Scanning Microscopy

Volume 5 | Number 2 Article 24

6-4-1991

Preparation of Inner Ear Sensory Hair Bundles for High Resolution Scanning Electron Microscopy

M. P. Osborne
University of Birmingham, United Kingdom

S. D. Comis
University of Birmingham, United Kingdom

Scanning Microscopy, Vol. 5, No. 2, 1991 (Pages 555-564) 0891-7035/91$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

PREPARATION OF INNER EAR SENSORY HAIR BUNDLES FOR HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY

M.P. Osborne* and S.D. Comis

Department of Physiology, School of Basic Medical Sciences, The Medical School, University of Birmingham, Birmingham B15 2TT, U.K.

(Received for publication March 26, 1991, and in revised form June 4, 1991)

Abstract

Chemical fixation techniques for preservation of sensory hair bundles in the mammalian inner ear for scanning electron microscopy (SEM) are reviewed. Fixatives employed were glutaraldehyde, glutaraldehyde-picrate, glutaraldehyde-tannic acid, glutaraldehyde-formaldehyde, glutaraldehyde followed by postfixation with osmium tetroxide and the osmium thiocarbohydrazide (OTOTO) method. Dehydration was routinely accomplished with ascending grades of acetone followed by critical point drying with liquid CO_2 or fluorocarbon sublimation. Specimens other than those prepared by the OTOTO method were metal coated with gold, gold-palladium or platinum. Material was viewed at high resolution (2-3 nm) in a transmission electron microscope (TEM) fitted with a scanning system and an LaB_6 filament. A few specimens, which were either coated with platinum, carbon or uncoated, were examined in a field emission SEM.

We have concluded that glutaraldehyde fixation followed by critical point drying with CO_2 and coating with platinum gives the best general preservation of stereocilia and their cross-links for routine high resolution SEM, but that carbon-coated or uncoated specimens offer potentially better results free from metal coating artifacts when viewed with field emission SEM.

These methods have enabled us to make novel observations upon the surface detail and cross-links of stereocilia which have helped considerably in understanding the mechanical properties of hair bundles particularly in relation to sensory transduction. We have found that stereocilial surface detail and cross-links are sensitive to fixation regimes. In particular they are degraded by exposure to osmium tetroxide; they are also highly labile since deleterious changes in their appearance can be detected as early as 15 minutes following death.

Key Words: Guinea pig, inner ear, hair cells, stereocilia, chemical fixation, critical point drying, fluorocarbon sublimation, scanning electron microscopy, field emission.

*Address for correspondence:
M.P. Osborne, Department of Physiology,
The Medical School, University of Birmingham.
PO Box 363, Birmingham B15 2TT, U.K.
Phone: 021-414-6917

Introduction

Sensory hair cells in the vertebrate acousticolateralis system have been examined by transmission electron microscopy (TEM) since the 1950's and their ultrastructure has been well characterized (see Engström *et al*, 1966; Wersäll *et al*, 1967). Examination by scanning electron microscopy (SEM) since the 1960's has revealed considerable detail of the topographical arrangement of stereocilia (e.g., Soudijn, 1976). In spite of several decades of study, little progress had been made, even by the early 1980's, in relating the structure of stereocilia (and kinocilium, if present) to the process of mechanoelectrical transduction (e.g., Hudspeth, 1983).

Conventional SEMs have a limiting resolution of approximately 5-6 nm with secondary electron detectors, but during the 1980's it became possible to fit scanning modules to TEMs. Such modules have an improved resolving power of 2-3 nm. By exploiting this higher resolving power in combination with glutaraldehyde-only fixation (i.e., by omitting postfixation in osmium tetroxide), we have been able to describe the surface detail and cross-linkages between stereocilia in both the organ of Corti and vestibular system in far greater detail than was previously possible. We also discovered a new type of cross-link, the tip link, between stereocilia; it projects from the apical point of each stereocilium to the lateral surface of the adjacent stereocilium in the next taller rank. Tip links are ideally placed to detect mechanical displacement of the hair bundles and could well be involved with mechanoelectrical transduction (for a full discussion of the arguments implicating tip links in sensory transduction see: Osborne *et al*, 1984, 1988; Pickles *et al*, 1984; Osborne and Comis, 1990a; Hudspeth, 1989).

The present paper is concerned with a description of those preparatory techniques which we have found by experience over the past eight years to give excellent preservation of sensory hair bundles suitable for examination by SEM at resolutions approaching 2 nm or better. We describe unpublished observations using fluoro-

carbon sublimation instead of the more conventional critical point drying (CPD) with liquid CO_2 and also present recent results obtained from metal coated, carbon coated and uncoated material examined by an ultrahigh resolution field emission SEM.

Material and Methods

Fixation

All observations made on hair bundles in this article are from the guinea pig inner ear. Guinea pigs were killed by administration of an overdose of pentobarbitone sodium injected intraperitoneally. Temporal bones were removed and the bullae were opened to expose the cochleae. A micropipette drawn to a fine point from borosilicate glass capillary tubing, 1.5 mm outside diameter, was coupled to a tuberculin 1 ml syringe with polythene tubing. The syringe was filled with fixative at 4°C, the tip of the pipette which had previously been broken such that its overall diameter was about 100 μm, was then inserted into the round window. Fixative, at a rate of 0.1 ml over three minutes, was perfused through the cochlea and labyrinth, the effluate escaping through a small hole drilled at the cochlea apex. This method enabled fixation of the inner ear within moments of removal from the animal. We have perfused-fixed *in situ*, but no differences in quality of preservation were detected between specimens prepared by either method. After perfusion, the ears were immersed in fresh, cold fixative. Several hours later the inner ears were opened under a dissecting microscope and the modioli and sensory areas from the vestibular system were removed for further processing. Particular care needs to be taken in removal of accessory structures such as cupulae and otolith masses in order to avoid mechanical disruption of hair bundles.

Glutaraldehyde. The majority of specimens were fixed in 2.5% glutaraldehyde in 0.05 M BES (NN-bis(2-hydroxyethyl)-2-aminoethane-sulphonic acid) buffer at pH 7.4 with the total molarity adjusted with sucrose to 0.35 M. BES was chosen because of its good ultrastructural preservation, excellent buffering capability at physiological pH, low effect upon the electrical activity of the cochlea and low chelating capacity for divalent cations including Ca^{++} and Mg^{++}. Material not destined for postfixation in osmium tetroxide was stored in fixative overnight at 4°C.

Glutaraldehyde-picrate. Fixation was achieved in a mixture of 1% glutaraldehyde and 15% saturated picric acid in 0.05 M phosphate buffer (Newman *et al*, 1983) for 1 hour at 4°C.

Glutaraldehyde-tannic acid. Specimens were fixed in 2.5% glutaraldehyde plus 2% tannic acid in BES buffer (pH 7.4) at 4°C. They were then washed in cold Millonig buffer for 10 minutes followed by immersion in 2% tannic acid in distilled water (pH adjusted to 7.0 with 0.1 M NaOH) in the cold for 30 minutes. This method is a modification of that used by Little and Neugebauer (1985).

Glutaraldehyde-formaldehyde. Cochleae were perfused with a mixture of 1.5% formaldehyde (prepared from paraformaldehyde) and 1% glutaraldehyde in 0.05 M BES buffer (pH 7.4) at 4°C. Fixation was in the cold for between 4-16 hours.

Glutaraldehyde-osmium tetroxide. After routine fixation in glutaraldehyde for 1-2 hours, tissues were postfixed in osmium tetroxide (1% w/v in Millonig buffer, pH 7.4) in the cold for times varying between 30 seconds and 24 hours.

Glutaraldehyde-osmium tetroxide-thiocarbohydrazide (OTOTO). This method is similar to that of Furness and Hackney (1986). Following perfusion with cold 2.5% glutaraldehyde buffered at pH 7.4 with 0.1 M sodium cacodylate plus 2 mM calcium chloride, unopened cochleae were kept in this fixative for a further 2 hours during which time they were allowed to warm up to room temperature. Specimens were washed in cacodylate buffer before postfixation in 1% osmium tetroxide in the same buffer. After immersion in 70% ethanol, cochleae were passed through 50 and 30% ethanol, 10 minutes in each, prior to placing in distilled water. Next, tissues were exposed to a saturated aqueous solution of thiocarbohydrazide for 20 minutes, washed 6 times in distilled water, transferred to 1% osmium tetroxide and passed through 6 changes of buffer. The thiocarbohydrazide treatment was repeated twice before removing the modioli.

Dehydration

A few specimens were passed through 70% ethanol prior to dehydration via 50, 70, 90 and 100% acetone, but the majority of the material was dehydrated in acetone only. Dehydration took place at 4°C, with tissues being allowed to warm to room temperature when in 100% acetone. OTOTO-fixed material was dehydrated in ethanol at room temperature.

Critical Point Drying (CPD)

CPD was usually carried out with liquid CO_2 in either Polaron E3000 or Emscope CPD 750 driers.

Fluorocarbon Sublimation (Peldri II) Drying

Peldri II (Kennedy *et al*, 1989) is a fluorocarbon that is solid at room temperature and liquid above 25°C. Tissues, after dehydration in ethanol or acetone are

embedded in Peldri II by immersion in the liquid phase and allowing it to solidify. Once solidified, sublimation dehydration will take place, with or without vacuum assistance.

With our material, after routine dehydration, tissues were placed in a 3:1 mixture of Peldri/acetone followed by 100% Peldri, both for 1 hour at 38°C. Surplus Peldri was decanted and the specimens were cooled over ice. Some specimens were dried at atmospheric pressure overnight between 0-5°C; others were vacuum dried for 2 hours at the same temperature.

Metal Coating

Prior to coating, sensory areas were glued to JEOL copper strip SEM TEMSCAN holders with Araldite Rapid (Ciba-Geigy). We used two coating systems in our studies, a Polaron E5400 using a current of 20 mA and high tension voltage of 1 kV, or an Emscope SC500 low voltage machine operated at 100-150 V with 10-20 mA. All specimens were coated to a nominal depth of 12-15 nm at ambient temperature with either gold, gold/palladium or platinum. Specimens were stored in desiccators over silica gel until examined by SEM.

Electron Microscopy

Most of the material was examined in a JEOL 120CXII TEM fitted with a scanning attachment (TEMSCAN, model EM-ASID-4D) and LaB_6 filament. This filament gave a less noisy image at higher magnifications combined with a slight improvement in depth of field compared with the conventional tungsten electron source. Specimens were examined in the secondary electron mode at accelerating voltages of 20 or 40 kV with a preference for the higher voltage. The beam current was set to 10-20 μA above the standing current. The second condenser aperture was selected to give the best depth of field allied with an adequate signal to noise ratio. The best compromise on our machine was 100 μm. The spot size was set to the "low magnification" mode; smaller spot sizes did not improve resolution and generally reduced even further the depth of field. Even in the low magnification mode, considerable care is necessary with focusing at magnifications > 20,000 X because of the very limited depth of field available. Most micrographs were taken at magnifications ranging between 500 and 30,000 X; only under very favourable conditions was it possible to take micrographs at usable magnifications above 30,000 X.

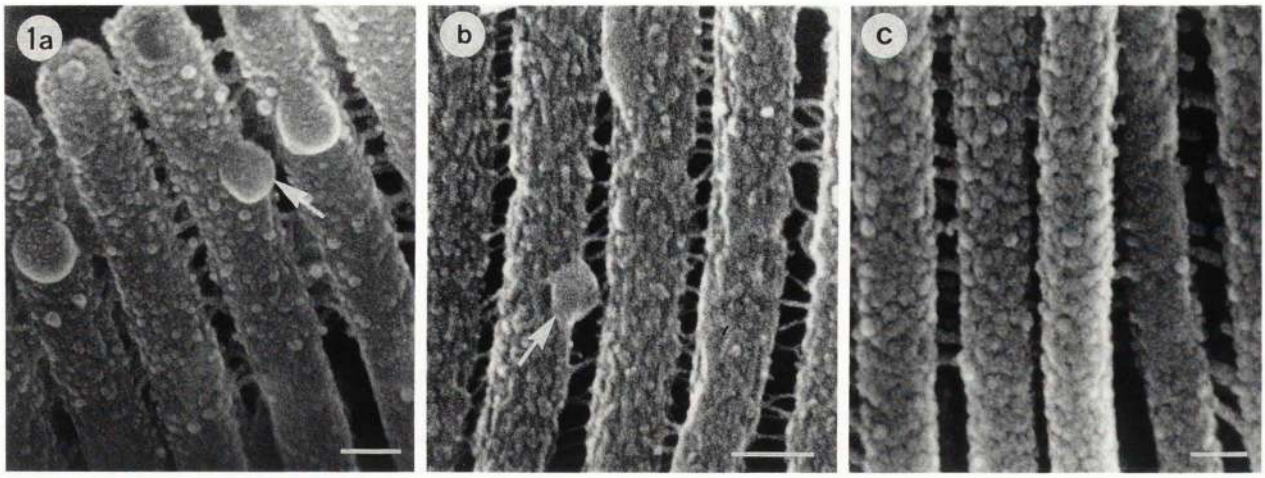
For field emission SEM observations, a JEOL JSM-890 was used. The beam and other operating parameters, apart from accelerating voltages, are unknown to us because specimens were sent to Japan for examination. However, perfectly acceptable micrographs could be taken at direct magnifications as high as 300,000 X with accelerating voltages as low as 6 kV.

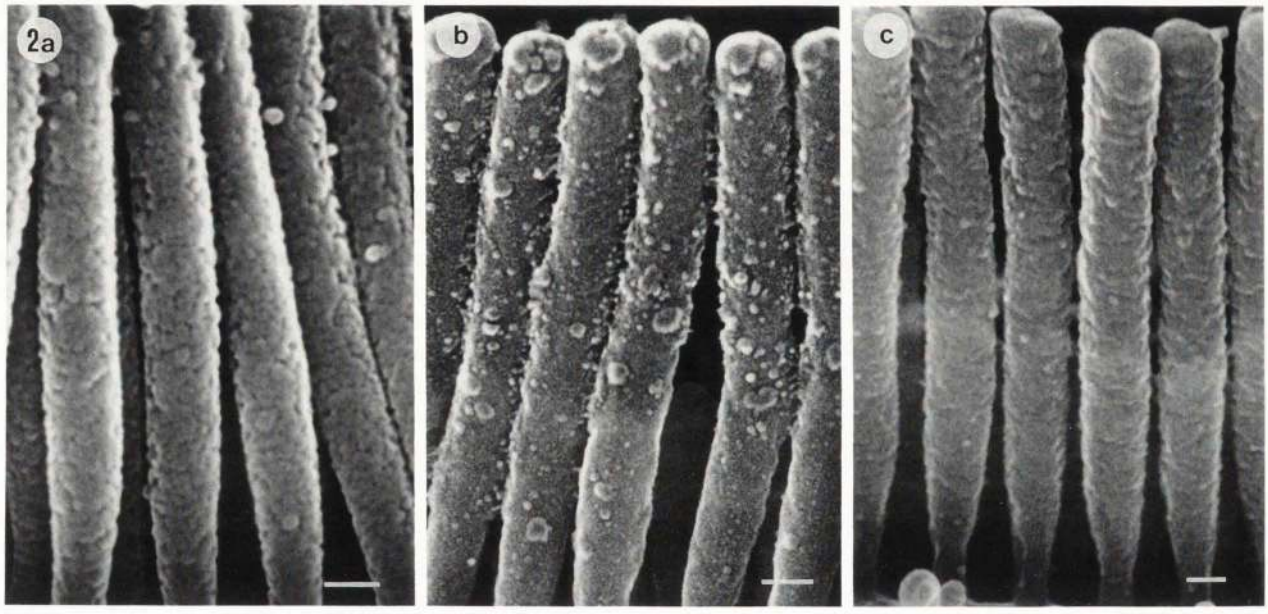
Results

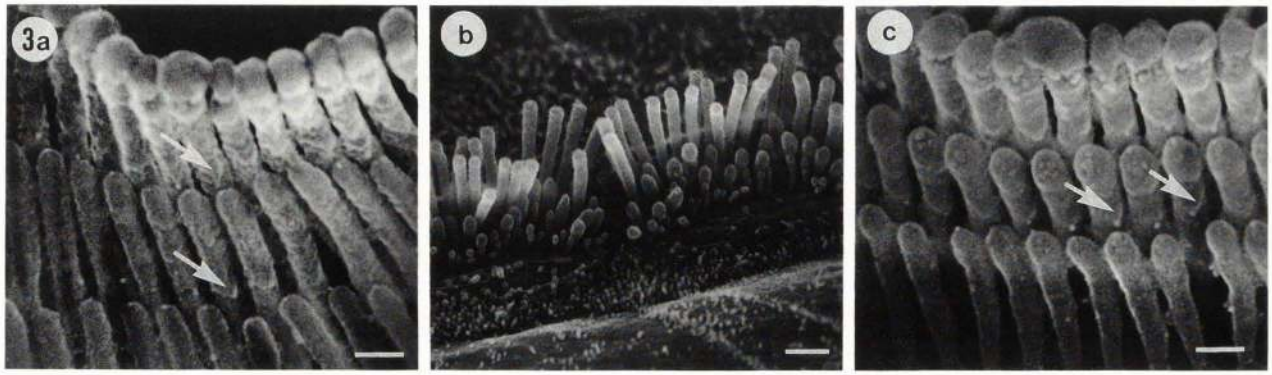
Critical Point Dried Material

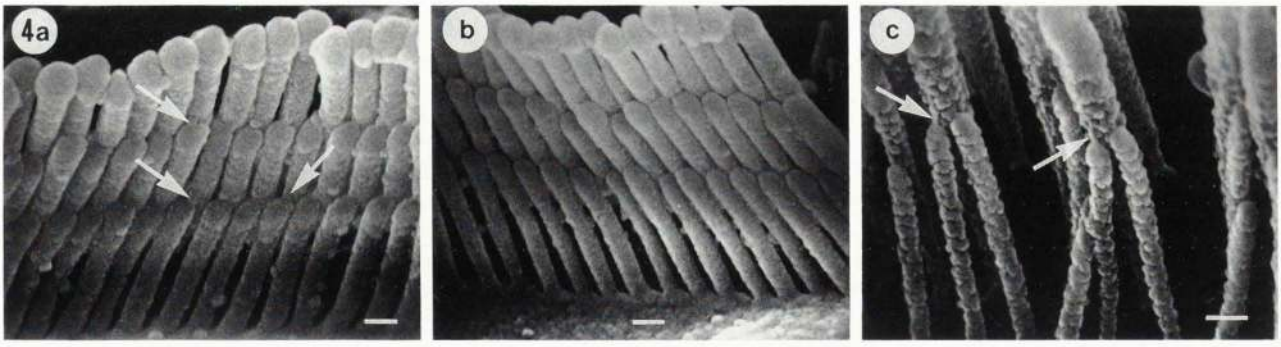
Gold coating usually gave a coarse-grained appearance to the surface of stereocilia. Gold/palladium gave less granular images, with platinum giving the best results. With this last metal, the granularity of the coating was sufficiently fine not to obscure surface details of specimens (Osborne and Comis, 1990a). Consequently, we now routinely use platinum for all our studies where metal coating is called for. A good internal control for assessing the quality of metal coating is afforded by swellings on the surface of stereocilia which we term 'blebs' (Figs. 1a, b). The surface of these structures is smooth by comparison with that of the stereocilia. Thus coating artifacts can easily be compared with and distinguished from the surface detail exhibited by the stereocilia and other cell surfaces. Our preference for a coating unit is one of the low voltage type, e.g., the Emscope SC500, since in our experience, it gives a more uniform deposition of metal over the surface of stereocilia.

We found that glutaraldehyde on its own, rather than in combination with other fixatives, gave the best results. With this fixative more subtle differences in the surface texture of the stereocilia were revealed, together with excellent preservation and retention of all types of cross-links between stereocilia. However, even with this fixative it is imperative to perfuse the labyrinth as soon as possible after death since changes in surface texture and cross-links of stereocilia were detected with 15 minutes of cardiac arrest (Osborne *et al*, 1989). The appearance of the tallest row of stereocilia of the inner hair cells was particularly vulnerable to changes in fixation regimes and can be used with advantage to assess the quality of preservation. With glutaraldehyde-only fixation, although the entire surface of this type of stereocilium appeared rough, zonation of the stereocilial shaft occurred, such that it was rougher in the apical region of the shaft where abundant lateral cross-links between adjacent stereocilia occurred (Fig. 1a). With glutaraldehyde fixation in combination with picrate (Fig. 1b), tannic acid (Fig. 2a) or formaldehyde the surface membrane of stereocilia appeared coarser and was more uniformly granular with less evidence of zonation between apical and proximal regions of the shaft. With picrate the lateral cross-links appeared thinner in diameter (Fig. 1b); with tannic acid they appeared thicker and fewer in number. Glutaraldehyde-formaldehyde produced shrinkage, i.e., a reduction in diameter of stereocilia, combined with thinning and fracture of lateral cross-links and thinning and/or considerable loss of tip links (cf


1a
b
c


2a
b
c


3a
b
c



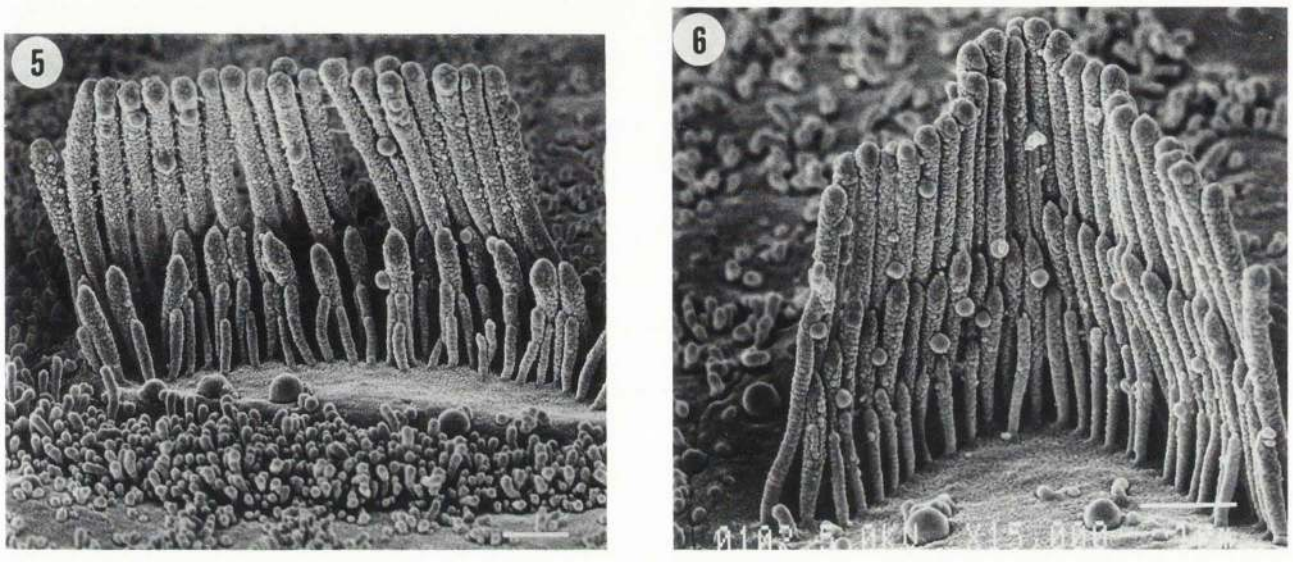


Figure 1. High magnification views of tallest rank of stereocilia from inner hair cells. **a**) Fixed in glutaraldehyde only. **b**) Fixed in glutaraldehyde-picrate. **c**) Fixed in glutaraldehyde only but dried with fluorocarbon (Peldri). Note presence of lateral cross-links between stereocilia; in a) and b) note swellings (blebs) on stereocilia (arrows). a) Zonation of stereocilial shaft is clearly shown; area associated with cross-links is rougher than rest of shaft. Scale bars = 200 nm.

Figure 2. High magnification views of tallest rank of inner hair cell stereocilia. **a**) Fixed in glutaraldehyde-tannic acid. Stereocilial membrane is uniformly rough. **b**) Fixed in glutaraldehyde and postfixed in osmium tetroxide for 30 seconds. Membrane is smoother and lateral cross-links thinner than with glutaraldehyde alone (see Fig. 1a). **c**) As in b except that postfixation in glutaraldehyde was prolonged for 24 hours. Surface membrane of stereocilia is 'wavy' rather than rough and cross-links are virtually absent. Scale bars = 200 nm.

Figure 3. **a**) Hair bundle of outer hair cell fixed in glutaraldehyde-formaldehyde. Note apparent shrinkage and increased roughness of stereocilial shafts. Many tip links have fractured with stress-lines (see text) prevalent where tip links were inserted on lateral surface of stereocilia (arrows). Scale bar = 200 nm. **b**) Hair bundles of inner hair cells fixed in glutaraldehyde and post-fixed in osmium tetroxide for 3 minutes. Stereocilia have splayed apart. Scale bar = 1 μm. **c**) Outer hair cell: hair bundle fixed with glutaraldehyde followed by post-fixation in osmium tetroxide for 30 seconds. Tip links are fractured leaving stress lines where they join lateral wall of stereocilia (arrows). Scale bar = 200 nm.

Figure 4. **a**) Outer hair cell stereocilia fixed in glutaraldehyde only and critical point dried. Note abundant presence of tip links (arrows). **b**) A similar field to a) but dried with the fluorocarbon (Peldri) method; few tip links are present. **c**) Glutaraldehyde-fixed, Peldri-dried inner hair cell stereocilia. Note shrinkage of stereocilial shafts and shrivelled appearance of tip links (arrows). Scale bars = 200 nm.

Figures 5-6. Field emission SEM images of inner hair cell stereocilial bundle (**Fig. 5**) and outer hair cell (**Fig. 6**) fixed in glutaraldehyde, critical point dried and coated with platinum. Note excellent clarity, resolution and tonal range of micrograph. Accelerating voltage 5 kV; scale bar = 1 μm.

Figs. 3a, 4a). Remnants of fractured tip links resembled those seen with short periods of postfixation with osmium tetroxide (see below) in that the upper point of insertion on the lateral wall of the stereocilium was often marked by 'stress lines' (cf Figs. 3a, c). These stress lines suggest that the tip links and side walls of stereocilia were under tension before fracture occurred. Such tension could well have been generated by the shrinkage of stereocilia possibly induced by hyperosmotic stress of glutaraldehyde-formaldehyde fixation mixtures (Bullock, 1984).

Postfixation of glutaraldehyde-fixed material with osmium tetroxide disrupted hair bundles, the extent of disruption being generally proportional to the duration of postfixation. Again by reference to inner hair cells, times of osmication as short as 30 seconds produced changes in surface texture of the stereocilia and their cross-links. The surface was smoother and the lateral cross-links were thinner and fewer in number (Fig. 2b). Longer times in osmium tended to make the stereocilial surface not only smoother but 'wavy' in appearance, together with more pronounced loss or virtual absence of lateral cross-links (Fig. 2c). Exposure to osmium tetroxide, as short as 3 minutes, so reduced the numbers of lateral cross-links that individual stereocilia splayed apart resulting in disorganization of hair bundles (Fig. 3b). Tip links were also reduced in number by osmication. Whether this loss was due to directly to osmium, or induced secondarily by splaying of stereocilia was not entirely clear. However, with short exposure times to osmium (30 seconds), the remnants of fractured tip links were seen on outer hair cells before noticeable separation of stereocilia occurred. Thus, as with glutaraldehyde-formaldehyde fixed material, 'stress lines' were seen at the points where tip links join the lateral surface of the stereocilium (Fig. 3c) indicating that these links were under tension before they fractured.

With the OTOTO method the cochleae were not opened; osmium tetroxide and thiocarbohydrazide gained entry via the holes made at the apex and base. There were obvious differences in the appearance of stereocilia according to their distance from the holes. Stereocilia from hair bundles at or near to the holes resembled those from material postfixed with osmium for 24 hours, whereas stereocilia from the central region resembled more closely those osmicated for 30 seconds (Osborne and Comis, 1990a).

Fluorocarbon (Peldri II) Dried Material

Preservation of the hair bundles appeared excellent at low magnification and was indistinguishable from those prepared by CPD. At higher magnifications (> 10,000 X) differences in texture of surface membranes and cross-linkages were found between Peldri- and CPD-prepared material. Perhaps the most striking difference was the relative paucity of the upward pointing tip links (Fig. 4b). Tip links even when present usually appeared distorted and shrivelled compared with CPD material (Fig. 4c). In inner hair cells (IHCs) the lateral cross-links between stereocilia of the tallest rank appeared in general thicker and the surface texture of all ranks of stereocilia looked more granular (Figs. 1c, 4c) with little evidence of the surface zonation characteristic of CPD material (cf Fig. 1a, c). In some IHCs the ranks of shorter stereocilia appeared thinner in diameter than with CPD (Fig. 4c). This was also the case with some outer hair cells (OHCs) which could account for the apparent wider separation of stereocilia (cf Figs. 4a, b).

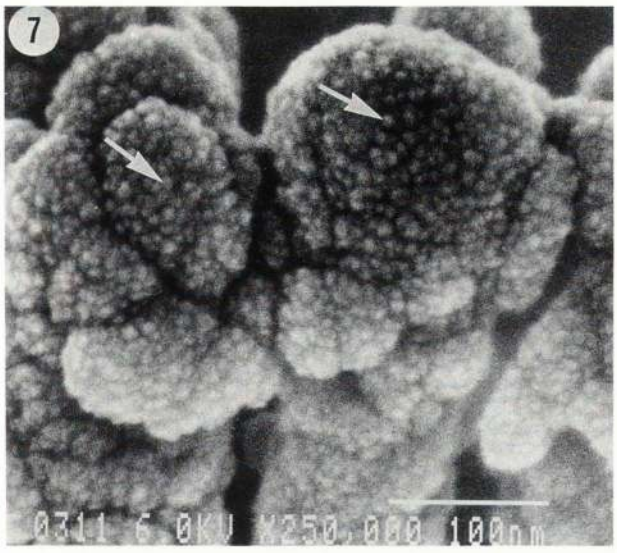
Field Emission SEM

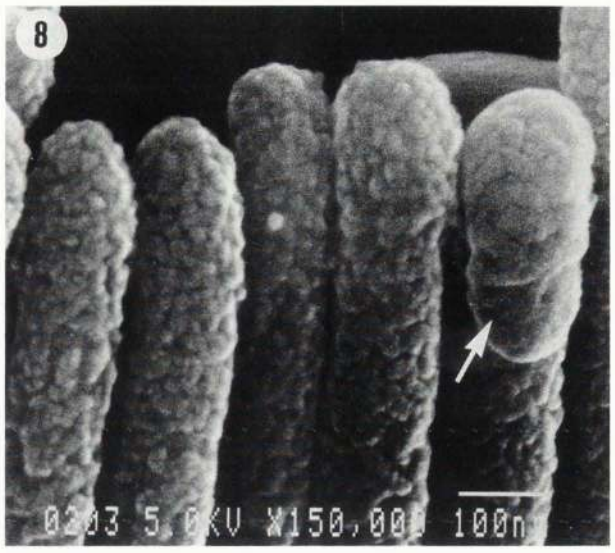
Metal Coated Specimens. These were all coated with platinum and even at low magnifications with an accelerating voltage of 5 kV gave a superior image particularly with regard to the clarity, tonal quality and depth of field of the micrographs compared with TEMSCAN results (Figs. 5, 6). At higher magnifications the resolution of the surface of the stereocilia and their cross-links was exceptional. Details of surface roughness and appearance of the cross-links confirmed those reported from TEMSCAN. At very high magnifications (250-300,000 X) individual platinum particles were easily resolved even at 6 kV (Fig. 7).

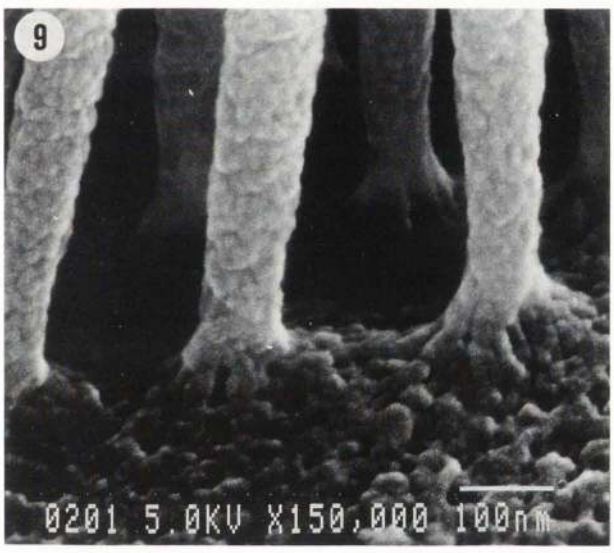
Carbon-coated and Uncoated Specimens. Carbon-coated material showed excellent detail at accelerating voltages as low as 1.5-5.0 kV. The surface of stereocilia, as with metal coated material, appeared rough in texture (Figs. 8, 9). Surface details of stereocilia were similar in both carbon-coated and uncoated specimens (cf Figs.9, 10); good resolution was achieved with the latter at 1.7 kV. Details of the points of insertion of the stereocilia into the apical surface of the sensory cells were particularly well shown in these specimens (Figs, 9, 10), which also revealed that the sensory-cell surface was more coarsely textured than that of the stereocilia. Blebs on the surface of stereocilia were also present in this material proving that they are not artifacts of metal coating. As with metal coated material the surface of the blebs was smoother than the stereocilial surface (Fig. 8).

Discussion

The techniques described above have enabled us to make detailed studies of the stereocilial bundles with SEM at resolutions in the range of 2-3 nm. Such studies have not only given greater insight into the structure and mechanical properties of hair bundles and how they relate to sensory transduction (Osborne *et al*, 1984; Osborne and Comis, 1990a), but they have also facilitated the investigation of a range of constraints, e.g.,







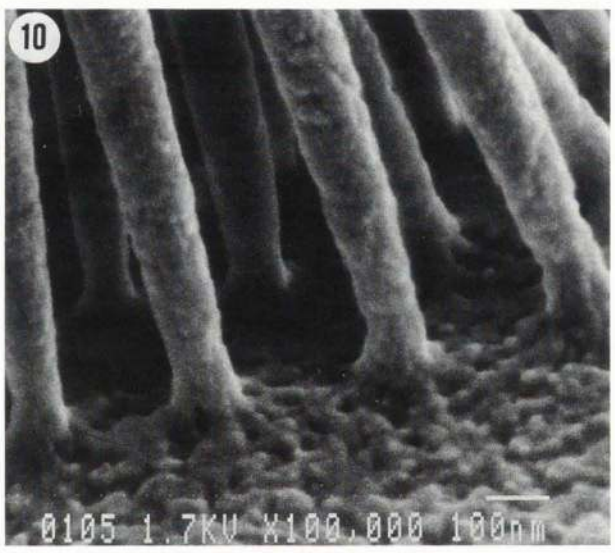


Figure 7. Field emission SEM; stereocilia from outer hair cell prepared as in Fig. 6. High magnification image showing rough surface of stereocilia. Individual particles of platinum coating are clearly resolved (arrows). Accelerating voltage 6 kV; scale bar = 100 nm.

Figures 8-10. Field emission SEM; fixed in glutaraldehyde, critical point dried and coated with carbon. Scale bar = 100 nm. **Figure 8**. Stereocilia of outer hair cell. Surface of stereocilia is clearly rough, although 'bleb' (arrow) has smoother texture. 5 kV. **Figure 9**. Basal region of stereocilia from outer hair cell. Root-like structure of stereocilia where they join apical surface of sensory cell is clearly visible. Surface of cell is rougher than that of stereocilia. 5 kV. **Figure 10**. Prepared as in Fig. 8 but uncoated. Outer hair cell showing point of insertion of stereocilia into cell surface. As in Fig. 9 root-like junctions are apparent and surface of sensory cell is rougher than that of stereocilia. 1.7 kV.

---

fixatives, acoustic (Pickles *et al*, 1987) and post-mortem insults (Osborne *et al*, 1989), drugs (Comis *et al*, 1986, 1990), bacterial endotoxin (Tarlow *et al*, 1991) and enzymes (Osborne and Comis, 1990b) upon these structures at a level of resolution approaching that of TEM. The advantage of SEM over TEM in these types of studies is that all hair bundles within an individual sensory area can be examined simultaneously; thus a much better overview can be obtained of the range and extent of the lesion than would be possible with TEM. The SEM can

therefore, with advantage, quickly direct TEM studies to the most fruitful areas for further detailed cytological investigation.

Our conclusion, that glutaraldehyde gave the best overall preservation of stereocilia must of necessity be somewhat subjective. It is based on the following observations:

1. Glutaraldehyde consistently revealed more subtle details in texture of cell coats including stereocilial surfaces and gave better retention and preservation of all types of cross-links than other fixatives we have used. Better retention of tip links may be obtained with short periods (approximately 30 seconds) of osmium tetroxide postfixation. However, this is obtained at the expense of a loss of lateral cross-links and reduction of cell-surface granularity (Comis *et al*, 1985).

2. Glutaraldehyde-fixed material is the best that we have used so far for the detection of changes in cross-links and texture of the surface membrane of stereocilia in response to a variety of insults (Osborne and Comis, 1990a).

Obviously, we do not know the real appearance of the surface of the stereocilia and their cross-links. One explanation of the differences seen in surface-appearance of glutaraldehyde-fixed stereocilia is that they reflect underlying differences in molecular architecture between various parts of the stereocilial membrane (Osborne and Comis, 1990a). However, we are almost certainly not directly visualizing the outer surface of the plasma membrane since it is coated with a carbohydrate-rich glycocalyx (Santi and Anderson, 1987; Takumida *et al*, 1988, 1990). Retention and appearance of the glycocalyx have been shown by TEM to be sensitive to fixation (see Takumida *et al*, 1988; Khan *et al*, 1990), pathological insult (Takumida *et al*, 1989a) and to changes in ionic composition of fluid bathing the hair bundles (Neugebauer and Thurm, 1987). Thus, it is likely that perturbations of the glycocalyx, rather than the outer surface of the plasma membrane, account for the variations in surface structure of stereocilia and their cross-links following different fixation regimes. Since glutaraldehyde shows more subtle details in surface texture of stereocilia than other fixatives this may indicate that it preserves the surface coat better.

In our material, osmium tetroxide, which is known to denature proteins (Itoh, 1982; Mauplin-Szamier and Pollard, 1978) and induce structural changes in membranes (Sjöstrand and Barajas, 1968; Luftig and MacMillan, 1981), destroys cross-links and changes the surface appearance of the stereocilia, although this has been challenged by others (Furness and Hackney, 1985; Little and Neugebauer, 1985). The OTOTO technique produces essentially similar results to those obtained with glutaraldehyde-osmium tetroxide fixation, although thiocarbohydrazide itself is thought to protect some proteins, e.g., it ameliorates osmium-induced disruption of actin filaments (Aoki and Tavassoli, 1981).

Osmium tetroxide postfixation and the OTOTO treatment both produce smoother cell surfaces than aldehyde fixation alone (Osborne and Comis, 1990a) or glutaraldehyde-tannic acid (Takumida *et al*, 1989b). This may be caused, as discussed above, by disruption and disintegration of the glycocalyx by osmium tetroxide (Takumida *et al*, 1988). All other fixation regimes used here produced cell surfaces that were rough including the surfaces of the stereocilia. Is this roughness real or artifactual? Clearly, the degree and patterning of this roughness is influenced by drug treatment, by post-mortem insult as short as 15 min (Osborne *et al*, 1989) and by acoustic overstimulation (Pickles *et al*, 1987; Takumida *et al*, 1989b). Thus the morphology of the roughness cannot entirely be attributed to the fixation process. Results from field emission SEM show that cell surfaces are rough even in the absence of metal coating. Therefore, this roughness is not due to coating artifacts. Examination of material by TEM following glutaraldehyde fixation and embedding in a water soluble resin also produced rough-surfaced membranes (Osborne *et al*, 1984; Pickles *et al*, 1984). Consequently, roughness per se does not appear to result from alcohol/acetone dehydration or from critical point or sublimation drying techniques, although the extent and appearance of the roughness is affected by these (cf CPD and Peldri II results). Deeply etched, freeze-fractured stereocilia from chick vestibular system (Hirokawa and Tilney, 1982) revealed irregular surfaces of cell membranes; by contrast freeze-fractured material in which the fracture plane ran obliquely or transversely across stereocilia showed that they had smooth external membranes (Jahnke, 1975; Bagger-Sjöbäck and Flock, 1977; Hamma and Saito, 1981). Possibly pretreatment with glycerol cryo-protectant, or bathing in artificial salines, prior to freezing as used in some freeze-fracture work (Forge *et al*, 1988) degrades the structure of the cell membrane and/or its glycocalyx resulting in a smoother appearance of the external coat of cell membranes.

From our studies we are inclined to accept that cell surfaces and/or their glycocalices are irregular (rough) in form and, moreover, that this form varies not only between cells but also between different areas of the same cell surface. However, the actual appearance of these irregularities must, to some extent, be compromised by chemical and other preparatory procedures. Clearly further work is necessary to resolve this issue, but we have little doubt that high resolution SEM, probably in combination with cryo-preservation techniques, will contribute significantly to the solution of this and other cell-membrane related phenomena.

Acknowledgements

We are grateful to the MRC, RNID, HAST, the Endowment Fund of the Area Health Authority and the Meningitis Trust for financial support. We are indebted to JEOL UK Ltd and JEOL Tokyo for use of their JSM-890 field emission SEM. We also thank Mr T. L. Hayward for expert technical assistance.

References

Aoki M, Tavassoli M. (1981). OTO method for preservation of actin filaments in electron microscopy. J Histochem Cytochem; **29**: 682-683.

Bagger-Sjöbäck D, Flock A. (1977). Freeze-fracturing of the auditory basilar papilla in the lizard *Calotes versicolor*. Cell Tissue Res; **177**: 431-443.

Bullock GR. (1984). The current status of fixation for electron microscopy: a review. J Microsc; **133**: 1-15.

Comis SD, Pickles JO, Osborne MP. (1985). Osmium tetroxide postfixation in relation to the cross-linkage and spatial organization of stereocilia in the guinea-pig cochlea. J Neurocytol; **14**: 113-130.

Comis SD, Rhys-Evans PH, Osborne MP, Pickles JO, Jeffries JR, Pearse HAC. (1986). Early morphological and chemical changes induced by cisplatin in the guinea pig organ of Corti. J Laryngol Otol; **100**: 1375-1383.

Comis SD, Osborne MP, Jeffries JR. (1990). Effect of furosemide upon morphology of hair bundles in guinea pig cochlear hair cells. Acta Otolaryngol; **109**: 49-56.

Engström H, Ades HW, Anderson A. (1966). Structural Pattern of the Organ of Corti. Stockholm: Almquist & Wiksell.

Forge A, Davies S, and Zajic G. (1988). Characteristics of the membrane of the stereocilia and cell apex in cochlear hair cells. J Neurocytol; **17**: 325-334.

Furness DN, Hackney CM. (1985). Cross-links between stereocilia in the guinea pig cochlea. Hear Res; **18**: 177-188.

Furness DN, Hackney CM. (1986). High-resolution scanning electron microscopy of stereocilia using the osmium-thiocarbohydrazide coating technique. Hear Res; **21**: 243-249.

Hamma K, Saito K. (1981). The fine structure of the sensory epithelium in the acoustico-lateralis system. Adv Neurol Sci; **25**: 765-776.

Hirokawa N, Tilney LG. (1982). Interactions between actin filaments and between actin filaments and membranes in quick-frozen and deeply etched hair cells of the chick ear. J Cell Biol; **95**: 249-261.

Hudspeth AJ. (1983). Mechanoelectrical transduction by hair cells in the acousticolateralis sensory system. Ann Rev Neurosci; **6**: 187-215.

Hudspeth AJ. (1989). How the ear's works work. Nature; **341**: 397-404.

Itoh M. (1982). Preservation and visualisation of actin-containing filaments in the apical zone of cochlear sensory hair cells. Hear Res; **6**: 277-289.

Jahnke K. (1975). The fine structure of freeze-fractured intercellular junctions in the guinea pig inner ear. Acta Otolaryngol Suppl; **336**: 1-40.

Kennedy JR, Williams RW, Gray JP. (1989). Use of Peldri II (a fluorocarbon solid at room temperature) as an alternative to critical point drying for biological tissues. J Electron Microsc Tech; **11**: 117-125.

Khan KM, Hatfield JS, Drescher DG. (1990). The cell coat of the sensory and supporting cells of the rainbow trout saccular macula as demonstrated by reaction with ruthenium red and tannic acid. J Histochem Cytochem; **38**: 1615-1623.

Little KF, Neugebauer DC. (1985). Inter-connections between the stereovilli of the fish inner ear. II. Systematic investigation of saccular hair bundles from *Rutilus rutilus*. Cell Tissue Res; **342**: 427-432.

Luftig RB, MacMillan PN. (1981). The importance of adequate fixation in preservation of membrane ultrastructure. Int Rev Cytol; **12**: 309-325.

Mauplin-Szamier P, Pollard TD. (1978). Actin filament destruction by osmium tetroxide. J Cell Biol; **77**: 837-852.

Neugebauer DC, Thurm U. (1987). Surface changes of the membrane and cell adhesion substances determine the structural integrity of hair bundles from the inner ear of fish. Cell Tissue Res; **249**: 199-207.

Newman GR, Jansani B, Williams ED. (1983) A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. Histochem J; **15**: 543-555.

Osborne MP, Comis SD, Johnson AP, Jeffries DJR. (1989). Post-mortem changes in hair bundles of the guinea pig and human cochlea studied by high resolution scanning microscopy. Acta Otolaryngol; **108**: 217-226.

Osborne MP, Comis SD. (1990a). High resolution scanning electron microscopy of stereocilia in the cochlea of normal, postmortem and drug-treated guinea pigs. J Electron Microsc Tech; **15**: 245-260.

Osborne MP, Comis SD. (1990b). Action of elastase, collagenase and other enzymes upon linkages between stereocilia in the guinea-pig cochlea. Acta Otolaryngol; **110**: 37-45.

Osborne MP, Comis SD, Pickles JO. (1984). Morphology and cross-linkage of stereocilia in the guinea-pig labyrinth examined without the use of osmium as a fixative. Cell Tissue Res; **237**: 43-48.

Osborne MP, Comis SD, Pickles JO. (1988). Further observations on the fine structure of tip links between stereocilia of the guinea pig cochlea. Hear Res; **35**: 99-108.

Pickles JO, Comis SD, Osborne MP. (1984). Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction. Hear Res; **15**: 103-112.

Pickles JO, Osborne MP, Comis SD. (1987). Vulnerability of tip links between stereocilia to acoustic trauma in the guinea pig. Hear Res; **25**: 173-183.

Santi PA, Anderson CB. (1987). A newly identified surface coat on cochlear hair cells. Hearing Res; **27**: 47-65.

Sjöstrand FS, Barajas L. (1968). Effects of modifications in conformation of protein molecules on structure of mitochondrial membranes. J Ultrastruct Res; **25**: 121-155.

Soudijn ER. (1976). Scanning electron microscope study of the organ of Corti in normal and sound-damaged guinea pigs. Ann Otol Rhinol Laryngol Suppl; **29**: 1-58.

Takumida M, Wersäll J, Bagger-Sjöbäck D. (1988). Stereociliary glycocalyx and inter-connections in the guinea pig vestibular organs. Acta Otolaryngol; **106**: 130-139.

Takumida M, Bagger-Sjöbäck D, Harada Y , Lim DJ, Wersäll J. (1989a). Sensory hair fusion and glycocalyx changes after gentamycin exposure in the guinea pig. Acta Otolaryngol; **107**: 39-47.

Takumida M, Fredelius L, Bagger-Sjöbäck D, Harada Y, Wersäll J. (1989b). Effect of acoustic overstimulation on the glycocalyx and the ciliary interconnections in the organ of Corti: high resolution scanning electron microscopic investigation. J Laryngol Otol; **103**: 1125-1129.

Takumida M, Suzuki M, Harada Y, Bagger-Sjöbäck D. (1990). Glycocalyx and ciliary inter-connections of the human vestibular end organs: an investigation by scanning electron microscopy. ORL J Otorhinolaringol Rel Spec; **52**: 137-142.

Tarlow MJ, Comis SD, Osborne MP. (1991). Endotoxin induced damaged to the cochlea in guinea pigs. Arch Dis Child; **66**: 181-184.

Wersäll J, Gleisner L, Lundquist PG. (1967). Ultrastructure of the vestibular end organs. In: Myotactic, Kinesthetic and Vestibular Mechanisms. Ciba foundation Symposium. De Reuck AVS, Knight J (eds.), pp 105-120. London: Churchill.

Discussion with Reviewers

R.V. Harrison: The authors are describing the fine surface structure and linkages between stereocilia in normal cochlear hair cells. One of the reported artifacts of post fixation of glutaraldehyde-fixed material with osmium tetroxide is a splaying of stereocilia. In this respect, it is rather important to know the recent history of the experimental animals under investigation. What are the ages and strains of the guinea pigs used? Because such splaying is also commonly associated with acoustic trauma, what are the levels of ambient noise exposure to the animals prior or during prefixation procedure? More specifically, is it possible that the observed splaying of stereocilia is not an artifact of fixation, but caused by overstimulation of the cochlea just prior to fixation?

Authors: The guinea pigs are aged from 10-12 weeks and are pigmented. The measured peak day-time level of ambient noise in the animal house is 73 dB SPL. Animals are fixed in a sound proof room. We do not think such sound levels would be sufficiently high to cause the stereocilial trauma seen in our material following post fixation with osmium tetroxide. In any case, stereocilial splaying is rarely found in glutaraldehyde-fixed control guinea pigs. Regarding your last point, it is possible that acoustic trauma weakens transverse cross-links such that osmium postfixation exacerbates their fracture and hence the separation of stereocilia.

S. Rydmarker: You describe the surface details in the organ of Corti with different methods for preparation. Are the hair bundles in the vestibular system affected in the same way? Do you prefer the same method for preparation in both the organ of Corti and in the vestibular system?

Authors: We have not made such detailed studies of vestibular hair bundles using different preparation regimes as we have with cochlear hair bundles. This is partly because vestibular bundles do not exhibit such a well-defined array of lateral cross-links as are found in IHCs and partly because most of our work has been involved with the organ of Corti. There is little doubt, in our opinion, that excellent preservation of vestibular hair bundles is achieved with glutaraldehyde-only fixation. However, where studies have been made by other workers upon vestibular bundles using different preparatory procedures, they too, find associated variations in the surface appearance of stereocilia (Khan *et al*, 1990; Takumida *et al*, 1988; Neugebauer and Thurm, 1987). For example, postfixation with osmium tetroxide, as with cochlear stereocilia, results in a smoother appearance of vestibular stereocilia.

S. Rydmarker: You describe dehydration with increasing concentrations of acetone. What is your comment to dehydration via increasing concentrations of ethanol?

Authors: We have used ethanol as well as acetone for dehydration of specimens. However, because specimens are always transferred to acetone prior to CPD with CO_2, we routinely dehydrate via acetone. The crucial point, in relation to your question, is that we did not see any obvious differences between ethanol- or acetone-dehydrated material.

S. Rydmarker: Following dehydration with Peldri II, you describe the stereocilia as thinner and more separated. Does this indicate more shrinkage than after CPD?

Authors: The 'thinning' of stereocilia reported following dehydration with Peldri II, we would agree, is probably caused by greater shrinkage than with CPD. We would admit though that we have not yet fully investigated subtle differences between these two drying techniques.