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RECENT ADVANCES IN LASER MICROPROBE MASS ANALYSIS (LAMMA) OF INNER EAR TISSUE

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Abstract

Maintenance of ionic gradients within the various fluids compartments of the inner ear requires transport active cellular systems at different locations. LAMMA analysis is ideally suited for detection of ions in microquantity on cellular levels overcoming many technical difficulties. The present paper summarizes the results of microprobe analysis obtained with laser induced mass spectrometry (LAMMA) supplemented by X-ray microprobe analysis of epithelial cell layers adjacent to the endolymphatic space (a) in the cochlear duct, (b) in the vestibular organ and (c) in the endolymphatic sac. The possible role of inner ear as well as ocular melanin in the mechanisms of active ion transport is discussed.

KEY WORDS: Microprobe analysis, Laser microprobe mass analyzer, X-ray, Inner ear tissue, Melanin granules, Trace elements.

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Introduction

The topographic inaccessibility of the inner ear in general and the rather small dimensions of specific structures (e.g. the stria vascularis) have so far prevented the application of conventional techniques usually employed in the experimental analysis of transepithelial transport mechanism. This lends itself to the application of so called microprobe technique to study cellular and/or subcellular ionic distributions.

Studies of that kind have been conducted by (2,3,4,38,39) using energy dispersive electron probe X-ray microanalysis and (1,33,34,35) employing the Laser Microprobe Mass Analyzer (LAMMA) technique.

While earlier LAMMA studies have been concentrated on the structures of the stria vascularis and the endolymphatic sac (1,29,31,33,34,35), recent work (still in progress) includes epithelial cells of the vestibular organ and sensory epithelium presumably involved in active ion transport.

The point of particular interest in this study is the possible role of pigment granula (-melanin)(48) found in cells which are in intimate topographic neighbourhood to transport active epithelial cells (13,20,21,40).

Fig. 1 shows a schematic representation of the cochlea, vestibular organ and endolymphatic sac and also the electrical potential of the endolymphatic space against the perilymphatic fluid (31,32).

The main aim of current microprobe investigations is the determination of intracellular electrolyte concentrations within epithelial cell layers (adjacent to the endolymphatic space) under physiological and experimental conditions, in order to shed some new light on cellular functions which are responsible for active maintenance of ionic gradients found in the various compartments of the inner ear.

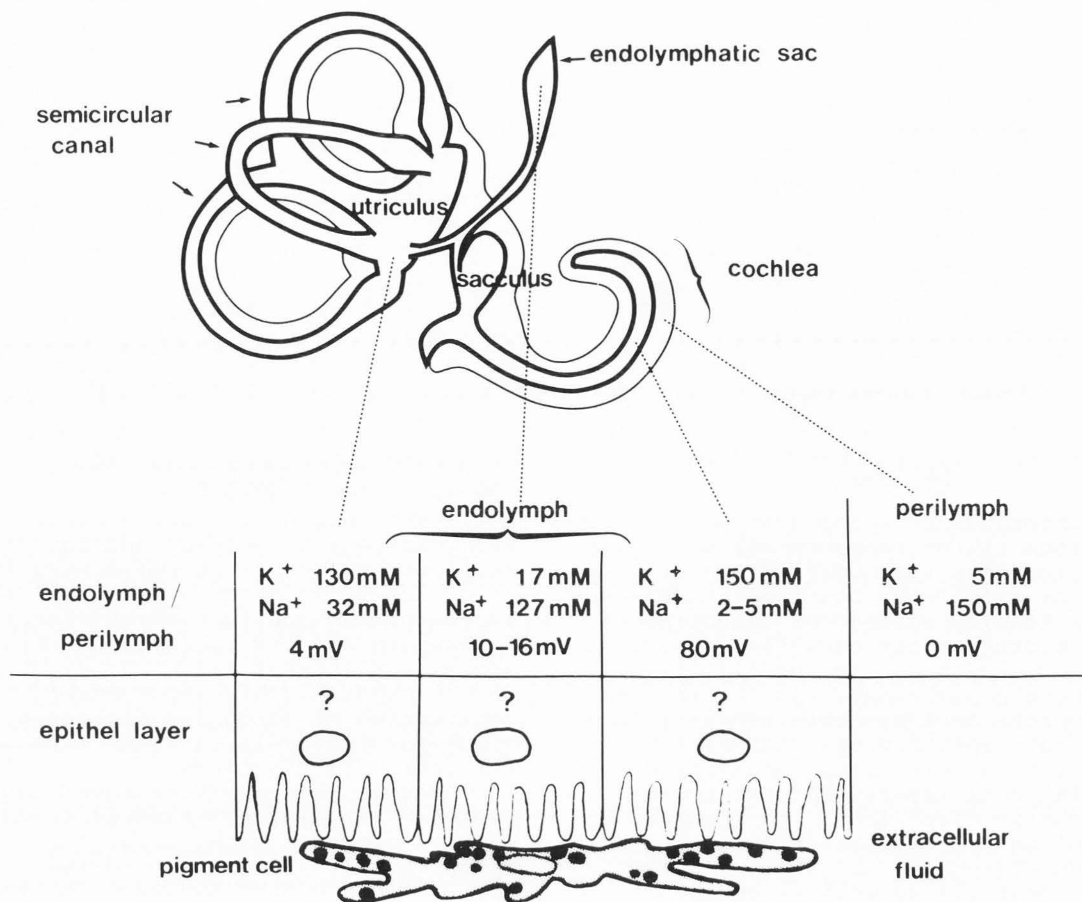

Fig. 1: a) Schematic diagram of the inner ear (after Anson et al. (5)) and
b) the ionic composition and DC-potential of the different endolymphatic spaces (Mori and Morgenstern (32), Morgenstern et al. (30)).

Materials and methods

Tissue preparation

Guinea pigs were anesthetized with 30 mg/kg Nembutal i.p. After opening the middle ear cavity, the perilymphatic space was perfused with oxygen saturated isotonic 0.32 mol sucrose solution at a temperature of 37°C. Under continued perfusion the bone of the cochlear wall was removed and the cochlear duct was quickly dissected.

The vestibular membranous labyrinth was dissected after removing the temporal bone in a Petri dish. The endolymphatic sac was removed in vivo via the posterior fossa approach (1). The tissue was immediately shock-frozen in propane slush, freeze-dried over a period of 2-3 weeks at -110°C and 10^{-6} mbar and finally embedded under vacuum in Spurr's low viscosity medium. Sections of about 0.7 - 1 µm were cut dry and mounted on 3 mm EM grids. Only samples with no apparent light microscopic signs of freezing damage were accepted for microprobe analysis.

The poor structural information obtained in semithin sections of unstained tissue specimen by light microscopy made it rather difficult to visualize structural details or areas of interest. Therefore, subsequent to analysis the sections were stained with 1% toluidine blue for topographical correlation and documentation.

In a recent modification (18) an ultraviolet (UV) transillumination device has been adapted into the LAMMA 500 instrument imaging the specimen at a wavelength of 250 ± 10 nm. This provides for a rather satisfactory absorption contrast (originating mainly from nucleic acid and some amino acid) in otherwise unstained plastic embedded tissue sections as demonstrated by Fig. 2.

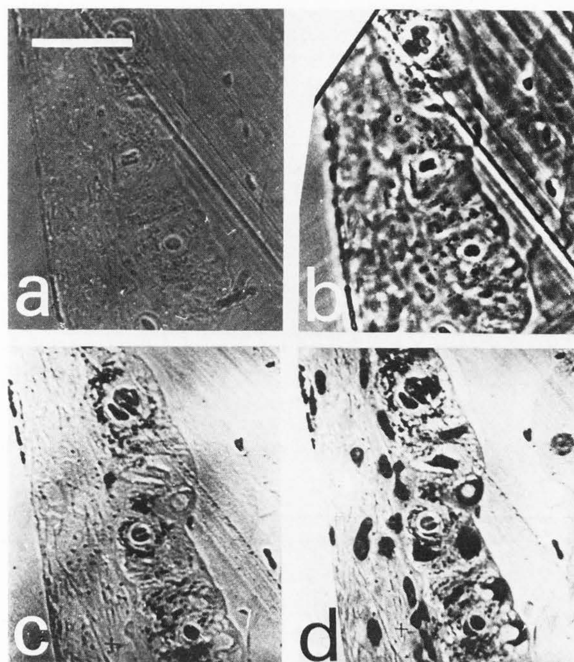

Fig. 2: Comparison of imaging capabilities employing various transillumination techniques in unstained (0.3 µm) sections of plastic embedded tissue specimen from the lateral cochlear wall; bar 25 µm.
a) Visible light, b) phase contrast, c) UV, λ = 350 nm, d) UV, λ = 250 nm.

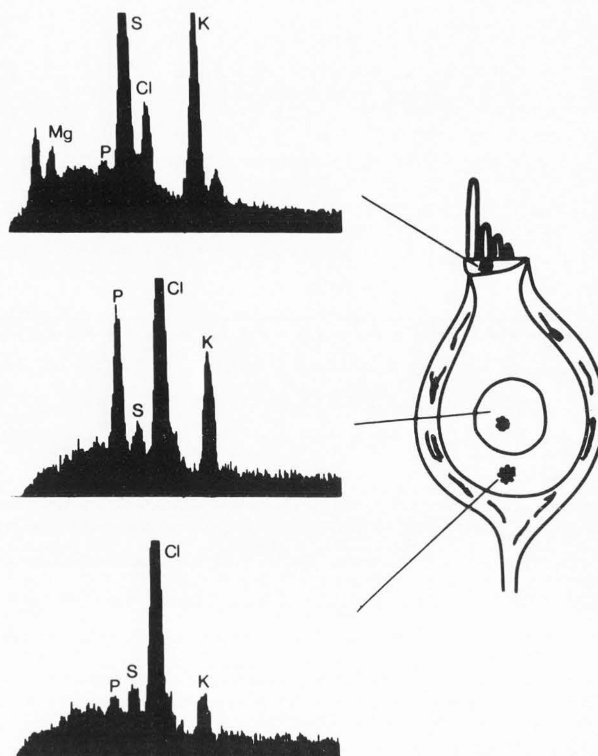

Fig. 3: Energy-dispersive electron probe X-ray spectra taken in a 1 µm thick section of freeze-dried, plastic embedded specimen (hair cell I).
a) Cuticula, b) nucleus, c) cytoplasma. Measurements were performed by Philips EM 420-STEM.

LAMMA technique

Technical features and performance data of the LAMMA instrument have been extensively published elsewhere (15,16, 18,42). Therefore we will restrict ourselves to some principal remarks related only to the problem of sensitivity and quantitative analysis.

In contrast to electron probe microanalysis the relative sensitivity of the LAMMA technique is distinctly higher, in particular for very light and heavy elements (e.g. Ba). This gives access to local distributions of trace elements such as Mg, Ca, Sr, Ba (pigment granules!) at concentrations which are inaccessible to electron probe instruments.

One of the shortcomings of LAMMA in exploring transport active biological structures is the fact that negative counter ions such as Cl cannot be measured in the mass spectrum of positive ions. Although the instrument can be switched to register negative ions it is rather difficult to estimate ion yield and sensitivity under these conditions. Here, electron probe X-ray microanalyses can ideally supplement LAMMA data as indicated by Fig. 3 demonstrating rather large changes of the Cl/K ratio across a single hair cell.

Absolute quantitation of results requires either the use of internal standards or employment of a theoretical approach based on a model for laser induced ion formation such as the LTE (local thermal equilibrium) model (12,28). However, since internal standards for plastic embedded tissue specimen are difficult to establish (44) and since the applicability of the LTE model appears so far to be restricted to inorganic materials only, no attempt for absolute quantitation has been undertaken in the present study.

Instead data refer to relative quantitation only as far as Na and K are concerned by setting arbitrarily the Na concentration to 1. For trace element data only rough quantitative classifications have been employed.

Table I: Data of main physiological cations in inner ear cells as determined by LAMMA analysis. *Standard error. **Data obtained in earliest measurements (Orsulakova and Morgenstern (33) and Orsulakova et al. (35) when specimens were exposed to Na-rich media during preparation.

	n	K / Na	Mg	Ca	Ba
I. COCHLEAR DUCT					
A) stria vascularis					
marginal cells	25	21.6 ± 2.5*	(+)	(-)	
marginal cells**	8	6.9 ± 0.5	+		
intermediate cells	47	9.2 ± 0.46	+	(+)	
intermediate cells**	10	6.8 ± 0.4	+		
basal cells	19	11.8 ± 0.9	(-)	(-)	
basal cells**	11	10.5 ± 0.3			
pigment granules		++	+	(-)	(-)
B) organ of Corti					
outer hair cells	126	13.4 ± 0.5	+	(+)	
inner hair cells	50	23.5 ± 1.9	+	(+)	
supporting cells (Hensen's)	60	10.0 ± 0.5	+		
II. VESTIBULAR ORGAN					
A) wall					
dark cells	41	44.0 ± 3.0	(+)	(-)	
light cells	14	20.7 ± 1.49	+	(+)	
transitional ep.	44	15.5 ± 1.0	+	(-)	
melanocytes		++	+++	(-)	(-)
B) sensory epithelium					
hair cells I	32	14.2 ± 1.6	+	(-)	
hair cells II	7	13.6 ± 2.0	+	(+)	
supporting cells	29	12.8 ± 1.6	(+)	++	
III. ENDOLYMPHATIC SAC					
epithelial layer	49	14.2 ± 1.2	+	(-)	
subepithelial layer	10	5.6 ± 0.56	+	(-)	
melanocytes		++	++	(-)	++

Results and discussion

Table I summarizes the data obtained by LAMMA analysis in various cell types at different locations throughout the inner ear. These results indicate that intracellular [K]/[Na] ratios are significantly different between various epithelial cells of the inner ear. It should be emphasized, however, that the differences behind the figures most probably reflect variation in the intracellular [Na^+] concentration rather than in the intracellular [K^+] content. Further, it appears worthwhile mentioning that the rather large standard deviation found for instance in the marginal cells of the stria vascularis must be at least partly attributed to preparation artifacts (35).

In the subsequent sections the results are presented in detail following a topographical order.

A) Cochlear duct

Stria vascularis. It is widely accepted that the stria vascularis is responsible for the generation of the positive endocochlear potential and the unique endolymphatic ionic composition. However, at the present time the transport mechanism is not yet fully understood. Several enzymes known to be usually involved in active ion transport such as K^+-Na^+-ATPase (19,27), adenylate cyclase (41) and carbonic anhydrase (22) were histochemically localized within cells of the cochlear duct especially in the stria vascularis. As to the fine structure of the stria vascularis one must bear in mind that marginal cells deeply penetrate in between the intermediate cells by protrusion of irregular protoplasmic processes which cannot be resolved in a light microscope. This, necessarily makes some of the LAMMA measurements unreliable.

The three cell types of the stria vascularis, i.e. marginal, intermediate and basal cells differ considerably with respect to their [K]/[Na] ratio. The highest ratio was found in marginal cells (21.6 ± 2.5). In intermediate cells the ratio declines to 9.2 ± 0.46 whereas, finally, in the basal cells [K]/[Na] ratio increases again to 11.8 ± 0.9.

Mg ions were detected in marginal and intermediate cells only the latter also containing Ca in small but detectable amounts.

Evidence of earlier work (34) indicates that this ionic profile in the stria vascularis reflects the presence of an energy dependent active transport mechanism. Marcus et al. (25) have considered the two ion pump models ("single-pump" and "two-pump") to explain the marginal cell function that induced transepithelial potassium movement. Because the potassium concentration is high in the marginal cells (31), they concluded that only the "two-pump" model is likely to be present.

Organ of Corti. The normal function of the inner and outer hair cells is still unknown. Differences of resting potentials reported in inner and outer hair cells respectively (9,45) have stimulated us to check for the ionic contents within cells of the organ of Corti (30).

The [K]/[Na] ratios of the inner and outer hair cells are significantly different. In the inner hair cells an average of 23.5 ± 1.9 and in the outer hair cells an average of 13.4 ± 0.5 were determined. In the area of the stereocilia of either the inner- and outer hair cells a high amount of K was detected (4).

It appears so far that there is a gradient (for either K or Na) along the outer hair cells or at least a clearcut difference in the [K]/[Na] ratio between the supra- and the subnuclear region. (It is well conceivable that this result reflects the rather steep K-concentration gradient existing between the two fluid components on both sides facing the hair cells and, thus, supports the suggestion that K is the ion involved in the transduction process (14). Divalent cations such as Mg and Ca were detected slightly above the detection limit in both types of hair cells. Their exact location needs further investigation. Interestingly enough complementary measurements by electron probe X-ray microanalysis in inner ear cells also demonstrated gradients (for K) when profiling across an individual cell (see Fig. 4).

B) Vestibular organ

Wall (presumably secretory part). The so called dark cells of the vestibular wall are structurally similar to other ion-transporting epithelia (basal foldings) and, hence, have been suspected to be involved in the maintenance of the vestibular endolymphatic composition (47). According to differences in the UV absorption contrast two different cell types could be distinguished along the single-layered vestibular wall: dark cells and irregularly distributed light cells. Underneath their common membrane a row of melanocytes is located (Fig. 5).

The [K]/[Na] ratio of the dark cells amounts to 44.0 ± 3.0 which is the highest figure found throughout the whole inner ear. In contrast to this, light cells (as defined above) had a [K]/[Na] ratio

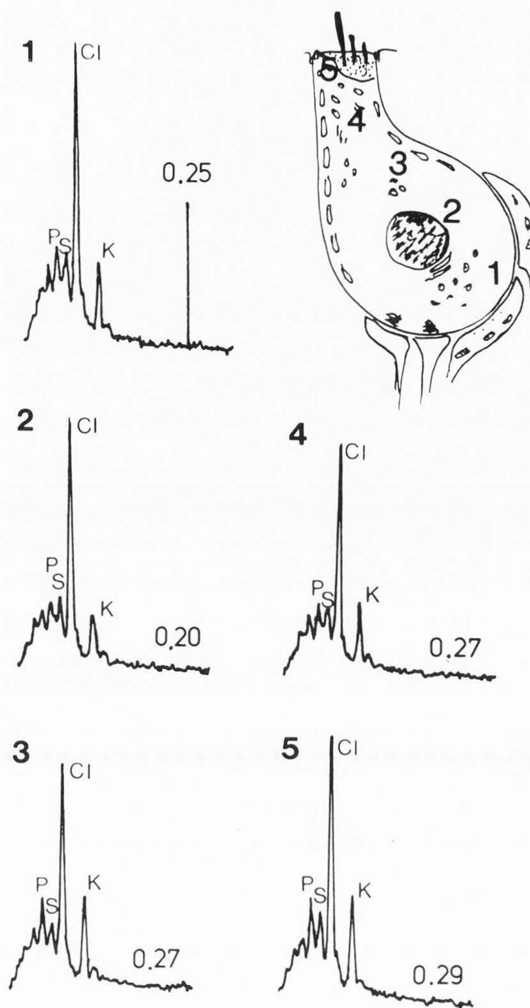

Fig. 4: Energy-dispersive electron probe X-ray spectra taken in a 1 µm thick section of freeze-dried, plastic embedded specimen (inner hair cell). Numbers below each spectrum indicate $\frac{K}{\text{background}}$ ratio for K_{α}-signal.

of 20.7 ± 1.49 only. Between the dark and the sensory epithelium is a layer of tall cylindrical cells containing unspecified secretory granules. The [K]/[Na] ratio of these transitorial cells was determined to be 15.5 ± 1.0.

Sensory epithelium. The sensory cells types I and II are both surrounded by supporting cells. There is generally a broad basal part of the supporting cells which contains the nucleus. The middle part, which protrudes between the sensory cells, is often flattened out to a thin prismatic portion extending to the epithelial surface.

With the LAMMA data so far available there is no significant difference between either type I hair cells (surrounded by a

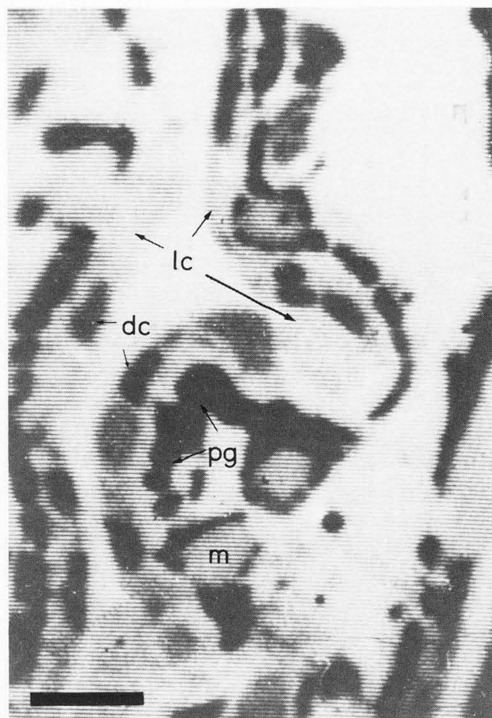

Fig. 5: UV imaging (λ = 250 nm) of utricular epithelial layer shows different absorption contrast of epithelial cells. dc = dark cell, lc = light cell, m = melanocytes, pg = pigment granules, bar = 15 µm.

nerve chalice) and type II hair cells on the one hand and between hair cells and supporting cells on the other hand. In the sensory epithelium of crista ampullaris and macula utriculi large amounts of Ca were found in supporting cells, especially in the area of thin prismatic portion extending between the subnuclear region of the hair cells (Fig. 6).

In this context the limitation of spatial resolution in the LAMMA instrument did not allow further (ultra)structural analysis of these findings. An attempt to employ electron probe X-ray microanalysis was unsuccessful due to the insufficient detection limits for low Ca-concentrations. However, the presence of high Ca sharply restricted to the subnuclear borderline between adjacent hair and supporting cells is rather puzzling and one may speculate about the transduction process occurring in the sensory hair cells. A hypothetical mechanism could, for instance, involve the entry of Ca ions as a trigger following the excitatory phase of their displacement (14). On the other hand the supporting cells contain a large amount of granules which increase in number toward the apical surface, their function is not yet fully understood. In these cells a high concentration of carbonic anhydrase, enzyme which has been discussed to be involved in the turnover of Ca (especially in immature inner ear), was demonstrated by Lim (22).

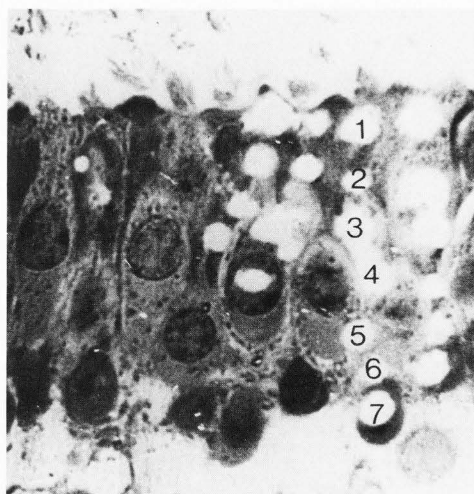

Fig. 6: Typical LAMMA spectra of sensory epithelium of crista ampullaris. The white spots indicate areas of LAMMA analysis. Ca was detected in the spots labelled 1, 5, and 6 respectively. For structural identification of these areas see text.

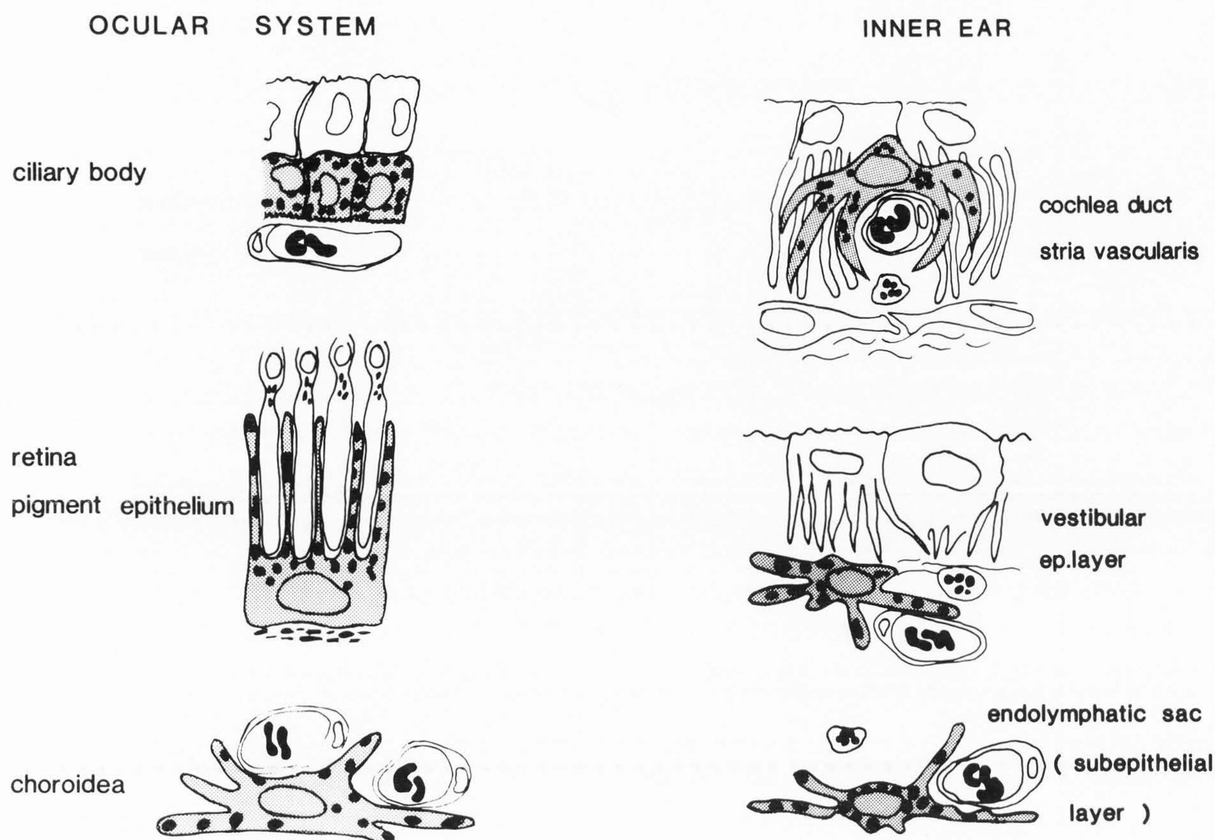

Fig. 7: Schematic diagram of typical pigment locations in the inner ear and eye of the guinea pig.

C) Endolymphatic sac

LAMMA measurements on the cation content were performed in the area of highest metabolic activity, that is the pars rugosa. The mean [K]/[Na] ratio in epithelial cells was 14.2 ± 1.2 (at the time the measurements were done the imaging system of LAMMA could not differentiate between dark and light cells) and differs significantly from the 5.6 ± 0.56 ratio found in the subepithelial layer.

Pigment granules

The presence of pigment granules in inner ear tissue has been known since Corti's publication in 1851. Pigment granules are found in most parts of the inner ear, especially in the neighbourhood of such epithelia, which are supposed to be involved in the secretion and/or absorption of the endolymphatic fluid.

In the stria vascularis the pigment granules are located in the intermediate cells which, according to Hilding and Ginsberg (13), are octopus-like melanocytes. The melanocytes in the utriculus and ampullar wall create some kind of a network located underneath the basement of the epithelial layer membrane. In the endolymphatic sac, finally, melanocytes are sparsely distributed within the subepithelial layer of the pars rugosa (Fig. 7). In all these locations the melanocytes are usually closely associated with capillaries and form extensive contacts via multiple dendritic processes.

A rather similar situation holds true for the ocular melanin which also appears to be located or concentrated in transport active areas.

The physiological role of melanin (except for skin) is by no means fully understood. With respect to the presence of free radicals Mason et al. (26) suppose that melanin protects tissue structures against reducing or oxidizing agents or conditions. On the other hand, the apparent affinity of melanin to bind certain drugs or toxic products (10,23,24,46) may be harmful in those areas where the turnover of melanin is slow.

Recent evidence from microprobe analysis including ion probe (8) and LAMMA (17,43) established a rather extreme affinity of some ocular melanin to accumulate divalent heavy metals, in particular Ba. It was later suspected By Brown and Fleming (7) that small amounts of Ba^{2+} ions modulate the intensity-response curve of rods in the process of dark adaptation. In the astacus retina Schroeder and Stieve

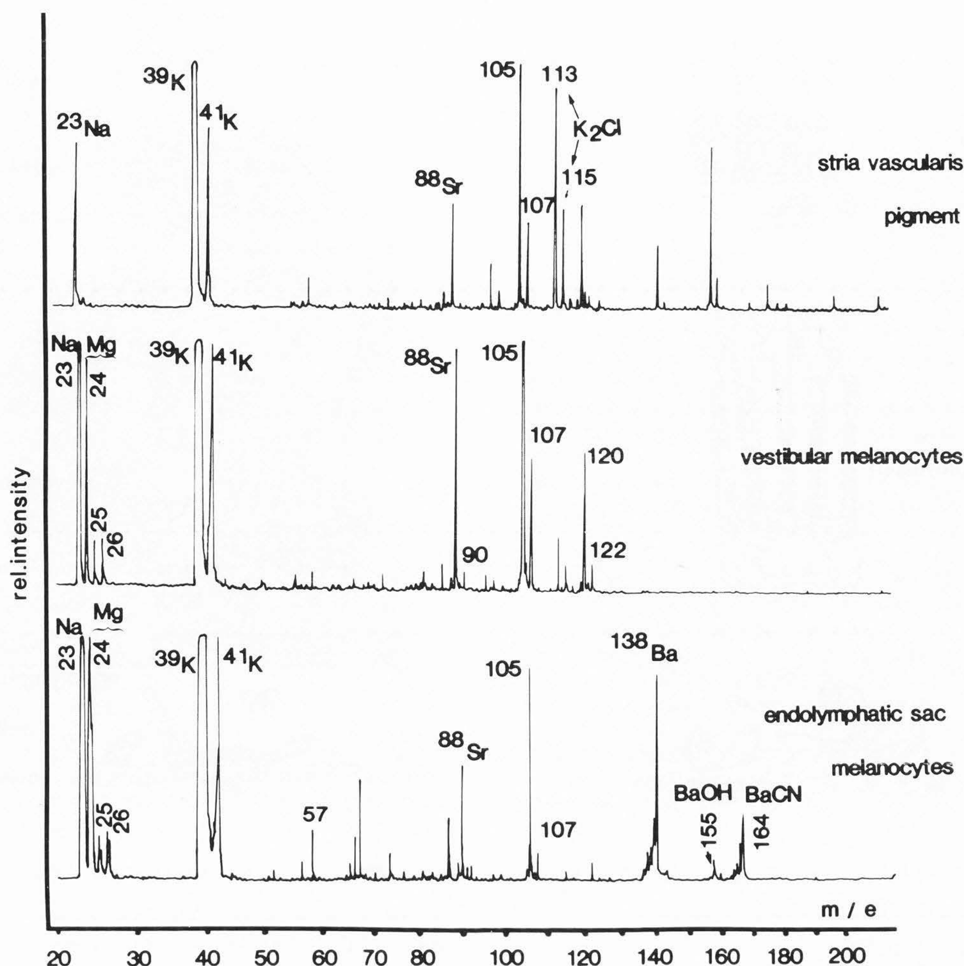

Fig. 8: LAMMA spectra of pigment granules from different locations in the inner ear (guinea pig).

(43) applying the LAMMA-technique in combination with stable isotope (44 Ca) dilution could demonstrate that, at least in this species, melanin serves as a storage compartment for Ca probably involved in the photoreceptor impulse transduction. One might be tempted to hypothesize that melanin serves as a reservoir for divalent inorganic cations, which are needed for neurotransmitter release or to modulate various metabolic processes, energy conversion and ion pumps.

To the best of our knowledge the widespread melanin of the inner ear has not been investigated so far with respect to its particular affinity for or ability to store specific cations. The results obtained in a preliminary LAMMA study can be summarized as follows:

Unlike ocular melanin melanocyte-bound melanin of the inner ear contains rather large amounts of Mg (see Figs. 8 and 9). This applies to both, vestibular melanocytes and to melanocytes of the endolymphatic sac. In addition a mass signal at mass number 88 appears to indicate the presence of Sr although the possibility has not yet fully ruled out that an organic fragment ion of normally the same m/e number hides behind this signal.

As to the presence of Ba there is no doubt that (amazingly enough) only melanin in the endolymphatic sac contains rather large amounts of Ba, whereas not even trace amounts of Ba could be detected in vestibular melanin (but also in stria vascularis melanin) (see Fig. 8). It might be worthwhile mentioning that also in ocular melanin, Ba uptake appears to be restricted to melanin of choroideal melanocytes only.

We come back to the suggestion that melanin indeed acts not only as a trap

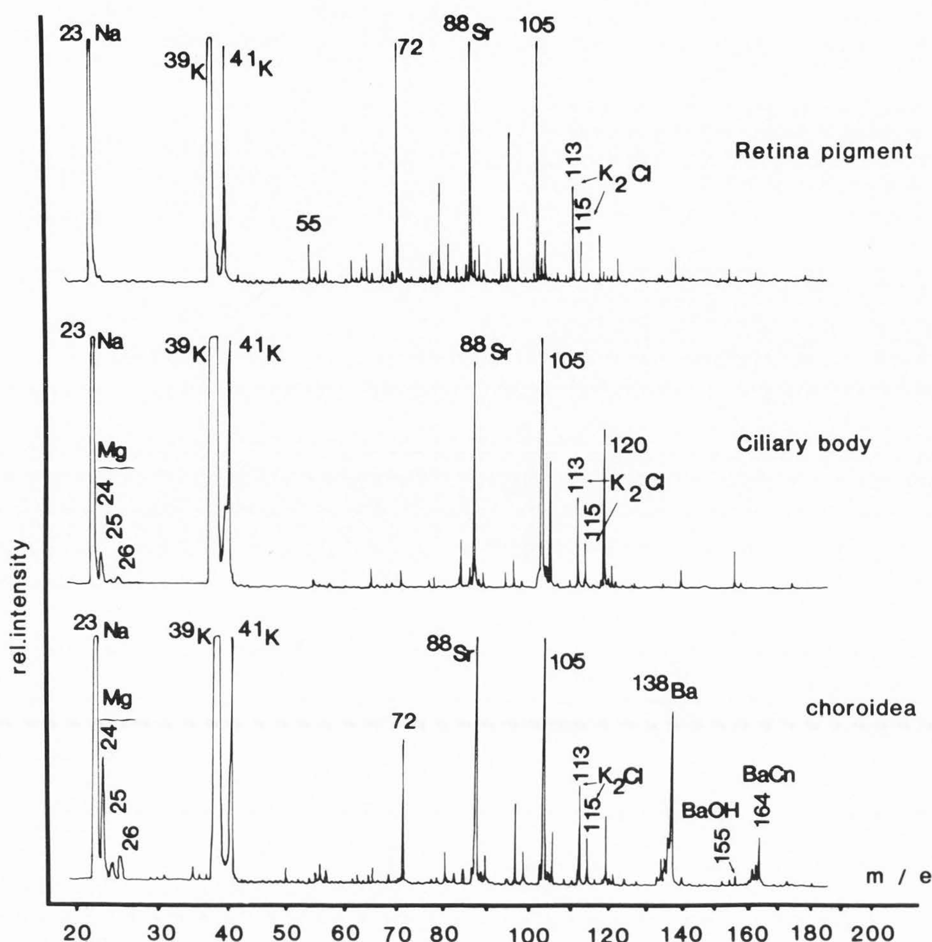

Fig. 9: LAMMA spectra of pigment granules from different locations in the ocular wall (guinea pig).

for drugs and (unwanted or unnecessary) cations but also serves as an important reservoir for essential trace elements or bioactive compounds. Thus, one may look for independent evidence as to possible interactions of unusual cations (such as Ba, Sr) with enzymatically controlled processes known to play a key role in sensory systems (maintenance of ionic gradients, impulse transduction, energy conversion).

Since an extensive review of the evidence so far available is beyond the scope of the present paper the reader may refer to review and/or articles of (6,11, 22,26,37,41).

However, a final observation made during this study (which will be published elsewhere) may be added. Subsequent to changes in the ionic concentration of the endolymph (especially elevated Na^+) pigment granules migrate from the perinuclear region into the dendritic processes. At the same time melanocytes tend to get closer to either secretory cells and cappillaries. This phenomenon can be taken as an attempt of the melanocytes to bring melanin into a shorter range to transport active cells (or sites)(36).

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Discussion with Reviewers

R.S. Kimura: The ratio of K/Na in the cochlear outer hair cells, vestibular type I cells and the endolymphatic sac epithelial cells is essentially the same (Table I). The explanation for the outer hair cells showing the gradient of K/Na from the cell apex to the base is given as the reflection of the high K^+ in the endolymph side and low K^+ in the Corti's lymph or perilymph side. How can one account for the similar K/Na ratio in the sac epithelium while K^+- and Na^+-concentration are reversed in the sac fluid in comparison to the cochlear and vestibular endolymph (Fig. 1)?

Authors: In both cases the data given in Table I represent mean values averaged over a large number of individual cells. The difference between cochlear hair cells and epithelial cells of the endolymphatic sac appears to be an intracellular K^+ gradient found exclusively along some (but not all) cochlear hair cells.

R.S. Kimura: In view of the fact that the albino guinea pig lacks pigment granules in the areas studied, how important are melanocytes in the function of the inner ear?

Authors: Significant differences between albino and pigmented animals have been reported in a number of experiments involving cochlear function (49). In albino animals melanocytes are present but contain no melanin. It would therefore not be surprising if at least some aspects of cochlear and vestibular functions were affectes by albino mutation.

D.J. Lim: According to Burgio, there is a very rapid sodium movement when the animal is killed, so he freezes the inner ear while the animal is under anesthesia. Because you are removing tissue before freezing, have you compared these data with freshly frozen tissue to see whether there is any possibility of electrolyte migration in your specimens?
Authors: Attempts to freeze cochlea in situ caused unacceptable damages and resulted in the development of large ice crystals which deteriorate the K/Na ratio (35). In a previous paper (35) we studied the redistribution of electrolytes in the stria vascularis as depending on the ionic composition of the medium used for dissection (artificial endolymph, perilymph and isotonic sucrose solution). From this study isotonic sucrose became the favoured incubation medium due to minimal changes in cellular ion distribution.

M. Anniko: For how long was the perilymphatic space perfused with oxygen saturated isotonic 0.35 mol sucrose solution?
Authors: Maximally for 2 minutes.

M. Anniko: How long do you estimate that you need for dissection of inner ear tissues after removing the temporal bone but before the tissues are shock frozen?
Authors: 1-2 minutes for the vestibular organ.

M. Anniko: Do you have any evidence if and in that case to what extent the elemental composition of the embedding medium Spurr can interfere with the results of elemental analysis?
Authors: One of the reasons for choosing Spurr's medium (instead of e.g. Epon) is its known low content of interfering elements. There are only trace amounts of K and Na in the commercially available Spurr's medium.

M. Anniko: I want to make a comment that the LAMMA technique has a higher sensitivity for detection of especially very light elements as compared with the energy dispersive X-ray microanalysis technique, but that spatial resolution is poorer as compared with the latter technique. The preparation of tissues for both techniques is similar and one should consider the two techniques as complementary to each other. I think that the preparation technique used for biological material at present is quite reliable. This is shown by differences in the elemental composition of the three cell types in the stria vascularis as documented both with the LAMMA technique and with the energy dispersive X-ray microanalysis technique.

Authors: We think that with respect to the comment of Anniko one must clarify that in X-ray microprobe analysis sensitivity and spatial resolution are not independent parameters. To make use of a high (analytical) spatial resolution in X-ray microanalysis ultrathin sections are needed but at the expense of detection limits whereas, with thicker sections, electron diffusion limits the analytical spatial resolution to about 1 µm which is essentially the same as in the LAMMA-technique.

R.W. Linton:
1) Details of LAMMA parameters should be specified (e.g. laser frequency, power density, mass resolution, etc).
2) Possibilities for preparative artifacts including the translocation of Na^+ or K^+ should be discussed.
3) The influence of dynamic range limitations on the absolute accuracy of K^+/Na^+ ratios should be mentioned. Is it possible to measure intensity ratios as high as 44 (Table I) without a significant attenuation of the K^+ signal? Combined electron multiplier saturation and transient digitizer limitations apparently reduce the effective dynamic range well below the maximum 8 bit value (D.S. Simons, Int. J. Mass Spec. Ion Proc. 55, 15-30, 1983/1984).
4) Do the authors have any information concerning possible relative ion yield variations for Na^+ vs. K^+ when comparing ratios from the various tissue components (Table I)?
5) Were any correlations made between LAMMA and electron microprobe Na/K ratio data?
Authors: Following the sequence of questions asked by the referee the subsequent responses are given:
1) LAMMA parameters are standard (as published elsewhere by the authors). To satisfy the referee's request: λ = 256 nm, power density is $\simeq 5 \times 10^8$ Wcm^{-2} corresponding to about 5 times the threshold irradiance for ion formation, mass resolution is typically 750 $m/\Delta m$.
2) See answers to referee Lim, Anniko and Ryan.
3) The dynamic range in the LAMMA-instrument is indeed limited to about 5 bits by the Biomation transient recorder. Fortunately enough the ^{41}K abundance is about 7% of total K which is about in the range of the Na-signal recorded at a given input sensitivity of the transient recorder. Therefore, no serious difficulties arise in the case of determining K/Na ratios at least within the limits of accuracy given by the transient recorder.
4) Relative ion yield for K and Na is not different in various tissue components. Matrix effects play only a minor role in LAMMA analysis (as compared with SIMS). With any plastic-embedded specimen the sensitivity factor for Na (with respect to K) was found to be constant at standardized irradiance conditions (see answer under 1)).

5) No correlations were made since the electron microprobe instrument at the Philips Company did not allow to determine Na at the given concentration level.

A.F. Ryan: There is evidence that plastic embedding can produce movement of ions, particularly sodium ions, in tissue. Have you prepared standards to test for the preservation of ion ratios during your plastic embedding procedure?
Authors: Studies (unpublished) have been made in embedded tissue culture cells as well as in erythrocytes or lympoid cells. With carefully controlled freeze-drying, sectioning and transfer procedures there was no "diffusion halo" of K^+ around such embedded individual cells. Furthermore, the K/Na ratio in red blood cells turned out to be exactly within the limits published in the literature.

A.F. Ryan: Several investigators (e.g. Marcus et al., Hearing Res. 4, 149, 1981) have shown that complete removal of potassium from the perilymphatic compartment abolishes the endolymphatic potential. This suggests that your sucrose perfusions result in a cochlea with a reduced or absent EP, depending upon the length of the perfusion period. Might this have influenced your observations of stria vascularis ion distribution?
Authors: The complete removal of potassium from the perilymphatic compartment abolishes the endolymphatic potential not before a period of 30 minutes (50). The first few minutes (our perfusion time is maximally 2 minutes) does not change the EP. The increased perfusion rates (20-25 µl/min) over the period of 2 min produced only minor changes of EP, probably due to the perfusion pressure.

A.F. Ryan: In the "LAMMA Technique" section the authors state that "internal standards for plastic embedded tissue specimens are difficult to establish". What does this mean? What are the difficulties?
Authors: The problem with internal standards is to fulfill the prerequisite of a homogeneous distribution of the reference element throughout the specimen. The only way to cope with this requirement is the vacuum deposition of, say a CaF layer of known thickness on top of the section (44). Unfortunately no K or Na salts are known which can be vacuum deposited to form a homogeneous molecular film.

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