown a linear relationship for calcium and potassium concentrations in both. However potassium has been found to be independent of calcium in both saccule and utricle (López-Escámez et al., 1992). The concentrations of phosphorus in the gelatinous membrane of the saccule and utricle are related by the polynomial function $y = 18.02x^2 + 133.0$ ($r = 0.83$, $P < 0.05$).

Lohdi et al. (1980) proposed that polyphosphoinositides (phosphatidylinositol phosphate and phosphate and phosphatidylinositol biphosphate) serve as *in vivo* receptors for aminoglycosides, suggesting that this interaction could be the mechanism of aminoglycoside ototoxicity. It is unknown whether phosphorus concentration in the gelatinous membrane is affected by aminoglycosides.

Quantitative EPMA used to investigate the otolithic membrane gelatinous layer can increase our understanding of otoconial biomineralization, and may elucidate whether this process is affected by the aminoglycosides.

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References

Anniko M. and Wroblewski R. (1981). The energy dispersive X-ray microanalysis technique in the study of fluids and tissues of the brain and inner ear. Histochemistry 72, 255-268.

Anniko M. and Wroblewski R. (1983). Qualitative and quantitative analysis of otoconia in normal and genetically deaf inner ears. Am. J. Otol. 4, 305-311.

Anniko M. and Wroblewski R. (1986). Ionic environment of cochlear hair cells. Hear. Res. 22, 279-293.

Anniko M., Ylikoski J. and Wroblewski R. (1984a). Microprobe analysis of human otoconia. Acta Otolaryngol. (Stockh.) 97, 283-289.

Anniko M., Lim D. and Wroblewski R. (1984b). Elemental composition of individual cells and tissues in the cochlea. Acta Otolaryngol. (Stockh.) 98, 439-443.

Anniko M., Lim D., Sobin A. and Wroblewski R. (1985). Applications of SEM and SEM-X-ray microanalysis in the inner ear patholgy. Scannin Microsc. 1, 345-355.

Anniko M., Wikström S.O. and Wroblewski R. (1987). X-ray microanalytic studies on developing otoconia. Acta Otolaryngol. (Stockh.)`104, 285-289.

Boekestein A., Stols A.L.H. and Roomans G.M. (1980). Quantitation in X-ray microanalysis of biological bulk specimens. Scanning Electron Microsc., II, 321-324.

Boekestein A., Stadhouders A.M., Stols A.L.H. and Roomans G.M. (1983). Quantitative biological X-ray microanalysis of bulk specimens: an analysis of inaccuracies involved in ZAF-correction. Scanning Electron Microsc. II, 725-736.

Campos A., Ciges M., Cañizares J. and Crespo P.V. (1984). Mineralization in the newborn rat statoconia. An EDAX study. Acta Otalaryngol. (Stockh.) 97, 475-478.

Campos A., López-Escámez J.A., Cañizares F.J. and Crespo P.V. (1992). Electron probe x-ray microanalysis of Ca and K distributions in the otolithic membrane. Micron Microscopica Acta 23, 349-350.

Cañizares F.J., Baeyens J.M., González M.R. and Campos A. (1990). Ototoxicity caused by gentamicin in the otolithic membrane of the saccule. In: Inner Ear Pathobiology. Adv. Otorhinolaryngol. vol. 45. Ciges M. and Campos A. (eds). Karger. Basel. pp 94-98.

Fermin C.D., Lovett A.E., Igarashi M. and Dunner K. (1990). Immunohistochemistry and histochemistry of the inner ear, gelatinous membrane and statoconia of the chick (*Gallus domesticus*). Acta Anat. 138, 75-83.

Glimcher MLJ. (1989). Mechanism of calcification: role of collagen fibrils and collagen-phosphoprotein complexes in vitro and in vivo. Anat. Rec. 224, 139-153.

Hall T.A., Anderson H.C. and Appleton T.C. (1973). The use of thin specimens for X-ray microanalysis in biology. J. Microsc. 99, 177-182.

Höhling H.J., Mishima H., Kozawa Y., Daimon T., Barckhaus R.H. and Ritchter K.D. (1991). Microprobe analyses of the potassium-calcium distribution relationship in predentine. Scanning Microsc. 5, 247-255.

Khan K.M. and Drescher D.G. (1990). Proteins of the gelatinous layer of the trout saccular otolithic membrane. Hear. Res. 43, 149-158.

Lim D.J. (1979). Fine morphology of the otoconial membrane and its relationship to the sensory epithelium. Scanning Electron Microsc. III, 929-938.

Lodhi S., Weiner N.D., Mechigian I. and Schacht J. (1980). Ototoxicity of aminoglycosides correlated with their action on monomolecular films of polyphosphoinositides. Biochem. Pharmacol. 29, 597-601.

López-Escámez J.A., Crespo P.V., Cañizares F.J. and Campos A. (1992). Dependence between saccule and utricle in Ca and K concentrations determined by electron probe X-ray microanalysis. Micron Microscopica Acta 23, 367-368.

Mann S. (1988). Molecular recognition in biomineralization. Nature 332, 119-124.

Morgan A.J. (1984). The localization of heavy metals in the tissues of terrestrial invertebrates by electron microprobe X-ray analysis. Scanning Electron Microsc., IV, 1847-1865.

Morgan A.J. and Winter C. (1991). Diapause in the earthworm, *Aporrectodea Longa*: morphological and quantitative X-ray microanalysis of cryosectioned chloragogenous tissue. Scanning Microsc. 5, 219-227.

Munyer P.D. and Schulte B.A. (1991). Immunohistochemical identification of proteoglycans in gelatinous membranes of cat and gerbil inner ear. Hear. Res. 52, 369-378.

Roomans G.M. (1981). Quantitative electron probe X-ray microanalysis of biological specimens. Scanning Electron Microsc. II, 345-356.

Roomans G.M. (1988). Quantiative X-ray microanalysis of biological specimens. J. Electron Microsc. Tech. 9, 19-43.

Sánchez-Quevedo M.C., Crespo P.V., García J.M. and Campos A. (1989). X-ray microanalytical histochemistry of human circumpulpar and mantle dentine. Bone Mineral. 6, 323-329.

Slepecky N.B., Cefaratti L.K. and Yoo T.J. (1992). Type II and Type IX Collagen from heterotypic fibers in the tectorial membrane of the inner ear. Matrix 11, 80-86.

Small J.A., Heinrick K.F.J., Newbury D.E. and Myklebust R.L. (1979). Progress in the development of the peak-to-background method for the quantitative analysis of single particles with the electron probe. Scanning Electron Micros. II 807-816.

Statham P.J. (1979). Measurements and use of peak to background rations in X-ray microanalysis to biological specimens. Mikrochim. Acta (Vienna) Suppl. 8, 229-242.

Sugiyama S., Spicer S.S., Munyer P.D. and Schulte B.A. (1992). Ultrastructural localization and semiquantiative analysis of glycocongugates in the tectorial membrane. Hear. Res. 58, 35-46.

Tomoda K., Yamashita T., Kumazawa T. and Yoo T.J. (1984). Type II collagen distribution in the middle and inner ear: Immuno-histochemical studies. Ear Res. Jpn. 15, 199-202.

Warley A. and Gupta B.L. (1991). Quantiative biological X-ray microanalysis. In: Electron Microscopy of Tissues, Cells and Organelles: A Practical Approach. Harris J.R. (eds). IRL Oxford University Press. Oxford. pp 243-281.

Wyess L.E., Morris J.A., Oates K., Staff W.G. and Huddart HG. (1987). Quantitative X-ray microanalysis of bulk hydrated specimens: a method using gelatin standards. J. Pathol. 153, 161-169.

Zs.-Nagy I., Piere C., Giuli C., Bertoni-Freddari C. and Zs.-Nagy V. (1977). Energy dispersive X-ray microanalysis of the electrolytes in biological bulk specimens. J. Ultrastruc. Res. 58, 22-33.

Zs.-Nagy I. and Casoli T. (1990). A review of the extension of Hall's method of quantification to bulk specimens X-ray microanalysis. Scanning Microsc. 4, 419-428.

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