CELL BIOLOGICAL ASPECTS OF GENTAMICIN COCHLEOTOXICITY

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CELBIOLOGISCHE ASPECTEN VAN GENTAMICINE COCHLEOTOXICITEIT
(met een samenvatting in het Nederlands)

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MANN SIEHT NUR, WAS MANN WEISS

J.W. von Goethe

In memoriam mijn vader
oom Henk, *pater familias*

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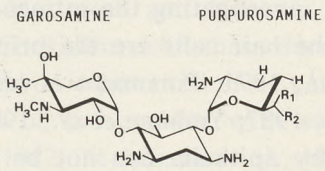
INTRODUCTION

CHAPTER 1

Aminoglycosides constitute a large group of polycationic compounds containing aminosugars as main components. Many members of this class of antibiotics feature an aminocyclitol moiety, hence the terms aminocyclitol glycosides and aminoglycoside-aminocyclitol antibiotics are also used to define this group [for general reviews see Umezawa and Hooper, 1982; Whelton and Neu, 1982].

One of the clinically (and commercially) most important groups among the aminoglycosides includes the gentamicins and related compounds. Unlike most aminoglycosides used in clinical practice, gentamicin is a well-defined mixture of the sulphates of the closely related gentamicins C_1, C_{1a}, and C_2, which are isolated from the fermentations of the actinomycetes *Micromonospora purpurea* and *M. echinospora*. Chemically, the gentamicins C are composed of pseudotrisaccharides being the aminosugars garosamine and purpurosamine joined via glycosidic linkage to the aminocyclitol 2-deoxystreptamine (Fig. 1).



	R_1	R_2	empirical formula	molecular weight
GENTAMICIN C_1	CH_3	$NHCH_2$	$C_{21}H_{43}N_5O_7$	477.60
GENTAMICIN C_2	CH_3	NH_2	$C_{20}H_{41}N_5O_7$	463.58
GENTAMICIN C_{1a}	H	NH_2	$C_{19}H_{39}N_5O_7$	449.55

Figure 1. Chemical structure, empirical formulas and molecular weights of the gentamicins C_1, C_{1a} and C_2.

Aminoglycoside cochleotoxicity

The aminoglycosides are broad-spectrum antibiotics used primarily for treating infections of gram-positive and gram-negative micro-organisms. The therapeutical efficacy of the aminoglycosides, however, is limited by the potential risks of bacterial resistance and nephro- and ototoxicity. Since the introduction of the first aminoglycoside - streptomycin - in the mid-1940s, cochlear ototoxicity (or cochleotoxicity) is recognized as a side effect of each aminoglycoside. Clinically, it is manifested as a progressive hearing impairment which will eventually result in complete loss of hearing [for general reviews see Brummett, 1980; Rybak, 1986; Griffin, 1988].

Microscopical observations of the cochlear lesions produced by chronic aminoglycoside treatment, both in humans and in animal models, reveal a selective destruction of the organ of Corti (viz., the hair cells), whereas the accessary epithelia stay intact for a considerable length of time. Most of the nerve fibers and spiral ganglion cells remain intact for a period of time, but subsequently will undergo secondary retrograde degeneration [Hawkins, 1976; Federspil, 1979; Hawkins and Johnsson, 1981; Wersäll, 1981; Tange *et al.*, 1982; Huizing and De Groot, 1987].

Notwithstanding differences in cochleotoxic potency, the pattern of hair cell degeneration is identical for all aminoglycosides. In general, the outer hair cells are more vulnerable than the inner hair cells, and the first row of outer hair cells is initially more affected than the second and third rows. Topographically, the hair cells of the basal turn of the cochlea are affected first; later on hair cell damage progresses towards the apex [Ylikoski, 1974; Federspil, 1979; Tange *et al.*, 1982].

Although cytochemical studies investigating the intracochlear distribution of aminoglycosides suggest that the hair cells are the primary site of cochleotoxic damage [Von Ilberg *et al.*, 1971; Portmann *et al.*, 1974; Hayashida *et al.*, 1985; Veldman *et al.*, 1985, 1987; Yamane *et al.*, 1988; Hayashida, 1989], an involvement of the accessary epithelia can not be excluded. Functional impairment of the stria vascularis and microvasculature alterations in both the stria vascularis and spiral ligament after aminoglycoside administration have been reported to occur in parallel to or even before actual hair cell degeneration [Müsebeck and Schätzle, 1962; Iinuma *et al.*, 1967; Saito and Daly, 1971; Mendelsohn and Katzenberg, 1972; Hawkins 1973; Tachibana *et al.*, 1978; Yamane *et al.*, 1983; Escoubet *et al.*, 1985; Forge and Fradis, 1985; Forge *et al.*, 1987]. Such changes could seriously impair aminoglycoside clearance from the cochlear fluids, resulting in a prolonged exposure of the hair cells to the drug [cf., Tran Ba Huy *et al.*, 1986; Tran Ba Huy and Deffrennes, 1988].

Cellular basis of aminoglycoside cochleotoxicity

Many researchers have attempted to elucidate the cellular mechanism(s) underlying the toxic action of aminoglycosides on the hair cells. This has been largely frustated by the myriad of metabolic and ultrastructural alterations observed after chronic exposure to aminoglycosides.

Recently, attention has been focused on the plasma membrane as a possible target for aminoglycoside action. The experiments of Saito *et al.* [1977] and Yung [1986, 1987] suggest an affinity of aminoglycosides to the plasma membrane, by reason of its net negative surface charge and the cationic nature of the drugs. According to Flock *et al.* [1977] and Takumida *et al.* [1989a-d] disorganization of the stereociliary glycocalyx will result in damage to the stereociliary bundle. Indeed, ultrastructural abnormalities of the stereocilia (e.g., stereocilia fusion) and even complete loss of stereocilia are common findings in aminoglycoside-intoxicated cochleas [Wersäll *et al.*, 1973; Leake-Jones and Vivion, 1979; Wersäll, 1981; Lim, 1986; Furness and Hackney, 1986; Pickles *et al.*, 1987]. But, as was pointed out by Harpur and Bridges [1979], structurally altered stereocilia are not seen until intracellular degeneration has progressed to a great extent.

Alternatively, the stereociliary glycocalyx is thought to sequester cations necessary for the mechano-electrical transduction process. Disorganization of the glycocalyx or binding of aminoglycosides to it would obviously result, by reason of a reduction of free binding sites, in a decreased uptake of cations. Indeed, it has been demonstrated that aminoglycosides, by reversible binding to ion-selective channels, interfere with the permeation of cations through these channels [Hayashi *et al.*, 1980; Sokabe *et al.*, 1982; Kroese and Van Den Bercken, 1982; Hudspeth and Kroese, 1983; Tachibana *et al.*, 1984; Kroese *et al.*, 1989]. Consistent with these data is the (reversible) suppression of the cochlear microphonic potential after perfusion of the perilymphatic or endolymphatic spaces of the cochlea with aminoglycosides [Konishi, 1979; Takada and Schacht, 1982; Tachibana *et al.*, 1983; Takada *et al.*, 1985].

However, although blockage of the ion-selective (or transduction) channels may very well explain the initial action of aminoglycosides on the hair cells, it is inconceivable that blockage will cause the hair cells to degenerate. This is supported by the finding that aminoglycoside binding takes place at the plasma membrane, and an intracellular pool [Williams *et al.*, 1987]; the uptake of the drug is an active, energy-dependent process [Takada *et al.*, 1985; Schacht and Van De Water, 1986].

In view of these findings, Lim [1986] has argued that the cochleotoxic action of the aminoglycosides is biphasic. In the acute (reversible) phase the drug interferes with the transduction channels. The chronic phase, which is

irreversible, comprises internalization of the drug followed by hair cell degeneration, presumably by interference with hair cell metabolism.

The intracellular action of the aminoglycosides was originally thought to be due to inhibition of protein synthesis, in analogy to their bactericidal activity [Spoendlin, 1966; Kraus and Doennig, 1969].
Richrath and Kraus [1973] have found a decrease of total protein in most cochlear tissues after chronic streptomycin administration. In addition, it has been reported that early ultrastructural changes in hair cells include the occurrence of Hensen's bodies and dilatation of (1) the Golgi saccules, (2) the subsurface cisternae of the endoplasmic reticulum, and (3) the nuclear envelope, suggesting an interaction with the cell's synthetic apparatus [Darrouzet and Guilhaume, 1974; Poch Broto *et al.*, 1980; Wersäll, 1981; Lim, 1986]. Histochemical studies, furthermore, have demonstrated a decrease in the amounts of nuclear and cytoplasmic RNA in the hair cells, the spiral ganglion and Reissner's membrane, which corresponds ultrastructurally with the disappearance of the ribosomes [Nakamura, 1957; Lundquist and Wersäll, 1966; Watanuki *et al.*, 1968; Richrath and Kraus, 1973; Jarlstedt and Bagger-Sjöbäck, 1977; Wersäll, 1981].

Besides interfering with protein synthesis, chronic aminoglycoside administration causes a significant decrease in the rate of cochlear oxygen consumption [El-Mofty and El-Serafy, 1966; Mizukoshi and Daly, 1967; Sato *et al.*, 1969]. This decrease is associated with the suppression of the activity of the mitochondrial enzyme succinate dehydrogenase in both the outer hair cells and the stria vascularis [Müsebeck and Schätzle, 1962; Koide *et al.*, 1966; Gozdzik-Zolnierkiewicz, 1969; Kaku *et al.*, 1973], and with reduced activities of the glycolytic enzymes phosphofructokinase and hexokinase in the hair cells [Tachibana *et al.*, 1976]. Furthermore, mitochondrial degeneration is frequently observed in the hair cells and the stria vascularis after aminoglycoside administration [Bagger-Sjöbäck and Wersäll, 1978; Gratacap *et al.*, 1985]. In addition, glycogen in the outer hair cells is depleted following aminoglycoside administration [Falbe-Hansen, 1963; Postma *et al.*, 1978].
Brown and Feldman [1978], therefore, have argued that inefficient energy utilization by the hair cells during intoxication will lead to exhaustion of intracellular energy reserves. This results in impairment of energy-dependent processes such as ATPase-regulated transport across membranes, and eventually in cell death.

In contrast Schacht c.s., after a series of mainly *in vitro* experiments, have presented a model based on the interaction of aminoglycosides with polyphosphoinositides sited in the inner leaflet of the plasma membrane [Schacht, 1974, 1976, 1979; Orsulakova *et al.*, 1976; Stockhorst and Schacht, 1977; Tachibana *et al.*, 1983; Williams *et al.*, 1987]. In this model it is hypothesized that the aminoglycosides, after internalization, specifically bind to phosphatidylinositol 4,5-biphosphate (or triphosphoinositide; TPI). They inhibit in this way TPI hydrolysis, thus preventing the formation of inositol triphosphate which acts as a second messenger in the phosphoinositide cascade. Since inositol triphosphate is thought to play a key role in the physiology of hair cells (e.g., motility), inhibition of the phosphoinositide cascade should inevitably lead to derangement of hair cell metabolism and function [Schacht, 1986; Zenner and Schacht, 1986].
Alternatively, biochemical and freeze-fracture studies on artificial liposomes containing TPI have demonstrated that aminoglycosides disturb membrane structure and fluidity [Wang *et al.*, 1984; Au *et al.*, 1986a, b; Forge *et al.*, 1989]. This in itself might result in an increased permeability of the plasma membrane to either ions and/or the drug itself [Dolev *et al.*, 1983; Schacht, 1986].

Among the earliest ultrastructural changes in the hair cells during aminoglycoside intoxication are the increased number of lysosomes and multivesicular bodies as well as the occurrence of residual bodies, particularly in the infracuticular region [Wersäll *et al.*, 1973; Darrouzet and Guilhaume, 1974; Wersäll, 1981; Gratacap *et al.*, 1985; Lim, 1986; Lenoir and Puel, 1987]. Histochemically, these findings correspond with the increase in staining for acid phosphatases after aminoglycoside administration [Ishii *et al.*, 1968; Federspil *et al.*, 1977].
In addition, immunoperoxidase studies show that specific labelling for aminoglycosides is most prominent in granules located particularly in the infracuticular region of the outer hair cells [Veldman *et al.*, 1985, 1987]. Although ultrastructural evidence is still lacking, these results suggest that aminoglycosides are accumulated within the lysosomal system of the hair cells. This is further supported by the observations of Siegel and Brownell [1986] and Leake and Snyder [1987] that perilymphatically applied macromolecular tracers such as horseradish peroxidase are internalized by coated vesicles and subsequently transferred to multivesicular bodies and lysosomes. Moreover, morphological and autoradiographical studies on the kidney demonstrate that aminoglycosides are rapidly accumulated within the lysosomes of the proximal tubule cells. This results eventually in a lysosomal

phospholipidosis and cell death [for reviews see Kaloyanides and Pastoriza-Munoz, 1980; Morrin and Fillastre, 1982; Walzyck *et al.*, 1986].
Clearly, these observations are indicative for an intralysosomal accumulation of the aminoglycosides. A direct relationship with hair cell degeneration, however, remains to be established.

Aim of this study

Although an interference with several subcellular processes has been demonstrated, the exact mechanism of aminoglycoside action on cochlear hair cells is still obscure. In particular the subcellular events taking place in the early stages of intoxication lack proper ultrastructural support. Therefore we have focused on these aspects of gentamicin intoxication in order to determine the intracellular target(s) of the drug in the hair cell.
Reviewing the literature, we concluded that most fixation methods for ultrastructural preservation of the cochlear tissues were insufficient. In *Chapter 2* we describe an improved fixation method comprising tri-aldehyde primary fixation and osmium tetroxide/potassium rutheniumcyanide post-fixation.
In *Chapter 3* this fixation method is used in a study of the ultrastructural changes in the hair cells during the early stages of gentamicin intoxication.
In *Chapters 4 and 5* the early effects of gentamicin on the glycocalyx of the hair cells and accessary epithelia in the cochlea are investigated. For this purpose the osmium tetroxide/potassium rutheniumcyanide method and the cationic probes colloidal thorium and cationized ferritin are employed to visualize the glycocalyx.
Finally, in *Chapter 6*, the ultrastructural distribution of gentamicin in the cochlea is investigated to determine its intracellular target(s) and to visualize the mechanism of aminoglycoside uptake. The localization of gentamicin in resin-embedded cochleas is immunocytochemically determined by the protein A-colloidal gold method.

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AN IMPROVED FIXATION METHOD FOR GUINEA PIG COCHLEAR TISSUES

CHAPTER 2

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AN IMPROVED FIXATION METHOD FOR GUINEA PIG COCHLEAR TISSUES

Since the introduction of electron microscopy into inner ear research by Engström and co-workers in the early 1950s, primary fixation of the cochlea has almost exclusively been performed by intralabyrinthine perfusion with buffered OsO_4 solutions. Today, OsO_4 fixation has been largely replaced by methods using primary aldehyde fixation followed by OsO_4 post-fixation. Iurato c.s. and Friedmann c.s. were among the first to use glutaraldehyde in their ultrastructural investigations of the cochlea [for a historical review see Engström, 1984].

Since then, various modifications have been proposed, mostly based on glutaraldehyde or glutaraldehyde-formaldehyde combinations. Occasionally, combinations such as glutaraldehyde-OsO_4 [Anniko and Lundquist, 1977], glutaraldehyde-acrolein [Shnerson *et al.*, 1982] and glutaraldehyde-formaldehyde-acrolein [Ekström von Lubitz, 1981a, b; Jørgensen, 1982] have been used.

However, most of the latter combinations have proved unsatisfactory, either because of their limited application or because of the frequent occurrence of fixation artefacts. Primary fixation artefacts have been reported to be caused by a variety of factors. The most influential appeared to be the method of fixation (i.e., intralabyrinthine *versus* intravascular perfusion) and the fixation agent itself [Anniko and Lundquist, 1977; Sparwald *et al.*, 1974; Merck *et al.*, 1974a, b, 1975, 1977; Santos-Sacchi, 1978; Anniko and Lundquist, 1980a, b; Park and Cohen, 1984].

Therefore we started a series of experiments to re-investigate the influence of various fixation methods on cochlear ultrastructure preservation. The individual efficacy of several primary fixatives currently in use in inner ear electron microscopy was also compared. In addition, the post-fixation with $OsO_4/K_4Ru(CN)_6$ and $OsO_4/K_4Fe(CN)_6$, reported for their contrast-enhancing properties [De Bruijn, 1968; Karnovsky, 1971], was tested.

MATERIALS AND METHODS

Healthy, female albino guinea pigs (strain GpHi65 *Himalayan*, weighing 200-350 g), which were housed under standard laboratory conditions and fed *ad libitum*, were anaesthetized by intraperitoneal injection of sodium pentobarbital (Triotal^{R}, 70 mg/kg).

Primary fixation was carried out by respectively:

I. *in situ* intravascular perfusion via the left ventricle with:
   1. modified Karnovsky fixative: 2.5% glutaraldehyde, 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.025% $CaCl_2$ [Karnovsky, 1965], or
   2. tri-aldehyde fixative: 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2.5% DMSO in 0.08 M sodium cacodylate buffer, pH 7.4 [Kalt and Tandler, 1971],

   followed by immersion of dissected cochleas in the same fixative for 3 h at room temperature (tri-aldehyde fixative) or for 2 h at 4°C (modified Karnovsky fixative), or

II. intralabyrinthine perfusion with:
   1. 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4,
   2. 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4,
   3. modified Karnovsky fixative,
   4. 3% glutaraldehyde, 1% acrolein, 2.5% DMSO in 0.08 M sodium cacodylate buffer, pH 7.4 [Bluemink *et al.*, 1976], or
   5. tri-aldehyde fixative,

   followed by immersion in the same fixative for 3 h at room temperature (tri-aldehyde fixative) or for 2 h at 4°C (all other fixatives).

Subsequently, cochleas were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4 (2x15 min), decalcified in 10% EDTA.2Na (pH 7.4) under constant agitation in a decalcifying device (LTI, Bilthoven, The Netherlands) for 4-5 days and rinsed in the same buffer (2x15 min), all at room temperature.

Post-fixation was carried out for 2 h at 4°C in:

1. 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4,
2. 1% OsO_4, 1% $K_4Ru(CN)_6$ (K & K Labs., ICN Biomedicals Inc., New York, USA) in 0.1 M sodium cacodylate buffer, pH 7.4 [De Bruijn, 1968; De Bruijn and Den Breejen, 1975], or
3. 1% OsO_4, 1.5% $K_4Fe(CN)_6.3H_2O$ in 0.1 M sodium cacodylate buffer, pH 7.4 [De Bruijn, 1968; De Bruijn and Den Breejen, 1975],

followed by several washes in distilled water. Cochleas fixed with OsO_4 were not post-fixed.

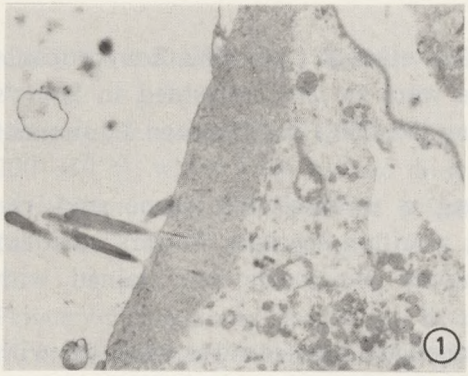
Dehydration was performed in a graded ethanol, 2,2-dimethoxypropane, propylene oxide series and the cochleas were *in toto* embedded in Spurr's low-viscosity resin, containing 1% silicone DC 200 fluid (Polaron Equipment Ltd., Bio-Rad Labs., Watford, UK).
Next, the cochleas were divided along a midmodiolar plane and re-embedded. For purposes of orientation, semithin sections (1 μm) were cut with glass knives on a Jung 1140 autocut microtome and stained with methylene blue and azure B in borax.
Subsequently, ultrathin sections of re-embedded quarter turns were cut with a diamond knife on an LKB Ultrotome V, collected on Pioloform-coated, single-slot copper grids and examined either without additional heavy metal staining or contrast-stained with 7% uranyl acetate in 70% methanol and lead citrate according to Reynolds in a Philips EM 201c transmission electron microscope operating at 60 kV.

RESULTS

Intravascular perfusion with modified Karnovsky fixative
Vacuolation of the cytoplasm, often in conjunction with membraneous whorls, was frequently seen in outer hair cells and nerve fibres. Severe extraction of the cell matrix, resulting in electron-lucent areas and a fuzzy appearance of the cytoplasm, was observed particularly in the outer hair cells (Fig. 1); but was also present in the nerve fibres, spiral ganglion cells, Schwann's cells, Huschke's cells in the spiral limbus and most supporting cells in the organ of Corti. Occasionally, shrinkage of the outer hair cells and loosely arranged myelin sheaths circumscribing the spiral ganglion cells were seen. In addition, swelling of mitochondria occurred, sometimes very extreme, particularly in nerve fibres, spiral ganglion cells and Huschke's cells in the spiral limbus.

Intravascular perfusion with tri-aldehyde fixative
All cells demonstrated a uniform preservation of their cytoplasm without any signs of swelling, shrinkage or extraction of matrix constituents (Fig. 2). Only some slight membrane distortion was infrequently seen in conjunction with the stereocilia (Fig. 2), Reissner's membrane, and the marginal cells of the stria vascularis.

Intralabyrinthine perfusion with OsO_4
All cells, and particularly the hair cells, the supporting cells and the nerve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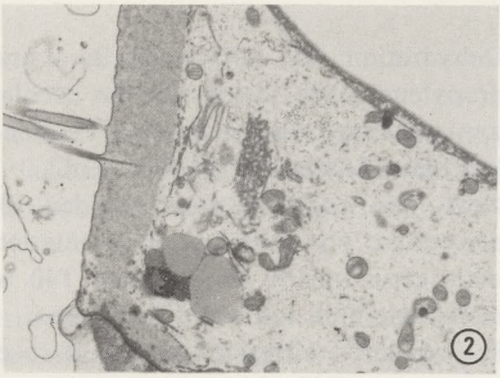
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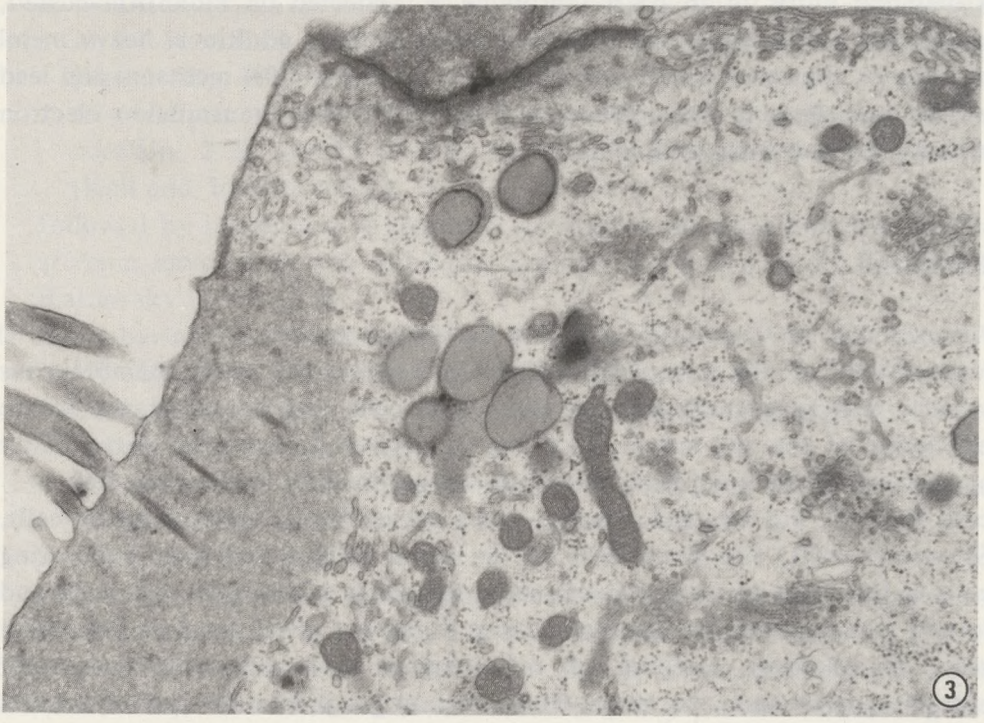
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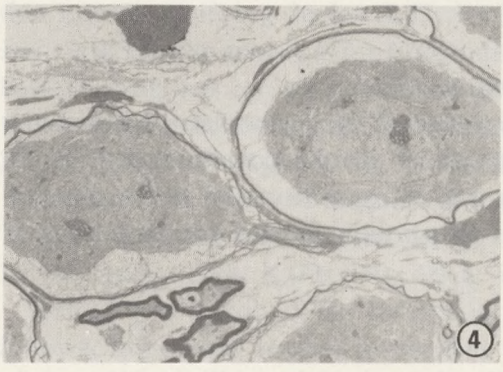
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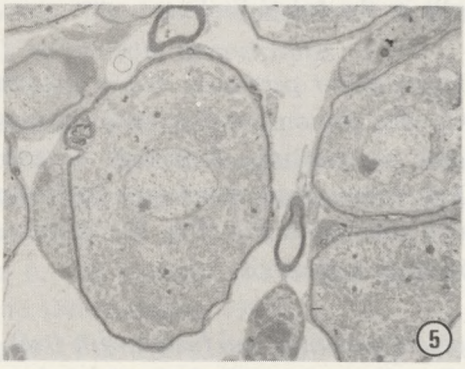
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fibres demonstrated a poorly preserved cell matrix and inadequate retention of glycogen. Also, deformation (i.e., angular-shaped instead of circular) of the large lipid inclusions in the Hensen's cells occurred, especially in the uppermost turns. In addition, the spiral ganglion cells demonstrated moderate shrinkage in combination with very loosely arranged myelin sheaths.

Intralabyrinthine perfusion with glutaraldehyde

Severe shrinkage was observed in the spiral ganglion (Fig. 4), the spiral limbus, the outer sulcus, the spiral ligament and the stroma of the spiral prominence together with a substantial enlargement of the extracellular spaces, most prominent in the spiral ligament. To a lesser degree, shrinkage was present in most of the supporting cells in the organ of Corti, the epithelial lining of the spiral prominence, and in the stria vascularis (viz., the intermediate cells). In addition, the marginal cells of the stria vascularis appeared very electron-dense. The outer hair cells also demonstrated signs of shrinkage resulting in an increased electron density. In contrast to the outer hair cells, the inner hair cells demonstrated signs of vacuolation and extraction of cell matrix constituents, e.g., glycogen and ribosomes. Sometimes, loss of glycogen was observed in the cells of Reissner's membrane. In addition, slight membrane distortion was infrequently seen in conjunction with the stereocilia, Reissner's membrane, and the marginal cells of the stria vascularis.

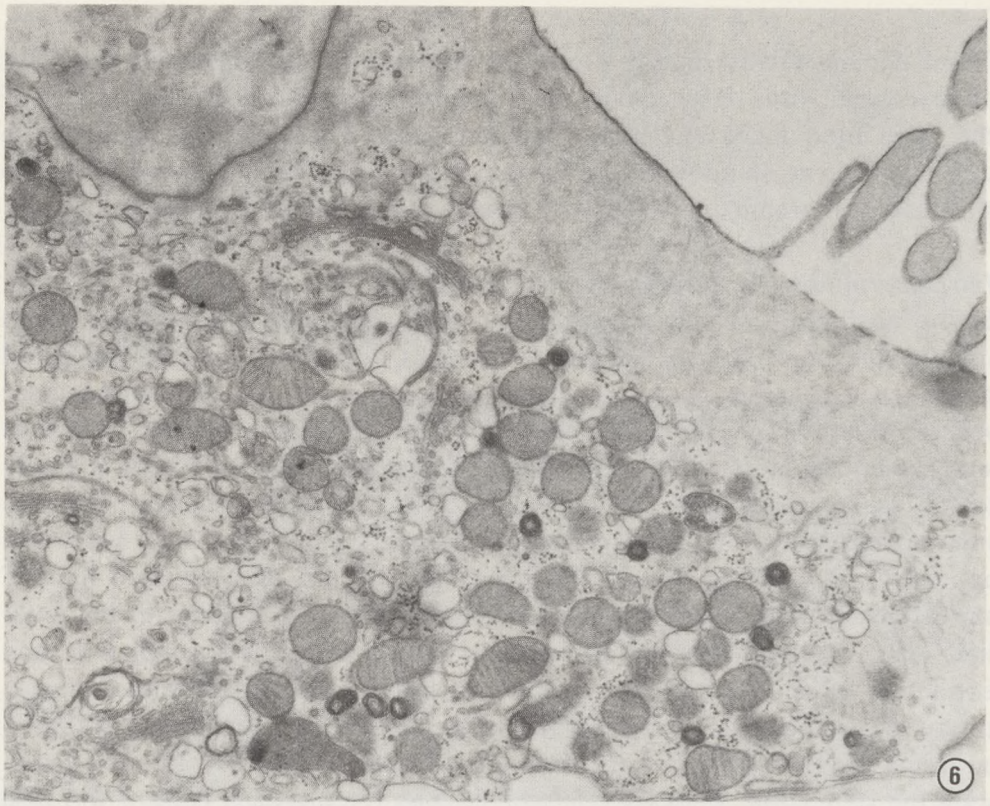
Figure 1. Outer hair cell, intravascular perfusion with modified Karnovsky fixative, OsO_4 post-fixation without uranyl acetate/lead citrate contrast-staining. Note the fuzzy appearance of the cytoplasm due to extraction (*x7,000*).

Figure 2. Outer hair cell, intravascular perfusion with tri-aldehyde fixative, $OsO_4/K_4Ru(CN)_6$ post-fixation, uranyl acetate/lead citrate contrast-staining (*x7,000*).

Figure 3. Outer hair cell, intralabyrinthine perfusion with tri-aldehyde fixative, $OsO_4/K_4Ru(CN)_6$ post-fixation, uranyl acetate/lead citrate contrast-staining (*x20,000*).

Figure 4. Glutaraldehyde primary fixation. Severe shrinkage of the spiral ganglion cells (*x2,000*).

Figure 5. Tri-aldehyde primary fixation. Normal appearance of the spiral ganglion cells (*x2,000*).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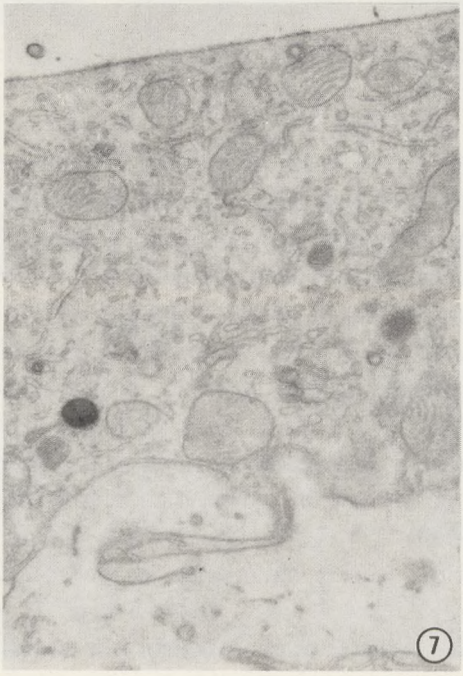
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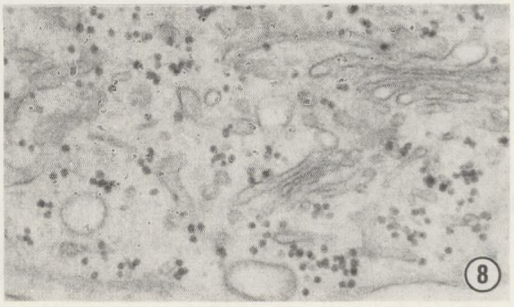
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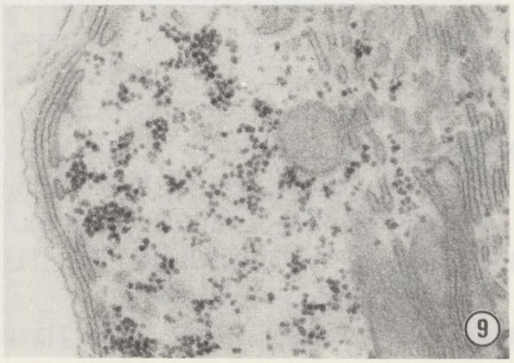
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Intralabyrinthine perfusion with modified Karnovsky fixative
No obvious signs of swelling, shrinkage or extraction of cell matrix constituents were observed. Here also some slight membrane distortion was seen infrequently.

Intralabyrinthine perfusion with glutaraldehyde-acrolein
Most cells demonstrated slight to moderate extraction of the cell matrix. However, extraction appeared more severe in the outer hair cells, resulting in electron-lucent areas and a fuzzy appearance of the cytoplasm. No signs of membrane distortion were observed.

Intralabyrinthine perfusion with tri-aldehyde fixative
All cells demonstrated a uniform preservation of their cytoplasm without obvious fixation artefacts (Figs. 3, 5-9). Only some slight membrane distortion was infrequently seen in conjunction with the stereocilia (Fig. 2), Reissner's membrane, and the marginal cells of the stria vascularis.

Post-fixation with OsO_4
OsO_4 post-fixation resulted in too weak a tissue contrast (Fig. 1), particularly of the membranes and glycogen particles; the contrast could not be enhanced adequately by means of heavy metal contrast-staining of ultra-thin sections.

Post-fixation with $OsO_4/K_4Ru(CN)_6$
After $OsO_4/K_4Ru(CN)_6$ post-fixation more contrast was impaired on the

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Figure 6. Inner hair cell, tri-aldehyde primary fixation followed by $OsO_4/K_4Ru(CN)_6$ post-fixation without additional uranyl acetate/lead citrate contrast-staining (*x20,000*).

Figure 7. Detail of the stria vascularis (viz., marginal cell), tri-aldehyde primary fixation followed by $OsO_4/K_4Fe(CN)_6$ post-fixation (*x20,000*).

Figure 8. Detail of Reissner's membrane (viz., epithelial cell). Note the enhanced membrane contrast after $OsO_4/K_4Ru(CN)_6$ post-fixation (*x42,000*).

Figure 9. Detail of an outer hair cell. Enhanced glycogen and membrane contrast after $OsO_4/K_4Ru(CN)_6$ post-fixation (*x42,000*).

membranes and the glycogen particles as compared with post-fixation with OsO_4 alone (Figs. 5, 6, 8, 9). However, the cells of the stria vascularis (viz., the marginal cells), the epithelial lining of the spiral prominence, the tympanal cells of the basilar membrane and the nerve terminals at the base of the hair cells appeared relatively more electron-dense as compared with the other cochlear tissues.

Post-fixation with $OsO_4/K_4Fe(CN)_6$

$OsO_4/K_4Fe(CN)_6$ post-fixation also resulted in a better general contrast of both the membranes and glycogen particles as compared with post-fixation with OsO_4 alone (Fig. 7). However, hair cells and supporting cells occasionally demonstrated extraction of ribosomes and glycogen, but also of the mitochondrial and nuclear matrices. The marginal cells, the epithelial lining of the spiral prominence, the tympanal lining of the basilar membrane, the Boettcher's cells and the nerve terminals were less electron-dense than after OsO_4 or $OsO_4/K_4Ru(CN)_6$ post-fixation, resulting in excellent cellular detail.

DISCUSSION

Primary fixation with OsO_4 has been reported to cause serious artefact formation in most cochlear tissues, e.g., splitting of the myelin sheaths in the spiral ganglion [Merck *et al.*, 1974b, 1975, 1977], cell matrix extraction, and disruption of the interdigitating cell membranes in the stria vascularis and the outer sulcus cells [Santos-Sacchi, 1978].

Glutaraldehyde primary fixation also resulted in apparent cell damage such as: severe cell shrinkage and a substantial increase in extracellular volume, predominantly in the spiral ligament, the spiral prominence and the spiral ganglion [Merck *et al.*, 1974a; Anniko and Lundquist, 1980b], and vacuolation and increased electron density in the hair cells [Anniko and Lundquist, 1977; Merck *et al.*, 1974b; Anniko and Lundquist, 1980a]. Glutaraldehyde-acrolein primary fixation, as used by Shnerson *et al.* [1982], also proved altogether unsatisfactory in our hands.

In contrast, after intralabyrinthine perfusion with tri-aldehyde and modified Karnovsky fixative, all cochlear tissues were equally well preserved, showing no obvious artefacts but for some membrane distortion. This latter phenomenon, although not reported earlier as such, is present occasionally in some published electron micrographs [cf., Engström *et al.*, 1972]. By our protocol it is not completely counteracted by adding $CaCl_2$, neither at low

nor at high concentrations, nor by changing the fixation buffer [De Groot, unpublished results]. Particularly with respect to hair cell preservation, our results are consistent with previous studies on the use of Karnovsky fixative [Anniko and Lundquist, 1977, 1980a], and with studies applying tri-aldehyde fixation to the cochlear tissues of a variety of species [Ekström von Lubitz, 1981a, b; Jørgensen, 1982].

Rapid penetration of fixatives into tissues is a critical requirement for good preservation. It is conceivable that too slow a permeation of the fixation agents might account for the observed artefacts following primary fixation with either OsO_4, glutaraldehyde or glutaraldehyde-acrolein. As both tri-aldehyde and modified Karnovsky fixatives contain formaldehyde, the presence of this aldehyde seems a prerequisite for optimal preservation of the cochlear ultrastructure.

Although primary fixation of the cochlea by intravascular perfusion has occasionally been used by several investigators, the results obtained proved unsatisfactory [Sparwald *et al.*, 1974; Merck *et al.*, 1974a; Anniko and Lundquist, 1980a, b; Smith *et al.*, 1985]. Primary fixation by intravascular perfusion with modified Karnovsky fixative resulted in inadequate preservation of the cochlear ultrastructure, with a multitude of fixation artefacts. However, after intravascular perfusion with tri-aldehyde fixative, we could not detect any fixation artefacts; the resultant fixation quality matched that obtained after intralabyrinthine perfusion. Additionally, intravascular perfusion with tri-aldehyde fixative is the method of choice when studying the most apical parts of the cochlea, as these are usually damaged during the microdissection procedure preceding intralabyrinthine perfusion of the cochlea.

When using tri-aldehyde fixation, by either intralabyrinthine or intravascular perfusion, we have obtained highly reproducible results, which indicates that the reliability of this method is absolutely more pertinent than with other fixation methods.

Post-fixation with $OsO_4/K_2Ru(CN)_6$ or $OsO_4/K_4Fe(CN)_6$ has been shown to impart a high electron density to glycogen and membranes of aldehyde-fixed tissues [De Bruijn, 1968; Karnovsky, 1971; De Bruijn and Den Breejen, 1975]. Although $OsO_4/K_4Fe(CN)_6$ post-fixation has occasionally been used in inner ear electron microscopy [Nijdam, 1982], $OsO_4/K_2Ru(CN)_6$ or $OsO_4/K_4Ru(CN)_6$ post-fixation never have been applied to cochlear tissues.

In general, cochlear tissues post-fixed with $OsO_4/K_4Ru(CN)_6$ or $OsO_4/K_4Fe(CN)_6$ exhibited more distinct membrane and glycogen contrast and greater cellular detail, as compared with tissues post-fixed with OsO_4

alone, thus making possible direct examination of ultrathin sections without additional heavy metal contrasting techniques.

Unfortunately, $OsO_4/K_4Fe(CN)_6$-enhanced contrast is sometimes accompanied by slight extraction of cell matrix constituents and of the mitochondrial and nuclear matrices, particularly in the outer hair cells. One of these appears to be glycogen, as it was not observed after $OsO_4/K_4Fe(CN)_6$, but was abundantly present after $OsO_4/K_4Ru(CN)_6$. Less extraction was reported by Neiss [1984], using lower concentrations of $K_4Fe(CN)_6$; but with concentrations lowered, membrane and glycogen contrast decreased accordingly [Neiss, 1984]. However, with respect to the degree of contrast-enhancement, distinct variations were observed between the two mixtures. $OsO_4/K_4Ru(CN)_6$ post-fixation resulted in excellent contrast, particularly of hair cells and their supporting cells, whereas the tympanal lining of the basilar membrane, the cells of the stria vascularis, the epithelial lining of the spiral prominence and the nerve terminals all displayed remarkably sharper contrast after $OsO_4/K_4Fe(CN)_6$ post-fixation. Most other cochlear tissues were equally well contrasted by both $OsO_4/K_4Ru(CN)_6$ and $OsO_4/K_4Fe(CN)_6$. This apparent selectivity may reflect the differing biochemical composition of the various cochlear tissues, which is directly related to their physical mass density.

In conclusion, our investigations have shown that, because of (1) the absence of fixation artefacts after both intralabyrinthine and intravascular perfusion, (2) the relatively sharp tissue contrast obtained, and (3) its reliability, primary fixation with tri-aldehyde fixative followed by post-fixation with $OsO_4/K_4Ru(CN)_6$ or $OsO_4/K_4Fe(CN)_6$ might well be useful as a routine procedure for optimal preservation of guinea pig cochlear ultrastructure.

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EARLY ULTRASTRUCTURAL CHANGES IN THE COCHLEAR HAIR CELLS AFTER GENTAMICIN ADMINISTRATION

CHAPTER 3

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EARLY ULTRASTRUCTURAL CHANGES IN THE COCHLEAR HAIR CELLS AFTER GENTAMICIN ADMINISTRATION

Although the cochleotoxic effects of the aminoglycosides have been extensively studied during the past decades, there still is no consensus of opinion with respect to the subcellular target of these drugs.

Electron microscopical examination of the cochlear lesions produced by chronic administration of aminoglycosides has revealed a multitude of ultrastructural changes (*vide infra*) in both the outer and inner hair cells preceding actual cell death [for reviews see Poch Broto *et al.*, 1980; Wersäll, 1981; Lim, 1986a].

The most dramatic - and clearly most documented - change observed in aminoglycoside-intoxicated cochleas is distortion of the stereociliary bundle resulting in collapse of the individual stereocilia, fusion of stereocilia and formation of giant stereocilia, and even complete loss of the stereocilia [i.a., Wersäll *et al.*, 1973; Lim, 1976; Theopold, 1977; Leake-Jones and Vivion, 1979; Yung, 1987]. Recently, it has been suggested that these changes are the consequence of disturbance of the stereociliary glycocalyx and/or of loss of the stereociliary cross-links [Flock *et al.*, 1977; Furness and Hackney, 1986; Pickles *et al.*, 1987; Takumida *et al.*, 1989]. However, stereociliary pathology is a relatively late event as structurally altered stereocilia are not seen until intracellular degeneration has progressed to a great extent [Lundquist and Wersäll, 1966; Ylikoski, 1974; Harpur and Bridges, 1979; Forge, 1985; Lenoir and Puel, 1987].

Intracellularly, the earliest recognizable changes known during aminoglycoside intoxication are the drastic increases in multivesicular bodies, lysosomes and residual bodies in the infracuticular region of the hair cells. Furthermore, numerous myelin figures are reported to be present throughout the cytoplasm which are generally believed to be degenerated mitochondria [Wersäll *et al.*, 1973; Ylikoski, 1974; Darrouzet and Guilhaume, 1974, 1976; Gratacap *et al.*, 1985; Lenoir and Puel, 1987].

Other changes include degranulation of the endoplasmic reticulum and loss of free ribosomes [Duvall and Wersäll, 1964; Lundquist and Wersäll, 1966; Ylikoski, 1974; Jarlstedt and Bagger-Sjöbäck, 1977], dissolution of glycogen

granules [Falbe-Hansen, 1963; Postma *et al.*, 1978] as well as mitochondrial damage such as swelling, loss of cristae and the formation of lamellar inclusion bodies [Friedmann and Bird, 1961; Lundquist and Wersäll, 1966; Friedmann *et al.*, 1966; Wersäll *et al.*, 1973; Ylikoski, 1974; Lim, 1976; Bagger-Sjöbäck and Wersäll, 1978; Harpur and Bridges, 1979].
A further typical feature observed during aminoglycoside intoxication is the formation of concentric membraneous bodies (or Hensen's bodies) in the infracuticular and supranuclear regions of the outer hair cells. In addition, pathological alteration of the cell's synthetic apparatus has been reported. This comprises proliferation and dilatation of the endoplasmic reticulum as well as the Golgi saccules, giving rise to extensive vacuolation of the cytoplasm [Lundquist and Wersäll, 1966; Friedmann *et al.*, 1966; Ylikoski, 1974; Darrouzet and Guilhaume, 1974, 1976; Lim, 1976; Harpur and Bridges, 1979; Forge, 1985].
However, attempts to distinguish - on morphological grounds - primary from secondary events have been greatly frustrated by the unfortunate fact that, in most studies, extensive damage to the hair cells had already occured. Moreover, inadequate tissue preservation has hampered reliable evaluation of the cellular changes. In this study we evaluate the ultrastructural changes after short-term gentamicin administration (viz., 1, 5, 10 and 15 days) in cochlear hair cells optimally fixed, as recommended by De Groot *et al.* [1987].

MATERIALS AND METHODS

Healthy, female albino guinea pigs (strain GpHi65 *Himalayan*, weighing 250-300 g) received gentamicin (Gentogram^{R}, 100 mg/kg) by daily intraperitoneal injections either for 1 day (n=3), 5 days (n=3), 10 days (n=3) or 15 days (n=3). The animals were sacrificed 24 h after the last application of the drug. Additionally, three animals were not treated with gentamicin and served as controls.
The animals were anaesthetized by intraperitoneal injection of sodium pentobarbitone (Nembutal^{R}, 60 mg/kg).

After decapitation, the cochleas were removed and immediately fixed by intralabyrinthine perfusion with a tri-aldehyde fixative followed by immersion in the same fixative for 3 h at room temperature. The fixative consisted of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2.5% DMSO in 0.08 M sodium cacodylate buffer, pH 7.4 [De Groot *et al.*, 1987]. Sub-

sequently, the cochleas were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) for 2x15 min, decalcified in 10% EDTA.2Na (pH 7.4) under constant agitation in a decalcifying device (LTI, Bilthoven, The Netherlands) for 4-5 days at room temperature and rinsed in the same buffer (2x15 min).

Post-fixation was carried out for 2 h at 4°C in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4), containing 1% $K_4Ru(CN)_6$ (K & K Labs., ICN Biomedicals Inc., New York, USA), followed by several washes in distilled water [De Groot *et al.*, 1987].

Dehydration was performed in a graded ethanol, 2,2-dimethoxypropane, propylene oxide series and the cochleas were *in toto* embedded in Spurr's low-viscosity resin, containing 1% silicone DC 200 fluid (Polaron Equipment Ltd., Bio-Rad Labs., Watford, UK).

Next, the cochleas were divided along a midmodiolar plane and re-embedded. For purposes of orientation, semithin sections (1 μm) were cut with glass knives on a Reichert-Jung 2050 microtome and stained with methylene blue and azure B in borax.

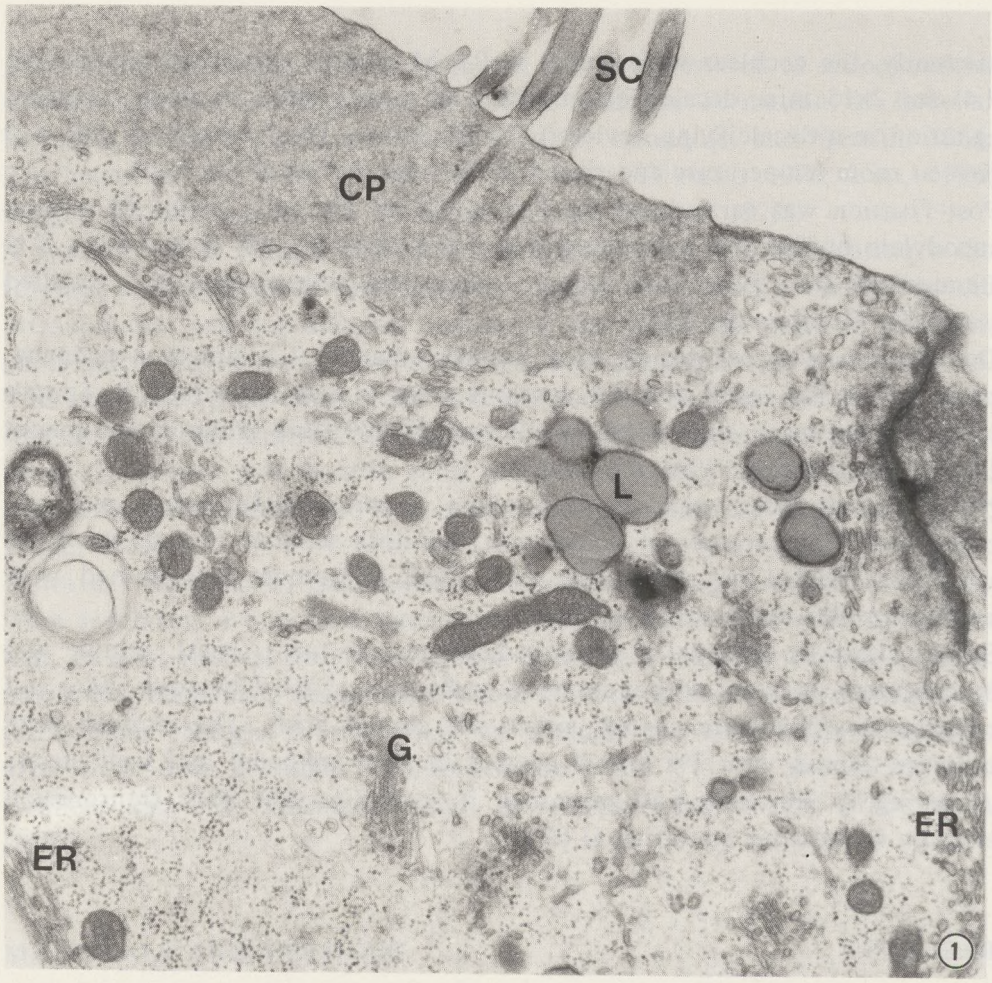
Serial, ultrathin sections of re-embedded quarter turns (basal, middle and apical turns) were cut with a diamond knife on an LKB Ultrotome *Nova* and collected on Pioloform-coated, single-slot copper grids. The sections were contrast-stained with 7% uranyl acetate in 70% methanol and lead citrate according to Reynolds and examined in a Philips EM 201c transmission electron microscope operating at 60 kV.

RESULTS

Normal cochleas

The *outer hair cells* are arranged in three parallel rows in the organ of Corti. They are cylindrical in shape with a flat apex from which protrude the stereocilia, and a rounded base where synaptic contacts with nerve endings are formed. The apex of the outer hair cells is attached to the apical processes of the Deiters' cells and to the outer pillar cells.

The apex of the outer hair cell (Fig. 1) contains the cuticular plate, which consists of a fine, homogeneous matrix interspersed with patches of electron-dense aggregates, and into which the stereocilia are inserted. The cuticular plate is not continuous throughout the entire apex. The cuticle-free region (or fonticulus) in which occasionally a basal body is observed, is filled with numerous smooth vesicles and apical cisternae of the endoplasmic reticulum. Junctional complexes composed of tight junctions, zonulae adherentes and gap junctions, linking the outer hair cell to the adjacent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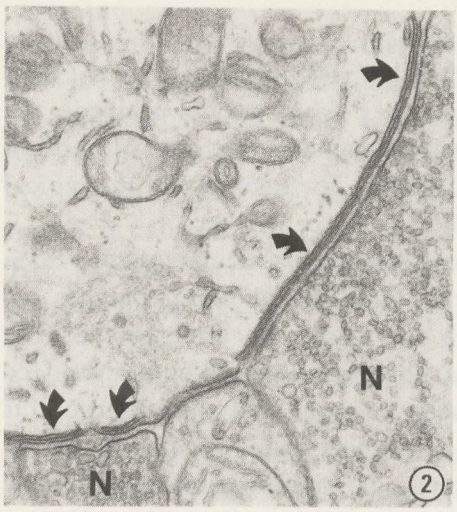
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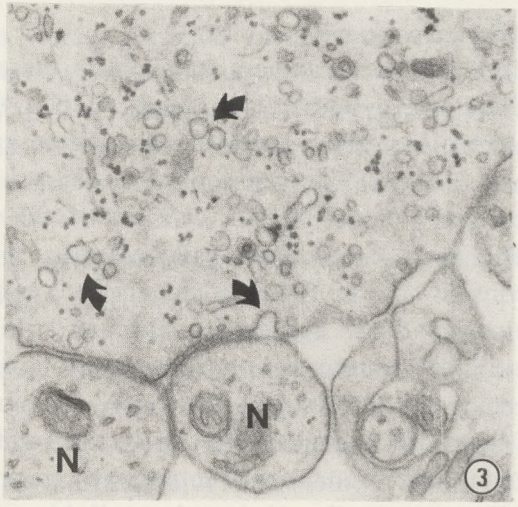
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supporting cells, are present.
The infracuticular region (Fig. 1) contains numerous mitochondria, lysosomes and residual bodies, monoparticulate glycogen, free ribosomes, Golgi saccules, and subsurface cisternae of the endoplasmic reticulum. The subsurface cisternae of the endoplasmic reticulum are free of ribosomes and form multiple (3-4) layers arranged along the entire cytoplasmic surface of the lateral membranes.
The nucleus is round or ovoid and located in the lower portion of the cell. The supranuclear region is thinly populated with organelles, but free ribosomes are scattered throughout the cytoplasm and monoparticulate glycogen is abundantly present.
The infranuclear region is characterized by the presence of mitochondria, subsynaptic cisternae of the endoplasmic reticulum and numerous smooth vesicles. The subsynaptic cisternae are more prevalent near the synaptic contacts between outer hair cell and efferent nerve endings (Fig. 2). The smooth vesicles are located near the sites where synaptic contacts are formed with afferent nerve endings (Fig 3).

The *inner hair cells* form a single row in the organ of Corti and are flask-shaped with a flat apex. In contrast to the outer hair cells, the inner hair cells are, along their lateral surfaces, completely surrounded by supporting cells.
The most prominent features in the apical region of the inner hair cell (Fig. 4) are besides the cuticular plate and the stereocilia, a well-developed Golgi

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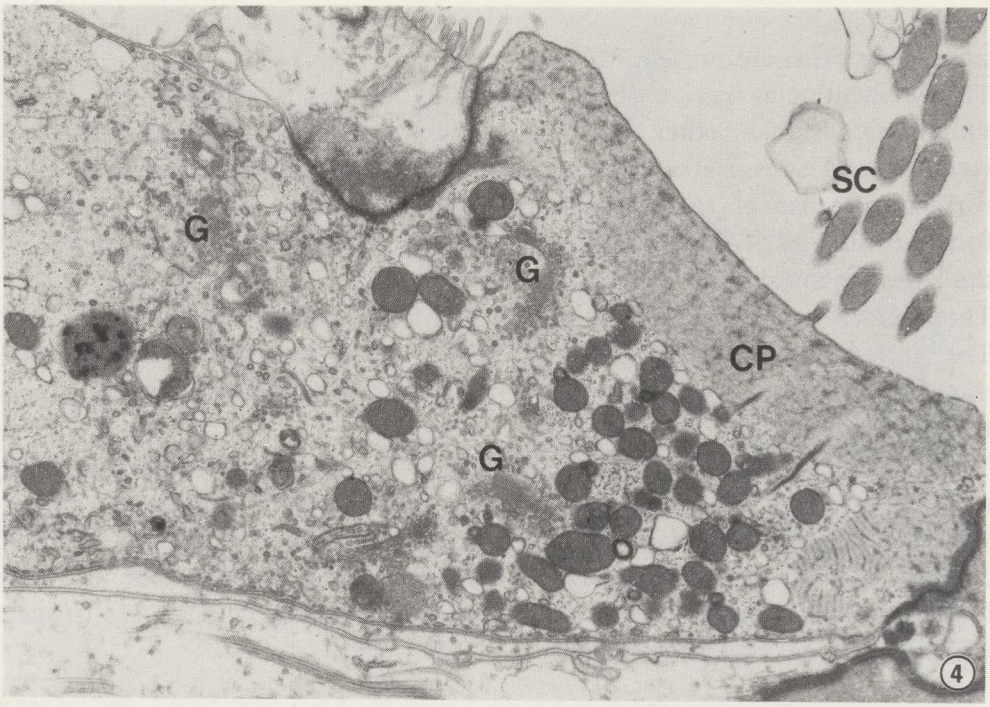
Figure 1. The apex of the outer hair cell is dominated by the cuticular plate CP into which the stereocilia SC are inserted. In the infracuticular region Golgi saccules G, mitochondria and lipofuscin granules L predominate. The endoplasmic reticulum ER is arranged along the lateral membranes (*x20,000*).

Figure 2. Conspicuous features in the infranuclear region of the outer hair cell are the subsynaptic cisternae of the endoplasmic reticulum (arrows) which are more prevalent near the synaptic contacts with the efferent nerve endings N (*x31,000*).

Figure 3. Smooth vesicles (arrows) predominate near the sites where synaptic contacts are formed with the afferent nerve endings N (*x31,000*).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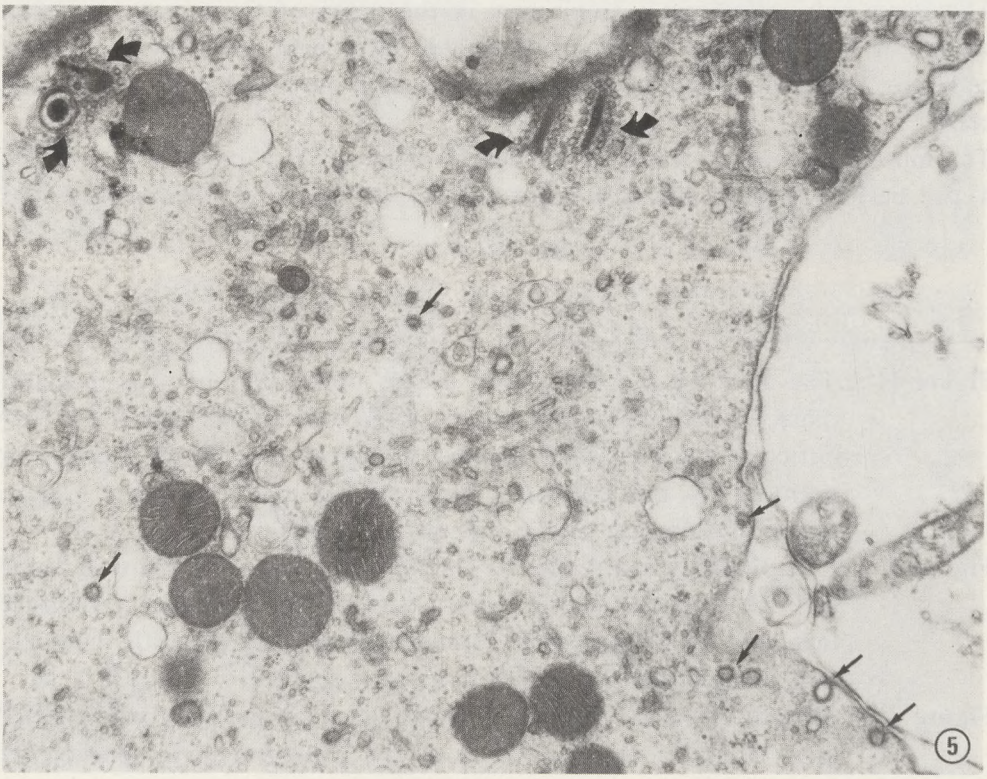
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complex, and numerous mitochondria and small vesicles. Occasionally multivesicular bodies, lysosomes and residual bodies are observed. Free ribosomes and glycogen particles are seldom seen. In contrast to the outer hair cell, the endoplasmic reticulum is dispersed throughout the cytoplasm as smooth-surfaced, sometimes dilated, tubules.
The nucleus of the inner hair cell is centrally located. The infranuclear region (Fig. 5) contains mitochondria and some parallel arrays of rough endoplasmic reticulum, and is dominated by coated pits and vesicles as well as smooth vesicles and so-called synaptic bodies. The latter are exclusively located near the synaptic contacts with the afferent nerve endings.

Gentamicin-intoxicated cochleas
No morphological alterations are observed after one day of gentamicin administration. After 5 days ultrastructural changes are observed only in the outer hair cells, varying from minimal to severe. Gentamicin administration for 10 or 15 days only differs from 5-day administration in the amount of cells affected. In all gentamicin-treated cochleas (5, 10 and 15 days) the individual outer hair cells (OHC_1, OHC_2 and OHC_3) are randomly affected. In addition, no gross differences in ultrastructural pathology are observed between the basal, middle and apical turns.

In the less affected outer hair cells ultrastructural changes are restricted to the infracuticular region. These changes comprise an increase in multivesicular bodies, secondary lysosomes, and in granules of medium electron density resembling primary lysosomes (Figs. 6-8). Autolysosome formation is regularly observed (Fig. 8). The endoplasmic reticulum displays proliferation of its subsurface cisternae (Fig. 6).
In the more affected outer hair cells Hensen's bodies and myelin-like figures appear in the infracuticular and supranuclear regions (Figs. 9, 10, 12 and

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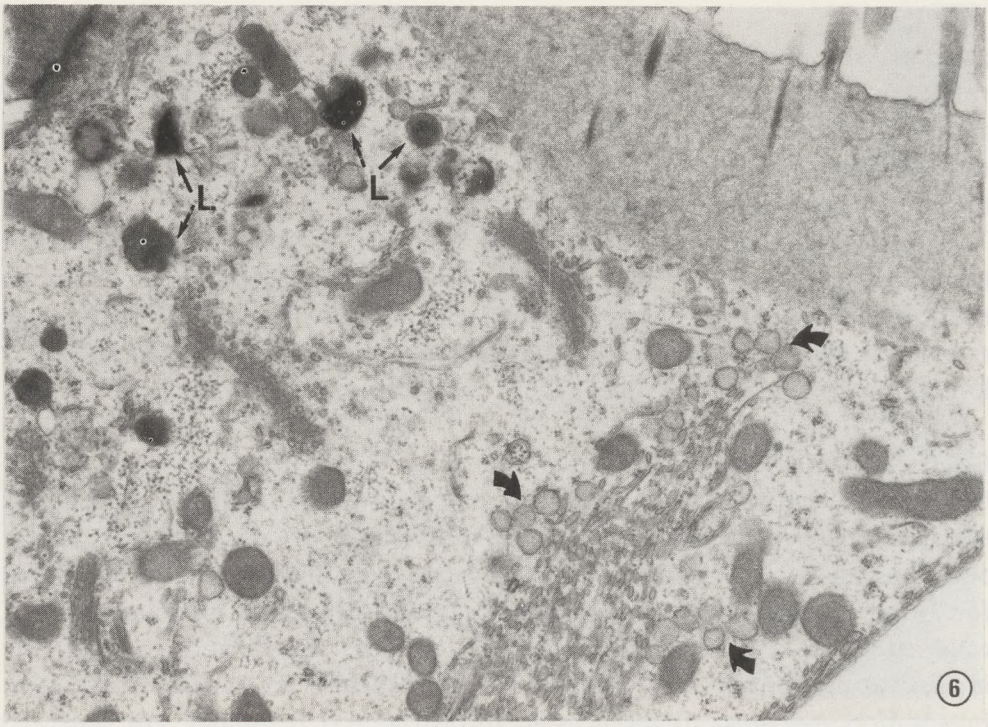
Figure 4. Most prominent features in the apical region of the inner hair cell are besides the cuticular plate CP and the stereocilia SC, the Golgi complex G, numerous mitochondria and vesicles, and tubular profiles of the endoplasmic reticulum (*x14,000*).

Figure 5. The infranuclear region of the inner hair cell is characterized by the presence of synaptic bodies (arrows) and the abundance of coated vesicles (small arrows) and pits (*x31,000*).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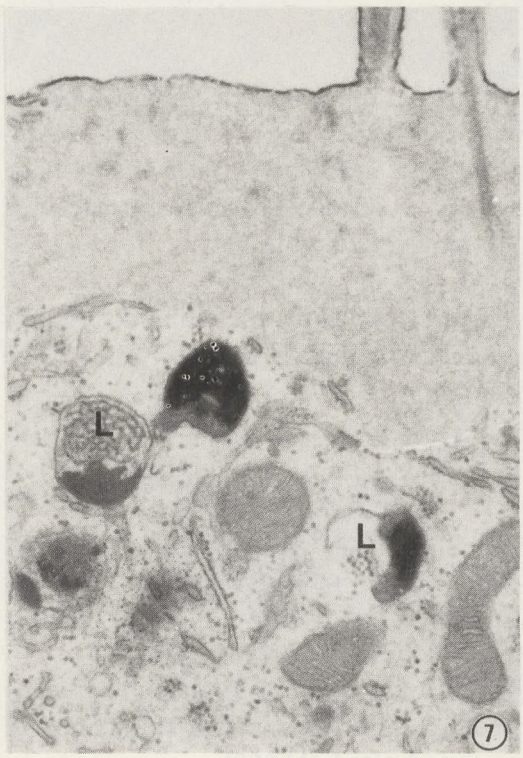
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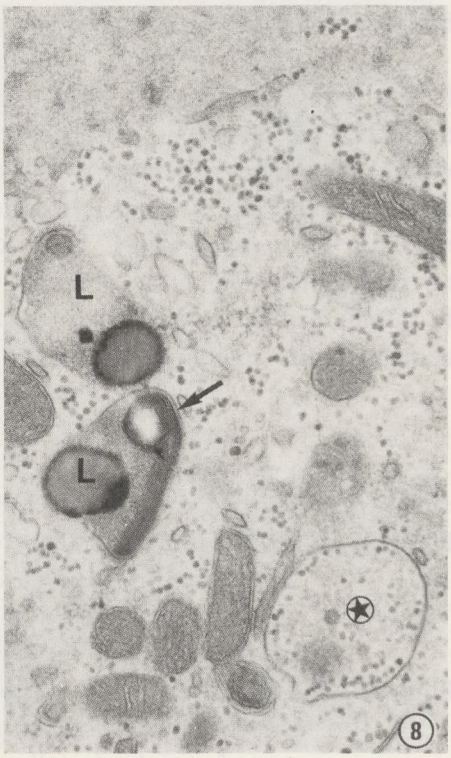
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13). In combination, invaginations of the lateral membranes are frequently seen (Fig. 11).

In the severely affected outer hair cells extensive dilatation of the cisternae of the endoplasmic reticulum and the nuclear envelope is added to the aforementioned changes, giving rise to vacuolation of the cytoplasm (Fig. 14).

The number and morphology of Golgi saccules remain unchanged in all but the most severely affected cells (Figs. 6 and 9). Free ribosomes and glycogen particles are still present and the mitochondria display a normal appearance (Figs. 6-13). No changes in the infranuclear region can be observed.

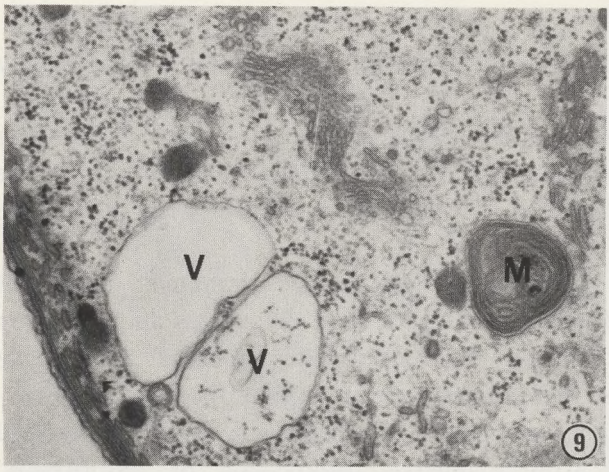
Mitochondrial alterations such as matrix condensation and intracristal swelling are observed only after extensive progression of cytoplasmic vacuolation (Fig. 14). At this stage, the outer hair cells demonstrate a shrunken appearance, the nucleus has undergone considerable changes (e.g., nuclear swelling and chromatolysis) and most cytoplasmic constituents such as glycogen and free ribosomes have disappeared (Fig. 14). Despite extensive intracellular damage, the cuticular plate and stereocilia have remained unchanged in most cells (Fig. 14).

In contrast to the outer hair cells, the inner hair cells do not exhibit any obvious ultrastructural changes, not even after 15 days of exposure to gentamicin (Fig. 15).

Figure 6. An increase in medium-density granules (arrows) and secondary lysosomes L, and a proliferation of the endoplasmic reticulum are the earliest identifiable changes seen in the outer hair cell after gentamicin administration (*x20,000*).

Figure 7. Electron micrograph demonstrating the pleomorphic nature of the secondary lysosomes L (*x31,000*).

Figure 8. Detail of the infracuticular region of an outer hair cell with autolysosome formation (asterisk) and lysosomes L containing lamellar inclusion bodies (arrow) after 5 days of gentamicin (*x42,000*).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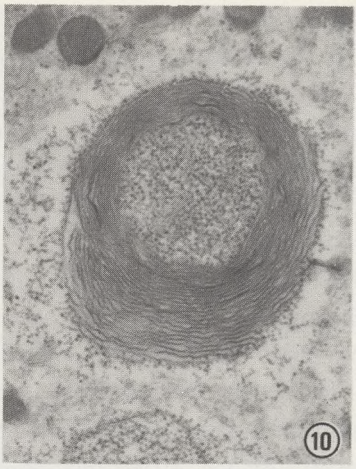
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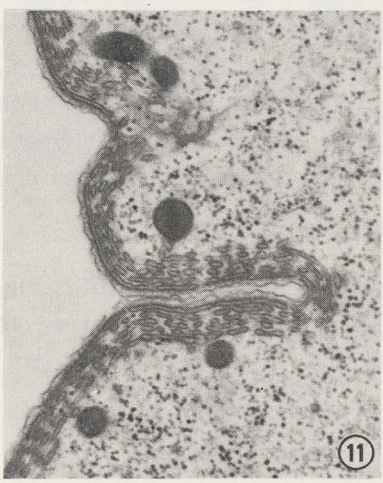
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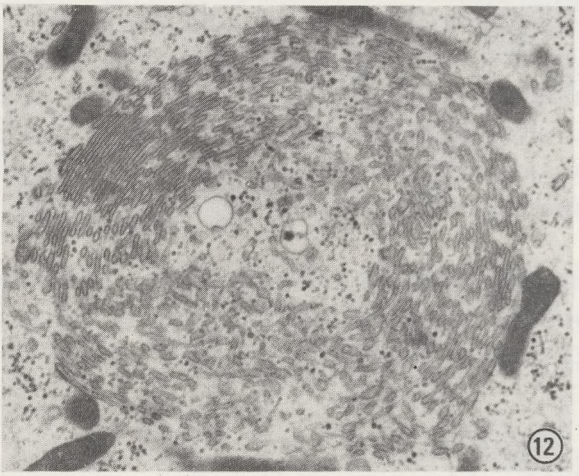
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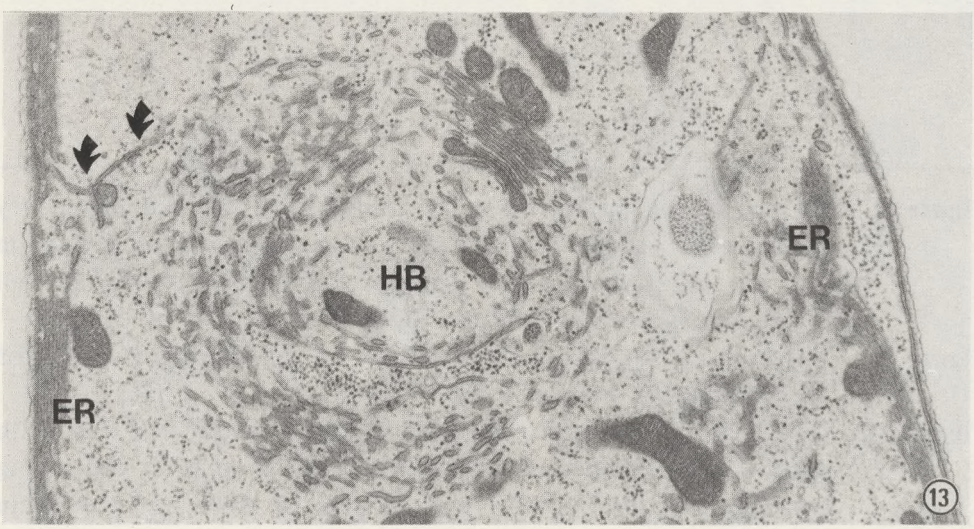
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DISCUSSION

When we compare the effects of gentamicin administration for 5, 10 and 15 days, there is no preference for any individual outer hair cell (OHC_1 *versus* OHC_2 *versus* OHC_3) in which pathological changes occur. In addition, with regard to hair cell degeneration, no preference for the basal turn of the cochlea could be discerned during the early stages of gentamicin intoxication. This is in disagreement with the data obtained from quantitative studies reporting a basal-apical gradient of hair cell degeneration [i.a., Ylikoski, 1974; Hawkins and Johnsson, 1981; Tange *et al.*, 1982]. However, it should be stressed that the pattern of degeneration is dependent on a multitude of factors such as interindividual variation [Ohtani *et al.*, 1982], the (total) dose of the drug administered as well as the duration of drug administration [Wersäll, 1981; Lenoir and Puel, 1987], and the survival time following drug administration [Theopold, 1977].
Although in our study the possibility of sampling error can not be excluded, our findings of an equally distributed degeneration pattern are supported by the biochemical studies of Tran Ba Huy and Deffrennes [1988] who deter-

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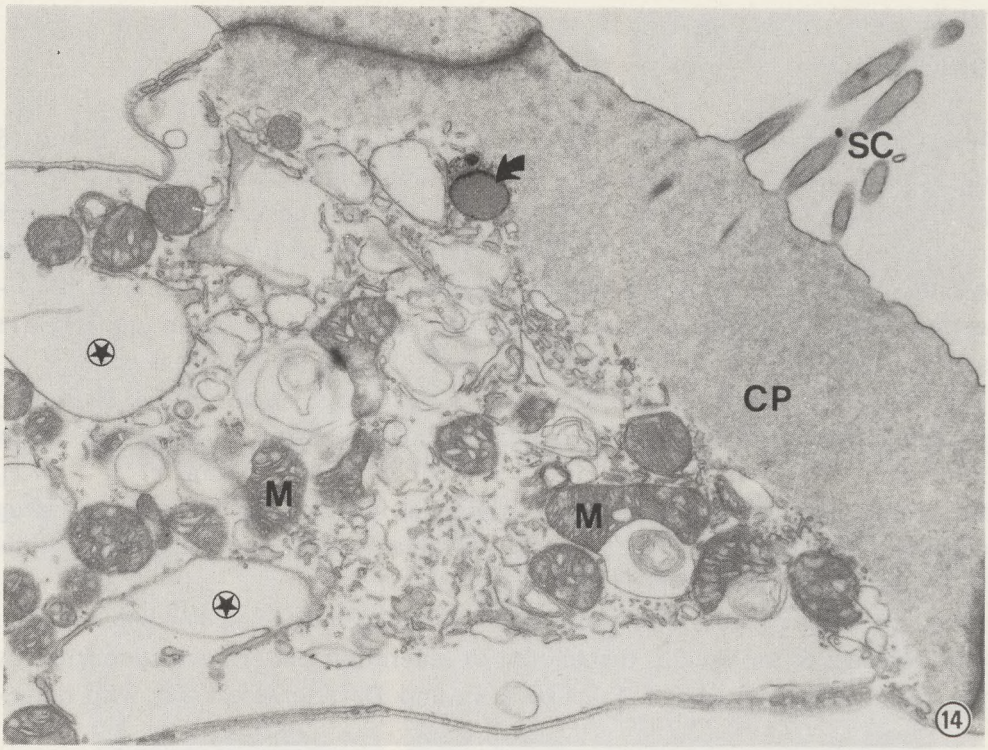
Figure 9. Portion of the supranuclear region of an outer hair cell after gentamicin administration. Vacuoles V and myelin-like figures M are present throughout the cytoplasm (*x20,000*).

Figure 10. Another myelin-like figure, varying in size and morphology as compared to that of Fig. 9, enclosing monoparticulate glycogen. These structures presumably represent degenerated Hensen's bodies (*x20,000*).

Figure 11. Invaginations of the lateral membranes are occasionally seen in outer hair cells during aminoglycoside intoxication. Noteworthy is the intimate relationship between the plasma membrane and the endoplasmic reticulum (*x20,000*).

Figure 12. Hensen's bodies are composed of concentric layers of smooth-surfaced membranes, enclosing a portion of the cytoplasm with one or more central vacuoles, and are frequently juxtaposed with mitochondria (*x20,000*).

Figure 13. Electron micrograph demonstrating the continuity (arrows) between the peripheral membranes of a Hensen's body HB and the cisternae of the endoplasmic reticulum ER (*x20,000*).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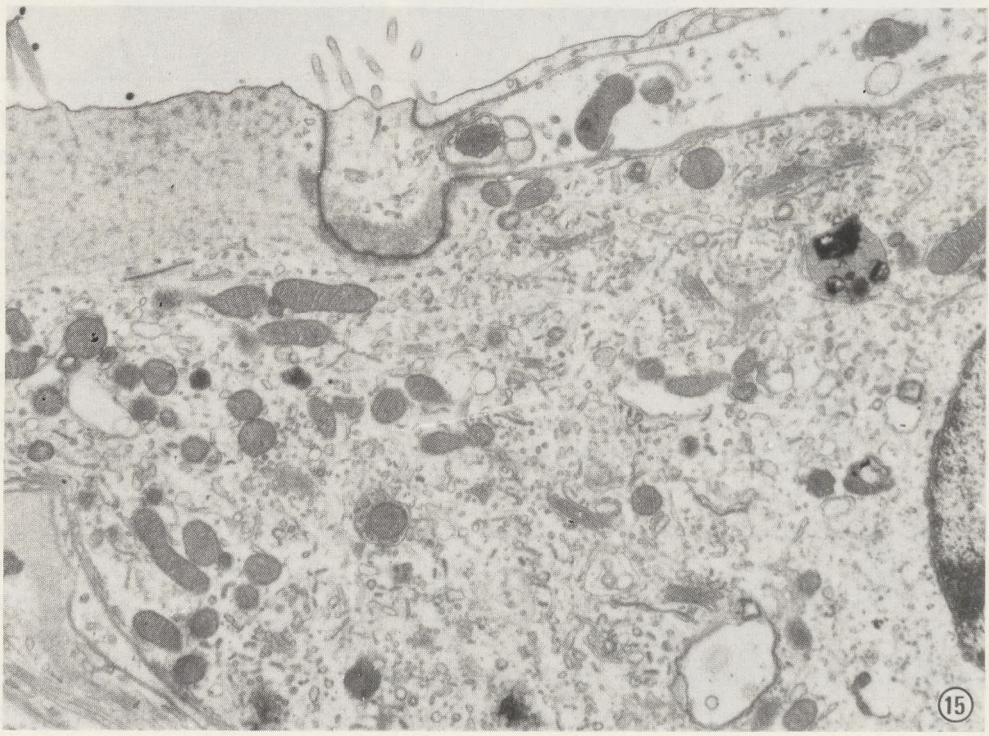
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mined tissue drug concentrations throughout the cochlea. Their data could not provide evidence for a preferential uptake of gentamicin either in the basal or in the apical turns.
Moreover, most studies which report a basal-apical gradient of hair cell degeneration are based on either light microscopical (i.e., Nomarski differential interference microscopy) or scanning electron microscopical evaluation of surface preparations. Harpur and Bridges [1979] consider an approach by scanning electron microscopy - and, hence, by light microscopy - as not suitable for identifying early degenerative changes in cochlear hair cells since surface (i.e., stereocilia) pathology is a relatively late event and occurs only after intracellular degeneration has progressed considerably [cf., Forge, 1985; Lenoir and Puel, 1987].

This study confirms the finding that outer hair cells are more vulnerable to the drug than inner hair cells [Ylikoski, 1974; Hawkins and Johnsson, 1981; Tange *et al.*, 1982; Lim, 1986a; Lenoir and Puel, 1987]. Even after 15 days the inner hair cells did not demonstrate any obvious ultrastructural changes. This phenomenon might be the consequence of the topographical anatomy of the organ of Corti. The outer hair cells are, in contrast to the inner hair cells, directly exposed to cortilymph which is, together with perilymph, considered to be the main route of access for the aminoglycosides to the organ of Corti [Tammela and Tjälve, 1986; Tran Ba Huy *et al.*, 1986].

In the present study, the outer hair cells display a great variety of ultrastructural changes ranging from mild to severe. We assume that these

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Figure 14. Morphological appearance of an outer hair cell after 15 days of gentamicin. The cytoplasm displays extensive vacuolation resulting from severe dilatation of the endoplasmic reticulum (asterisks). Damage to the mitochondria M is limited to intracristal swelling. Free ribososmes and glycogen particles have disappeared. Residual bodies (arrow) are still present and intact. Despite extensive intracellular damage, the cuticular plate CP and stereocilia SC have remained intact (*x20,000*).

Figure 15. After 15 days of gentamicin, no obvious signs of degeneration are present in the topographically corresponding inner hair cell (*x13,000*).

changes reflect a time-sequence, and that the changes observed in the less affected hair cells precede those encountered in the severely affected hair cells. In the light of this assumption, the earliest identifiable ultrastructural changes following gentamicin administration are an increase in secondary lysosomes and medium-density granules, and a proliferation of the endoplasmic reticulum. This is in close agreement with other ultrastructural studies [Darrouzet and Guilhaume, 1974, 1976; Poch Broto *et al.*, 1980; Lenoir and Puel, 1987].

An increase in the number of secondary lysosomes (e.g., residual bodies) is a commonly observed feature in hair cells after chemical or mechanical injury, and has generally been related to autophagocytosis of degenerated mitochondria [Wersäll, 1981; Lim, 1986a]. However, in our study, at this stage of intoxication there is no morphological evidence for mitochondrial degeneration. Moreover, degeneration of mitochondria is thought to be a relatively late event since they display no morphological changes until severe vacuolation of the cytoplasm or even disruption of the hair cell has commenced [Darrouzet and Guilhaume, 1974, 1976]. Autophagocytotic activity can not be excluded, however, since autolysosomes are frequently observed in this stage.

The abundance of medium-density granules as well as the proliferation of the subsurface cisternae indicates an increase in the activity of the cell's synthetic apparatus, i.e., the endoplasmic reticulum and the Golgi complex, as suggested by Lim [1986a]. Concomitantly, this could explain the observed increase in secondary lysosomes especially since the medium-density granules resemble primary lysosomes.

During the initial stages of intoxication no changes in the morphology of the Golgi complex were observed (Figs. 6 and 9; compare with Fig. 1). In a later stage, however, distinct Golgi saccules could not be identified anymore due to the extensive cytoplasmic vacuolation [cf., Harpur and Bridges, 1979; Poch Broto *et al.*, 1980]. Unlike Darrouzet and Guilhaume [1974, 1976] and Lim [1986a], we could not discern a proliferation of the Golgi complex in the infracuticular region of the outer hair cells. It should be noted that, in general, the Golgi complex is one of the cell organelles which is less susceptible to cellular damage. This may be due to its biochemical 'downstream' function by acting as a modifier of proteins as opposed to that of the endoplasmic reticulum as a primary synthesizer.

A further change during gentamicin intoxication is the formation of Hensen's bodies. These organelles are frequently observed in outer hair cells after acoustic trauma [Lim, 1976], aminoglycoside administration [i.a., Wersäll, 1981] and obliteration of the endolymphatic sac [Albers *et al.*, 1988].

It is assumed that these structures are also present in normal outer hair cells [Lim, 1986b]. In our preparations, however, Hensen's bodies could only be discerned in affected hair cells.

The mechanism underlying the formation of Hensen's bodies is incompletely understood. Morphologically, the membranes of the Hensen's bodies resemble the smooth-surfaced cisternae of lamellar endoplasmic reticulum which is typical for outer hair cells. Moreover, the observation that the subsurface cisternae are continuous with the peripheral membranes of the Hensen's bodies (see Fig. 13) strongly suggests that these structures arise from the endoplasmic reticulum [cf., Lim, 1986a, b]. This is further supported by the observation that Hensen's bodies are never found in the inner hair cells which lack a lamellar arrangement of the endoplasmic reticulum.

Likewise, the functional significance of Hensen's bodies remains obscure. In analogy to the concentric membraneous bodies which are formed in hepatocytes after exposure to inhibitors of protein synthesis [Hwang *et al.*, 1974] and a variety of other xenobiotics [for a review see Ghadially, 1982], they may be looked upon as a specialized type of smooth endoplasmic reticulum involved in the detoxification of drugs. Wersäll [1981] has stated that Hensen's bodies, in a later stage, degenerate into accumulations of lamellar bodies, of a varying size and structure; these structures resemble the myelin-like figures which are frequently seen in our preparations (Figs. 9 and 10).

In conclusion, the results obtained in this study demonstrate that gentamicin-induced changes in the outer hair cells primarily involve the endoplasmic reticulum. All further changes occur at a much later stage and therefore are to be considered secondary events.

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GLYCOCALYX HETEROGENEITY IN THE GUINEA PIG COCHLEA. EFFECTS OF GENTAMICIN ADMINISTRATION

CHAPTER 4

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GLYCOCALYX HETEROGENEITY IN THE GUINEA PIG COCHLEA. EFFECTS OF GENTAMICIN ADMINISTRATION

The hair cells and the accessary epithelia of the cochlea, similar to other epithelial or to free-floating cells, exhibit a glycocalyx on the external surfaces of their plasma membranes. This delicate layer is not uniformly distributed throughout the entire cochlea, but is present only on the free luminal surfaces. In other words, it is present on those cell surfaces which are directly exposed to either perilymph or endolymph [Spoendlin, 1968; Küttner and Geyer, 1972, 1974; Küttner, 1974; Saito *et al.*, 1977; Forge, 1981; Slepecky and Chamberlain, 1985; De Groot, 1986; Lim, 1986; Prieto and Merchan, 1986, 1987; Santi and Anderson, 1987].

The glycocalyx mainly consists of oligosaccharide side chains of the glycolipids and the integral glycoproteins forming the outer leaflet of the plasma membrane, and is to be considered as a part of the plasma membrane. It is further composed of acidic glycosaminoglycans and small peripheral proteins, which are produced by the cell itself or, more or less permanently, absorbed from the cell's environment. Since many of these so-called glycoconjugates contain anionic groups (viz., carboxyl, sulphate and phosphate groups), the plasma membrane demonstrates a net negative surface charge [Spicer *et al.*, 1981; Temmink, 1981; Seno, 1987].

In general, the glycocalyx lacks sufficient electron density to be detected by routine electron microscopy. Hence, various methods have been developed to visualize the glycocalyx, most of which specifically detect the oligosaccharide moiety of this layer [for reviews see Luft, 1976; Schrével *et al.*, 1981; Spicer *et al.*, 1981; Temmink, 1981; Seno, 1987].

In inner ear research the cationic dyes ruthenium red and Alcian blue have been frequently used to visualize the glycocalyx in the cochlea. In our hands, however, the results obtained with both dyes proved to be erratic. Therefore we looked for an alternative method which would give reproducible results. In an earlier study we had found that post-fixation with either $OsO_4/K_4Ru(CN)_6$ or $OsO_4/K_4Fe(CN)_6$ resulted in impaired glycogen and membrane contrast in aldehyde-fixed cochleas [De Groot *et al.*, 1987]. In the same preparations, serendipitously, glycocalyx contrast appeared to be

enhanced as well. Consequently, we further investigated the contrast-enhancing properties of $K_4Ru(CN)_6$ and related compounds (*post-fixation experiment*) as well as the influence of primary fixation (*primary fixation experiment*). Furthermore, making use of $OsO_4/K_4Ru(CN)_6$ post-fixation, we determined the effect of gentamicin administration on the glycocalyx of both the hair cells and the accessary epithelia of the cochlea (*gentamicin experiment*).

MATERIALS AND METHODS

For all experiments we used healthy, female albino guinea pigs (strain GpHi65 *Himalayan*, weighing 200-350 g) which were housed under standard laboratory conditions and fed *ad libitum*.
One group of animals was treated with gentamicin (Gentogram^R, 100 mg/kg) by daily intraperitoneal injections for 5 days. The animals were sacrificed 24 h after the final injection of the drug.
Anaesthesia was performed by intraperitoneal injection of sodium pentobarbital (Triotal^R, 70 mg/kg) followed by decapitation and removal of the cochleas.

Post-fixation experiment
Cochleas were immediately fixed by intralabyrinthine perfusion with a trialdehyde fixative followed by immersion in the same solution for 3 h at room temperature. The fixative consisted of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2.5% DMSO in 0.08 M sodium cacodylate buffer, pH 7.4 [De Groot *et al.*, 1987]. Subsequently, the cochleas were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4 (2x15 min), decalcified in 10% EDTA.2Na (pH 7.4) under constant agitation in a decalcifying device (LTI, Bilthoven, The Netherlands) for 4-5 days and rinsed in the same buffer (2x15 min), all at room temperature.
Cochleas were post-fixed for 2 h at 4°C in respectively:

1. 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4,
2. 1% OsO_4, containing $K_4Ru(CN)_6$ (K & K Labs., ICN Biomedicals Inc., New York, USA) in varying concentrations (0.1%; 1%; 1.5%; or 2%), either in distilled water or in 0.1 M sodium cacodylate buffer, pH 7.4 [De Bruijn, 1968],
3. 1% OsO_4, 1.5% $K_4Fe(CN)_6.3H_2O$ in 0.1 M sodium cacodylate buffer, pH 7.4 [De Bruijn, 1968; Karnovsky, 1971],
4. 1% OsO_4, 1.5% $K_3Fe(CN)_6$ in 0.1 M sodium cacodylate buffer, pH 7.4

[De Bruijn, 1968], or

5. 1% OsO_4, 1.5% $K_3Co(CN)_6$ (K & K Labs., ICN Biomedicals Inc., New York, USA) in 0.1 M sodium cacodylate buffer, pH 7.4 [De Bruijn, 1968],

followed by several washes in distilled water. All solutions were freshly prepared.

Dehydration was carried out in a graded ethanol, 2,2-dimethoxypropane, propylene oxide series and the cochleas were *in toto* embedded in Spurr's low-viscosity resin, containing 1% silicone DC 200 fluid (Polaron Equipment Ltd., Bio-Rad Labs., Watford, UK).

Ultrathin sections of re-embedded quarter turns were examined without additional heavy metal staining in a Philips EM 201c transmission electron micoscope operating at 60 kV.

Primary fixation experiment

In order to investigate the influence of primary fixation on the contrast-enhancing properties of $K_4Ru(CN)_6$, a number of cochleas were fixed by intralabyrinthine perfusion with respectively:

1. 2.5% glutaraldehyde, 2% formaldehyde in 0.08 M sodium cacodylate buffer (pH 7.4) containing 0.025% $CaCl_2$ [modified from Karnovsky, 1965],
2. 3% glutaraldehyde, 1% acrolein, 2.5% DMSO in 0.1 M sodium cacodylate buffer (pH 7.4), or
3. 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4),

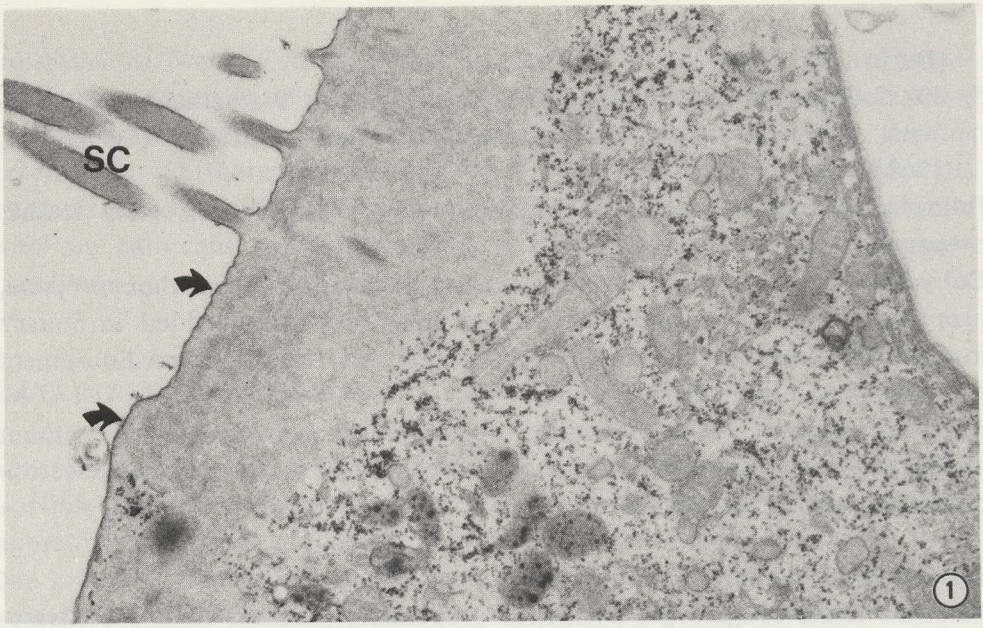
followed by immersion in the same fixative for 2 h at 4°C and subsequent rinses in 0.1 M sodium cacodylate buffer, pH 7.4 (2x15 min).

After EDTA-decalcification, the cochleas were post-fixed for 2 h at 4°C in 1% OsO_4, 1% $K_4Ru(CN)_6$ in 0.1 M sodium cacodylate buffer (pH 7.4) followed by several washes in distilled water. Dehydration and embedding were as described above.

Gentamicin experiment

Cochleas of gentamicin-treated animals were fixed by intralabyrinthine perfusion with tri-aldehyde fixative [De Groot *et al.*, 1987] followed by immersion in the same fixative for 3 h at room temperature and subsequent rinses in 0.1 M sodium cacodylate buffer, pH 7.4 (2x15 min).

After EDTA-decalcification, the cochleas were post-fixed for 2 h at 4°C in 1% OsO_4, 1% $K_4Ru(CN)_6$ in 0.1 M sodium cacodylate buffer (pH 7.4) followed by several washes in distilled water. Dehydration and embedding were as described above.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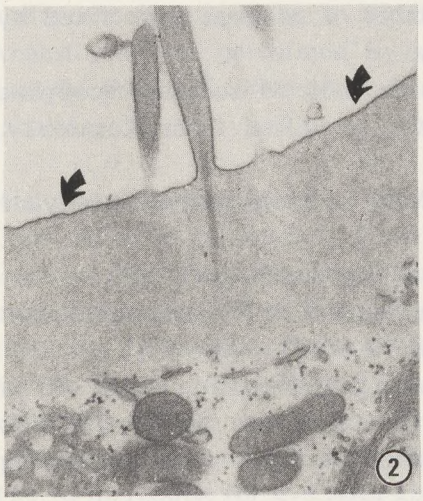
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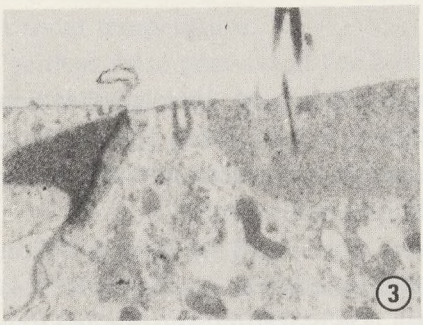
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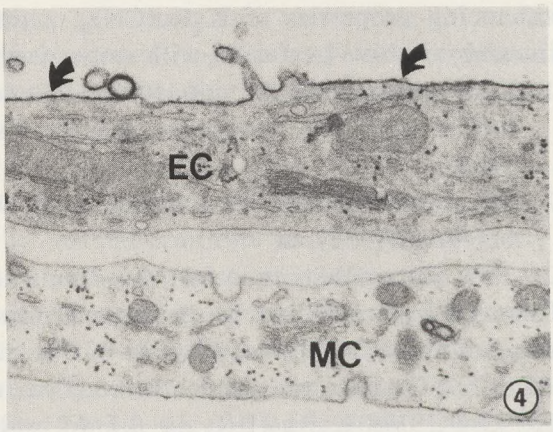
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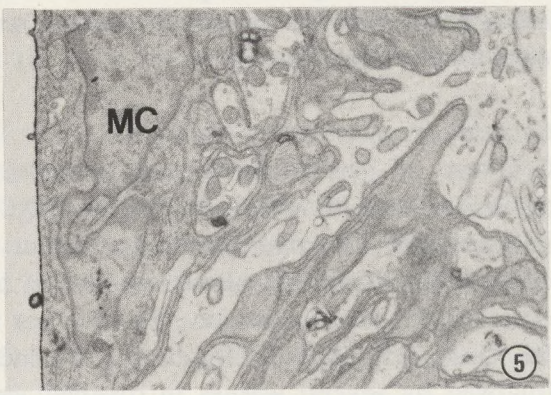
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RESULTS

Post-fixation experiment

The results of this experiment are summarized in Table 1. After post-fixation with $OsO_4/K_4Ru(CN)_6$ a continuous, thin electron-dense layer (approximately 7 nm in thickness) was seen covering the apical surfaces of the hair cells and the supporting cells of the organ of Corti (Figs. 1 and 2), as well as the outer sulcus, the epithelial lining of the spiral prominence, the marginal cells of the stria vascularis (Fig. 5), and the epithelial lining of Reissner's membrane (Fig. 4), i.e., those membranes directly exposed to the scala media. No gross differences in thickness of the glycocalyx between the different epithelial cells could be detected.

No layer was visible on the basolateral membranes of the hair cells and supporting cells (Fig. 1), the membranes of the tunnel-crossing fibres, nor on the mesothelial lining of Reissner's membrane (Fig. 4) and the mesothelial cells of the basilar membrane, i.e., the cells delineating the scala vestibuli, the scala tympani and the membranes directly exposed to Nuel's space.

The contrast of this layer was also enhanced after post-fixation with either $OsO_4/K_4Fe(CN)_6$ or $OsO_4/K_3Fe(CN)_6$ (Fig. 2), but was completely absent after $OsO_4/K_3Co(CN)_6$ post-fixation or when using OsO_4 alone (Table 1).

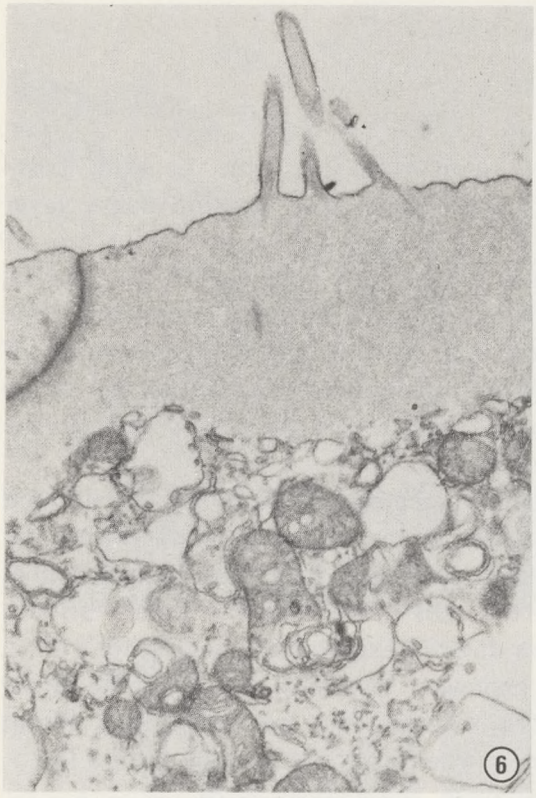
Figure 1. Outer hair cell demonstrating glycocalyx contrast-staining on the apical membranes (arrows) as well on the stereocilia SC; tri-aldehyde fixation, $OsO_4/K_4Ru(CN)_6$ post-fixation. Note absence of contrast-staining at the lateral membranes (*x20,000*).

Figure 2. Outer hair cell; contrast-staining of the glycocalyx (arrows) is also present after $OsO_4/K_4Fe(CN)_6$ post-fixation (*x20,000*).

Figure 3. Outer hair cell; glycocalyx contrast-staining is absent after glutaraldehyde-acrolein primary fixation (*x9,000*).

Figure 4. Reissner's membrane demonstrating glycocalyx contrast-staining (arrows) only on the membranes of the epithelial cells EC. The membranes of the mesothelial cells MC, which are exposed to perilymph, are lacking contrast-staining (*x20,000*).

Figure 5. Stria vascularis demonstrating the glycocalyx on the apical membranes of the marginal cells MC (*x9,000*).



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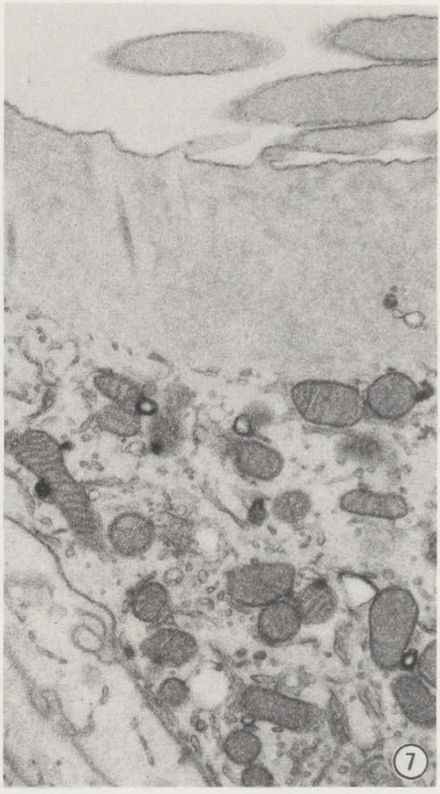

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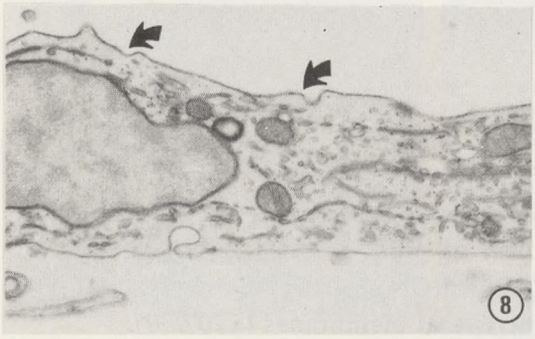

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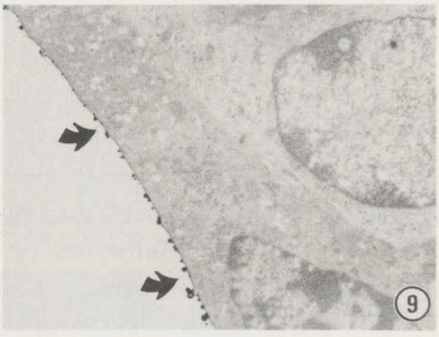

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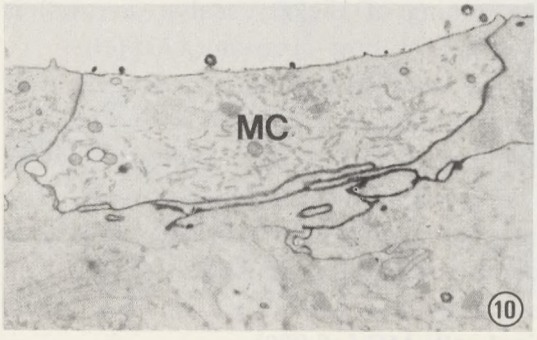

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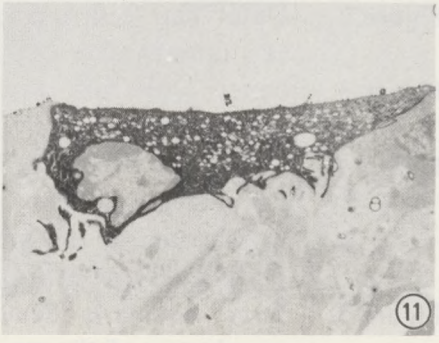

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Primary fixation experiment

The observed contrast-enhancement was present only after primary fixation with tri-aldehyde or modified Karnovsky fixative. No glycocalyx contrast was observed after primary fixation with glutaraldehyde alone or with glutaraldehyde-acrolein (Fig. 3).

Gentamicin experiment

Contrast-staining of the glycocalyx on the apical membranes of the hair cells and supporting cells was not affected by gentamicin administration (Figs. 6 and 7). Glycocalyx contrast-staining of the epithelial surfaces of Reissner's membrane, also, remained unchanged (Fig. 8).

Contrast-staining of the glycocalyx lining the spiral prominence and the stria vascularis had become irregular and of a flocculent appearance (Fig. 9). Occasionally, the basolateral membranes of the marginal cells were prominent in contrast (Fig. 10), whereas the marginal cells sometimes demonstrated an extremely electron-dense cytoplasm (Fig. 11).

Figure 6. Outer hair cell after 5 days of gentamicin administration. Glycocalyx contrast is not affected, although the hair cell shows signs of excessive damage (*x20,000*).

Figure 7. Inner hair cell after 5 days of gentamicin administration (*x20,000*).

Figure 8. Reissner's membrane after 5 days of gentamicin administration. Glycocalyx contrast-staining (arrows) of the epithelial cells remains unaffected (*x20,000*).

Figure 9. Stria vascularis after 5 days of gentamicin administration. Glycocalyx contrast-staining (arrows) appears irregular and flocculent (*x7,000*).

Figure 10. Stria vascularis showing additional contrast-staining of the basolateral membranes of the marginal cells MC (*x10,000*).

Figure 11. Stria vascularis demonstrating a marginal cell with electron-dense cytoplasm (*x4,500*).

post-fixatives	perilymphatic surfaces	endolymphatic surfaces
1% OsO_4	-	-
1% OsO_4 + 0.1% $K_4Ru(CN)_6$	-	-
1% OsO_4 + 1% $K_4Ru(CN)_6$	-	+
1% OsO_4 + 1.5% $K_4Ru(CN)_6$	-	+
1% OsO_4 + 2% $K_4Ru(CN)_6$	-	+
1% OsO_4 + 1% $K_4Ru(CN)_6$; buffered	-	+
1% OsO_4 + 1% $K_4Ru(CN)_6$; no buffer	-	+
1% OsO_4 + 1.5% $K_4Fe(CN)_6$	-	+
1% OsO_4 + 1.5% $K_3Fe(CN)_6$	-	+
1% OsO_4 + 1.5% $K_3Co(CN)_6$	-	-

(+ = positive reaction; - = no reaction)

Table 1. Glycocalyx contrast-enhancement after post-fixation with OsO_4/$K_4Ru(CN)_6$ and related post-fixatives.

DISCUSSION

The results of this study show that addition of either $K_4Ru(CN)_6$, $K_4Fe(CN)_6$ or $K_3Fe(CN)_6$ to aqueous OsO_4 solutions not only imparts a high electron density to glycogen and membranes [cf., De Bruijn, 1968; Karnovsky, 1971; De Bruijn and Den Breejen, 1975], but also selectively enhances glycocalyx contrast in aldehyde-fixed cochleas. Glycocalyx contrast-staining by OsO_4/$K_4Fe(CN)_6$ has been reported already by Karnovsky [1971], Dvorak *et al.* [1972] and Neiss [1984]. Unlike the former authors, Neiss [1984] observed glycocalyx contrast-staining only at low concentrations (<0.25%) of $K_4Fe(CN)_6$ and argued that glycocalyx contrast-

staining at high concentrations (>0.75%) of $K_4Fe(CN)_6$, as found by Dvorak *et al.* [1972], is mainly due to the use of phosphate buffers at low pH. This is not compatible with our results as in this study cacodylate-buffered solutions of high concentrations of $K_4Fe(CN)_6$ (=1.5%) or $K_4Ru(CN)_6$ (=1%) were used at neutral pH. Moreover, glycocalyx contrast-staining even occurs when using (un)buffered solutions at concentrations as high as 2%, whereas contrast-staining is absent at a concentration of 0.1% [De Groot, this study].
At present, however, it remains unclear why glycocalyx contrast-staining was not obtained after post-fixation with $OsO_4/K_3Co(CN)_6$, the more so as this mixture has been proven to selectively enhance glycogen contrast [De Bruijn, 1968; De Bruijn and Den Breejen, 1975].
Our results further suggest that contrast-staining is fixation-dependent, since glycocalyx contrast was observed only after primary fixation with formaldehyde-glutaraldehyde-containing fixatives. Although tissue-specific differences or topographical heterogeneity can not be excluded [Spicer *et al.*, 1981], primary fixation may well account for the different results obtained by Neiss [1984] as compared to this study.
The mechanism of the reaction underlying selective glycogen contrast-staining with solutions of OsO_4 and $K_4Ru(CN)_6$ (or related compounds) has been extensively investigated by De Bruijn and Den Breejen [1975] and Riemersma *et al.* [1984]. Their findings suggest that OsO_4 is reduced to osmate, and that this osmate reacts with the hydroxyl groups of glycogen to form glycogen osmate; other polyhydroxyl compounds such as oligosaccharides appear to react in a similar way. Since oligosaccharide-containing macromolecules are abundantly present in the plasma membrane in general and in the cell membranes of the organ of Corti in particular [Tachibana *et al.*, 1987a, b; Gil-Loyzaga and Brownell, 1988; Rueda and Lim, 1988], these macromolecules may contribute to the glycocalyx contrast-staining in the cochlea.

Glycocalyx contrast-staining by either $OsO_4/K_4Ru(CN)_6$, $OsO_4/K_4Fe(CN)_6$ or $OsO_4/K_3Fe(CN)_6$ was limited to the membranes directly exposed to the scala media, i.e., the endolymphatic compartment. It should be noted that with this visualization method no glycocalyx was visible on the membranes facing either Nuel's space or the scala tympani and scala vestibuli, i.e., the perilymphatic compartment.
This selectivity conceivably reflects biochemical differences between these two layers and may be of functional significance, especially since endolymph differs both in electrolyte and protein composition from perilymph [cf., Arnold and Vosteen, 1979]. Hence, we propose the terms '*endolymphatic*

glycocalyx' indicating the layer lining the endolymphatic compartment, and '*perilymphatic glycocalyx*' for the layer present on the membranes directly exposed to perilymph.
Apparently this heterogeneity is in disagreement with the studies of Küttner [1974] and Küttner and Geyer [1974], who found no differences in reactivity between both layers using ruthenium red and colloidal iron, respectively. However, the notion that the endolymphatic glycocalyx differs in reactivity and/or biochemical composition from the perilymphatic glycocalyx has been recently confirmed by studies using the probes tannic acid [Prieto and Merchan, 1986], Alcian blue [Santi and Anderson, 1987] and colloidal iron [Prieto and Merchan, 1987]. Actually, glutaraldehyde fixation extracts some membrane constituents (i.a., glycoconjugates) and modifies cell surface charge, resulting in an increase or decrease in contrast-staining [Schrével *et al.*, 1981]. This may account for the positive reaction of both the endo- and perilymphatic glycocalyx as seen with ruthenium red [Küttner, 1974] and colloidal iron [Küttner and Geyer, 1974].
Regional differences in glycocalyx contrast-staining between hair cells and supporting cells have been reported by Lim [1986], Santi and Anderson [1987] and Prieto and Merchan [1987]. According to these authors, reactivity with cationic probes of the endolymphatic glycocalyx of the hair cells is more pronounced than that of the supporting cells, which they relate to its supposed (ion-sequestering) role in the mechano-electrical transduction process. However, their findings could not be corroborated by our investigations, nor are they consistent with earlier observations [Küttner and Geyer, 1972; Küttner, 1974; Slepecky and Chamberlain, 1985; De Groot, 1986; Prieto and Merchan, 1986].

The present investigation seems to indicate that gentamicin administration does not affect the endolymphatic glycocalyx of the hair cells and supporting cells in the organ of Corti. The (endolymphatic) glycocalyx of the marginal cells, however, did demonstrate irregular contrast-staining of a flocculent appearance (Fig. 9; compare with Fig. 5). Disorganization of the glycocalyx lining the stria vascularis has also been observed after ethacrynic acid administration [Forge, 1981].
Evidently, the plasma membranes themselves are affected by gentamicin as well since some marginal cells demonstrated a highly electron-dense cytoplasm [cf., Forge et al., 1987]. As aminoglycosides have been demonstrated to alter membrane structure [Forge and Fradis, 1985; Forge *et al.*, 1989], the above phenomenon might be due to 'leaky' plasma membranes. Moreover, junctional permeability of the stria vascularis seems to be

altered as evidenced by the contrast-staining of the basolateral membranes of the marginal cells (Fig. 10; compare with Fig. 5). The apical membranes of the marginal cells and the junctional complexes (i.e., tight junctions) between the individual marginal cells are part of the blood-strial (or blood-endolymph) barrier, which regulates the electrolyte composition of the endolymph and is involved in endolymph clearance [Jahnke, 1980; Juhn and Rybak, 1981; Duvall and Klinkner, 1983]. Disorganization of this barrier due to gentamicin administration could lead not only to a disturbance of cochlear fluid balance - and cochlear homoeostasis - but could also impair endolymph clearance resulting in a prolonged exposure of the cochlear tissues to the drug. This is supported by the observations that both strial capillary transport and strial membrane-ATPase activity are altered by aminoglycoside administration [Iinuma *et al.*, 1969; Yamane *et al.*, 1983].

In conclusion, our results seem to indicate that the endolymphatic glycocalyx of the hair cells is not affected by gentamicin as opposed to that of the stria vascularis. However, although $OsO_4/K_4Ru(CN)_6$ post-fixation is useful as a general method to visualize the glycocalyx, its use might be limited by its apparent non-selectivity. Therefore it is premature to draw definite conclusions from the presented results, and we consider it essential to investigate further the possible effects of gentamicin on the glycocalyx of the hair cells using other - preferably cationic - probes to visualize the glycocalyx (cf., Chapter 5).

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EARLY EFFECTS OF GENTAMICIN ON COCHLEAR GLYCOCALYX CYTOCHEMISTRY

CHAPTER 5

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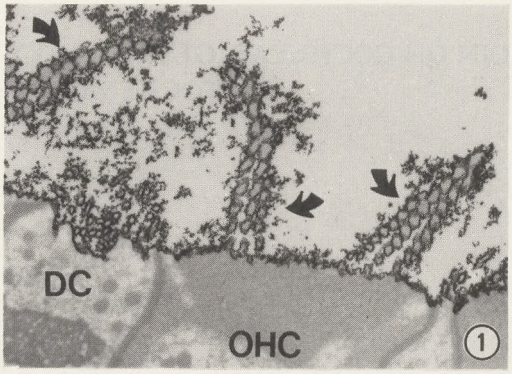
EARLY EFFECTS OF GENTAMICIN ON COCHLEAR GLYCOCALYX CYTOCHEMISTRY

During the past decades various cytochemical studies have shown that a glycocalyx is present on the free luminal surfaces of the hair cells and the accessary epithelia of the cochlea [Spoendlin, 1968; Küttner and Geyer, 1972, 1974; Küttner, 1974, 1977; Saito *et al.*, 1977; Forge, 1981; Slepecky and Chamberlain, 1985; Lim, 1986; cf., the electron micrograph on p. 17 of Geyer, 1977]. Most of these investigators have studied primarily the glycocalyx which is present on the stria vascularis and the endolymphatic surfaces of the organ of Corti and Reissner's membrane, i.e., the endolymphatic glycocalyx. Less attention has been paid to the glycocalyx lining the perilymphatic compartments, i.e., the perilympatic glycocalyx.

Recent studies, however, have demonstrated that the endolymphatic glycocalyx differs from the latter both in ultrastructural appearance and in cytochemical reactivity [De Groot, 1986; Prieto and Merchan, 1986; Santi and Anderson, 1987; Prieto and Merchan, 1987], which may reflect functional differences between the two layers.

Although the function of the glycocalyx in the cochlea has not been established as yet, it could act as a barrier which maintains the microenvironment of the cochlear cells and generates a net negative surface charge of the cells. More particularly, the endolymphatic glycocalyx of the hair cells is thought to play an important role in the mechano-electrical transduction process by sequestering cations [Slepecky and Chamberlain, 1985; Lim, 1986; Santi and Anderson, 1986], and it may be responsible for the maintenance of stereocilia integrity [Flock *et al.*, 1977; Slepecky and Chamberlain, 1986], while the perilymphatic glycocalyx seems to be involved in the absorption of macromolecules [Leake and Snyder, 1987].

Consequently, disturbance of the glycocalyx could eventually lead to serious damage of the hair cells. Disorganization of the endolymphatic glycocalyx, in particular, has been extensively reported to occur under pathological conditions, especially after ethacrynic acid administration [Brummett *et al.*, 1977; Forge, 1981], but also after endolymphatic sac obliteration [Albers *et al.*, 1987]. Although it has been frequently suggested that the glycocalyx is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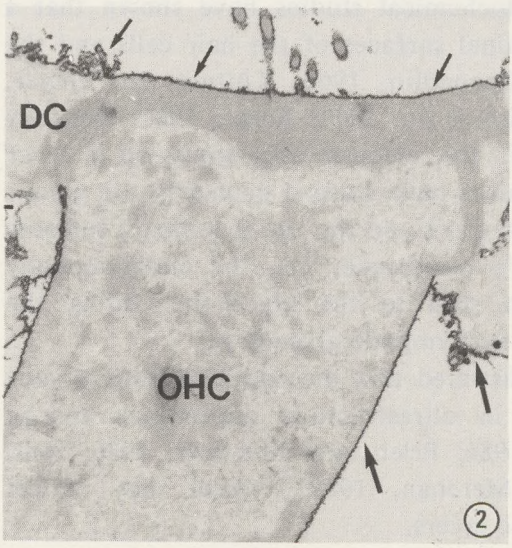
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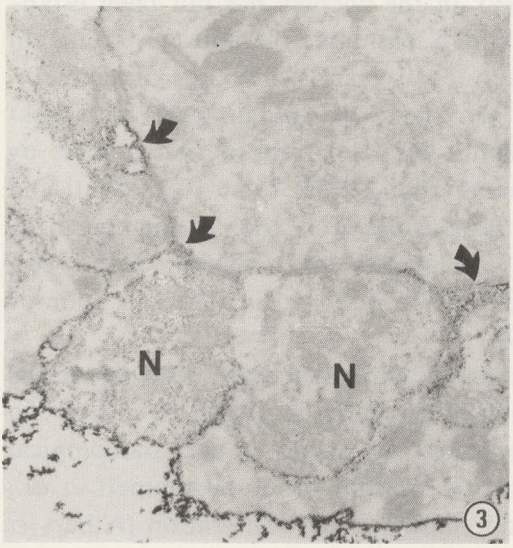
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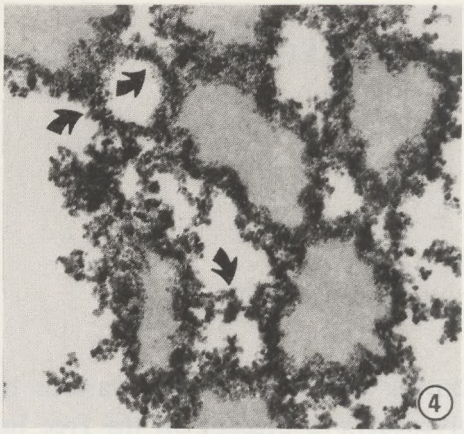
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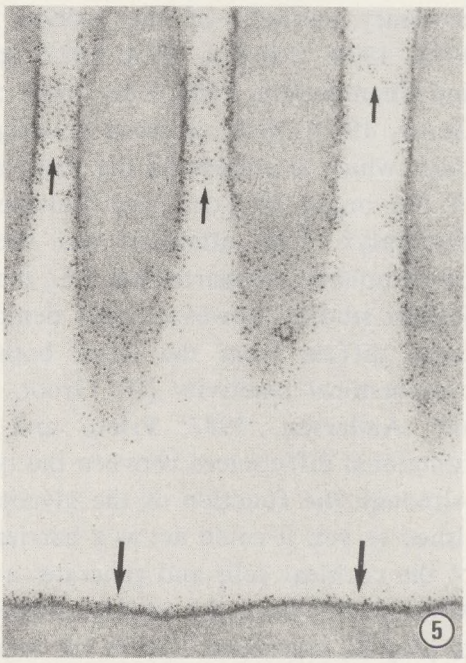
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implicated in aminoglycoside cochleotoxicity, direct experimental evidence is limited. Therefore we have investigated the early effects of gentamicin on both the endolymphatic and the perilymphatic glycocalyx, using the cationic probes colloidal thorium and cationized ferritin.

MATERIALS AND METHODS

Six healthy, female albino guinea pigs (strain GpHi65 *Himalayan*, weighing 250-300 g) were injected intraperitoneally with gentamicin (Gentogram^{R}, 100 mg/kg/day) either for 1 day (n=3) or for 5 days (n=3). Additionally, three animals were not treated with gentamicin and served as controls.

Twenty-four hours after the final injection of the drug, the animals were sacrificed and the cochleas were fixed by intralabyrinthine perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and immersed in the same fixative for 2 h at 4°C. Subsequently, the cochleas were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C followed by microdissection.

Figure 1. Thorium reactivity on the endolymphatic surfaces of the normal organ of Corti. Note the uniform distribution of thorium on the outer hair cells OHC, stereocilia (arrows) and Deiters' cell DC (*x10,000*).

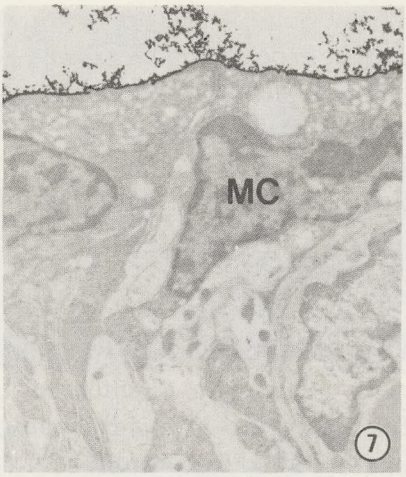
Figure 2. Thorium reactivity on the apical (small arrows) and lateral membranes (large arrows) of an outer hair cell OHC and Deiters' cells DC (*x9,000*).

Figure 3. Presence of thorium reactivity on the basal membranes of an outer hair cell (arrows) and the synaptic membranes N (*x20,000*).

Figure 4. Transverse section through the stereocilia. Note the enhanced sub-structure of the stereociliary cross-links (arrows) after incubation with colloidal thorium (*x67,000*).

Figure 5. Apical part of outer hair cell (large arrows), stereocilia and the cross-links (small arrows) demonstrating CF reactivity (*x67,000*).

Figure 6. Transverse section through the stereocilia demonstrating reactivity of the stereociliary cross-links (arrows) after CF incubation (*x95,000*).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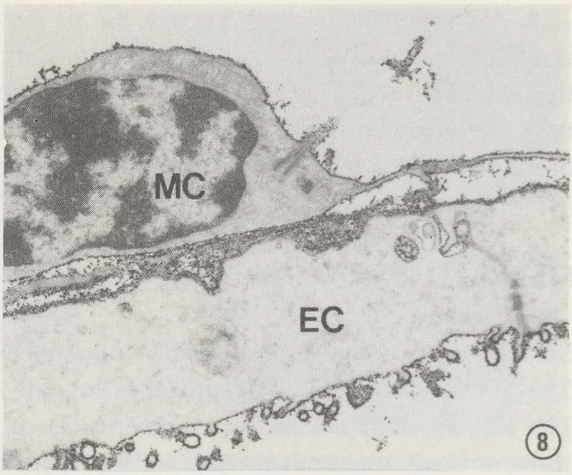
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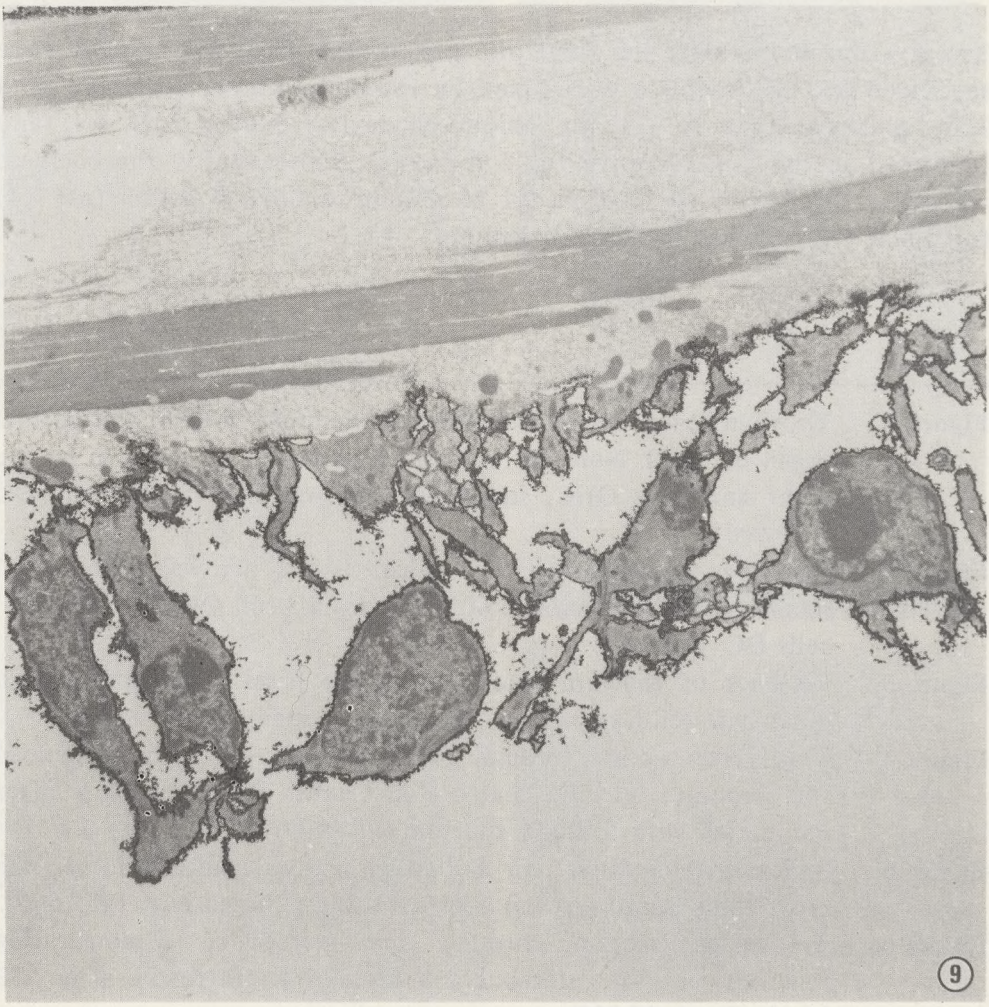
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For cytochemical visualization of the glycocalyx, microdissected cochlear turns were incubated *en bloc* either with 1% colloidal thorium (Thoria-sol; Polysciences, Warrington, USA) in 3% acetic acid (pH 2-3) for 24 h or with 5 mg/ml cationized ferritin in physiological saline (pH 7.4) for 1 h. All incubations were carried out at room temperature.
Dehydration was carried out in a graded ethanol, 2,2-dimethoxypropane, propylene oxide series and the cochleas were embedded in Spurr's low-viscosity resin, containing 1% silicone DC 200 fluid (Polaron Equipment Ltd., Bio-Rad Labs., Watford, UK).
Ultrathin sections were examined without additional heavy metal staining in a Philips EM 201c transmission electron microscope operating at 60 kV.

RESULTS

Normal cochleas
In non-treated animals, both the endolymphatic and perilymphatic glycocalyx reacted heavily with colloidal thorium. They appeared as a continuous, electron-dense layer with a thickness of 25-90 nm, which was present on the free luminal surfaces of all cochlear cells (Figs. 1-4 and 7-9, Table 1). A more delicate reaction was manifest after incubation with cationized ferritin (Figs. 5 and 6). With either probe, no apparent regional differences in reactivity or thickness of the endolymphatic glycocalyx could be observed, neither on one and the same cell nor among the various cell types. In addition, no significant differences in reactivity or thickness were present between apical and basal turns.
With cationized ferritin, no gross differences in reactivity or thickness could be discerned between the endolymphatic and perilymphatic glycocalyx. With colloidal thorium, however, the perilymphatic glycocalyx occasionally

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Figure 7. Stria vascularis demonstrating thorium reactivity on the apical membranes of the marginal cells MC (*x7,000*).

Figure 8. Reissner's membrane demonstrating thorium reactivity on the membranes of both the epithelial cells EC and the mesothelial cells MC (*x10,000*).

Figure 9. Basilar membrane demonstrating thorium reactivity on the membranes of the tympanal cells (*x6,000*).


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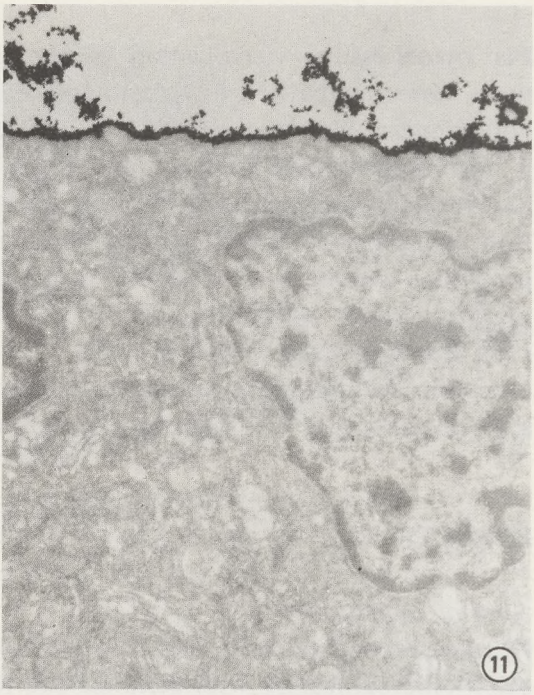
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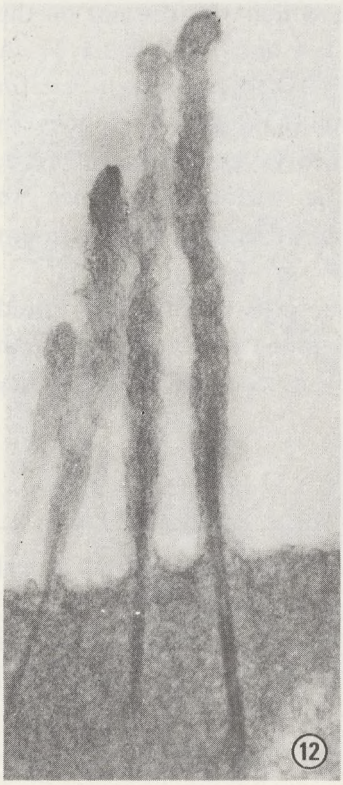
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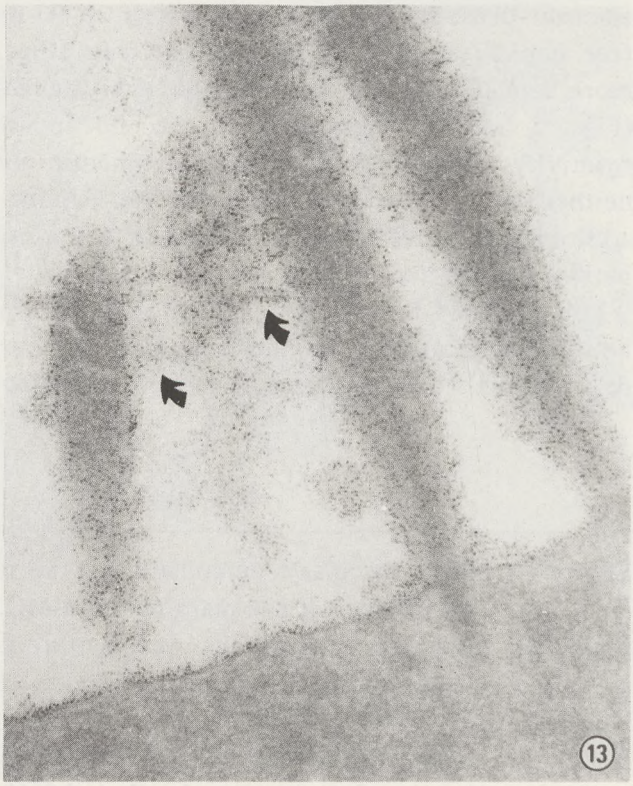
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appeared to be thinner than the endolymphatic, but this could well be due either to poor penetration of the probe into Nuel's space or to oblique-sectioning.

Gentamicin-intoxicated cochleas

After one day of gentamicin administration, the endolymphatic glycocalyx of the hair cells and the supporting cells demonstrated a diminished reactivity with colloidal thorium (Fig. 10, Table 1), whereas reactivity of the perilymphatic glycocalyx of the hair cells, the supporting cells and the tympanal cells of the basilar membrane was completely abolished. The stria vascularis, spiral prominence and Reissner's membrane showed no differences in reactivity as compared to non-treated cochleas (Table 1).

After 5 days thorium reactivity of the endolymphatic glycocalyx of the hair cells and supporting cells was completely abolished, as well as that of the stereocilia and stereociliary cross-links (Fig. 12, Table 1). Again, the stria vascularis, spiral prominence and Reissner's membrane did not show any differences in reactivity as compared to non-treated cochleas (Fig. 11, Table 1).

With cationized ferritin, no differences in reactivity of the endolymphatic and the perilymphatic glycocalyx as well as the stereociliary cross-links were observed, neither after one day nor after 5 days (Fig. 13).

Figure 10. Apical part and stereocilia of outer hair cell showing diminished thorium reactivity after 1 day of gentamicin (*x32,000*).

Figure 11. Thorium reactivity of the stria vascularis remains unaffected after 5 days of gentamicin administration (*x14,000*).

Figure 12. Thorium reactivity of the endolymphatic glycocalyx of the outer hair cell and the stereocilia is completely abolished after 5 days of gentamicin (*x32,000*).

Figure 13. CF reactivity of the stereocilia and stereociliary cross-links (arrows) is not affected after 5 days of gentamicin administration (*x67,000*).

cochlear tissues	- GM	1d GM	5d GM
Outer hair cells:			
* apical membranes	+	#	-
* stereocilia	+	#	-
* basolateral membranes	+	-	-
Inner hair cells:			
* apical membranes	+	#	-
* stereocilia	+	#	-
Deiters' cells:			
* apical membranes	+	#	-
* basolateral membranes	+	-	-
Basilar membrane:			
* tympanal cells	+	#	-
Stria vascularis:			
* marginal cells	+	+	+
Spiral prominence:			
* epithelial lining	+	+	+
Reissner's membrane:			
* endolymphatic surfaces	+	+	+
* perilymphatic surfaces	+	+	+

(+ = positive reaction; # = weak reaction; - = no reaction)

Table 1. Cytochemical visualization of the inner ear glycocalyx with colloidal thorium. Effects of gentamicin (GM) administration.

DISCUSSION

In the present study, colloidal thorium and cationized ferritin (CF) were used as cationic probes to detect negatively charged groups on the cochlear epithelia under normal and pathological conditions.

In normal cochleas the endolymphatic glycocalyx reacted with both probes and appeared to be uniformly distributed. This is in agreement with the studies of Slepecky and Chamberlain [1985], De Groot [1986], Prieto and Merchan [1986] and Takumida [personal communication]. In contrast to our earlier observations [De Groot, 1986] and those of Prieto and Merchan [1986, 1987], no gross differences in reactivity or thickness could be discerned between the endolymphatic and the perilymphatic glycocalyx with either probe. Similar results have been obtained with ruthenium red [Küttner, 1974]. Glutaraldehyde fixation extracts some membrane constituents, such as glycoconjugates, and modifies cell surface charge, resulting in a decrease or increase in reactivity [Schrével *et al.*, 1981]; and this may explain the differences in reactivity as seen with the various visualization methods.

The stereociliary cross-links reacted in a similar way with both probes as did the endolymphatic glycocalyx [cf., Prieto and Merchan, 1986; Csukas *et al.*, 1987]. This could imply that the stereociliary cross-links are biochemically identical to the endolymphatic glycocalyx and that they are to be considered as mere specializations of the latter. This is further supported by our observations that after gentamicin treatment both the stereociliary cross-links and the endolymphatic glycocalyx of the hair cells could not be visualized with colloidal thorium [De Groot and Veldman, this study].

Following gentamicin administration thorium reactivity of the glycocalyx of the hair cells and supporting cells was completely abolished, suggesting that gentamicin induces in some way a loss of membrane glycoconjugates.

In this study, with colloidal thorium, the outer hair cells did not seem to react differently from the inner hair cells, nor were there any differences in reactivity between the basal and apical turns. This may be explained by a rapid uptake and accumulation of the drug in the cochlear tissues and by the fact that all hair cells, regardless of their position along the basilar membrane, receive equal amounts of gentamicin [cf., Tran Ba Huy and Deffrennes, 1988]. This implicates that the distinct degeneration pattern of the organ of Corti, reported in many histological studies, is the net result of regional differences in vulnerability between the outer hair cells.

However, with CF, no alterations in glycocalyx reactivity could be observed after gentamicin intoxication [De Groot and Veldman, this study]. Similar results were obtained in an earlier study [De Groot and Veldman, 1987]

using $OsO_4/K_4Ru(CN)_6$ post-fixation to visualize the glycocalyx after gentamicin administration. This discrepancy may be explained by the fact that these methods are carried out at a different (i.e., physiological) pH and, more importantly, detect other moieties of the glycocalyx.

Incubation with colloidal thorium was performed at pH 2-3. The only groups which are ionized at this pH are the carboxyl groups of sialic acid and hyaluronic acid, and the sulphate groups of sulphated glycoconjugates [Schrével *et al.*, 1981; Seno, 1987]. Preliminary studies seem to indicate that thorium reactivity of the glycocalyx of the hair cells and supporting cells is mainly due to sialic acid residues, as it was entirely eliminated after neuraminidase digestion [De Groot, unpublished results].

How gentamicin induces a loss of glycoconjugates in the hair cells is subject to speculation. The experiments of Saito *et al.* [1977] and Yung [1987] suggest that the aminoglycosides interact with the plasma membrane by binding to its anionic groups. However, although this may be a first step in the cochleotoxic mechanism, it most certainly does not mean that gentamicin directly affects the glycocalyx.

Immunoperoxidase and immunofluorescence studies have demonstrated that gentamicin is accumulated in the hair cells [Veldman *et al.*, 1985; Hayashida *et al.*, 1985]. Moreover, recent biochemical evidence suggests that gentamicin binding takes place at the plasma membrane and an intracellular pool [Williams *et al.*, 1987], and that the uptake of gentamicin is an active, energy-dependent process [Schacht and Van De Water, 1986].

From our experiments it is likely that the effects of gentamicin on the glycocalyx are not due to masking of anionic groups, as non-internalized gentamicin molecules should be cleared from the cochlear fluids during the 24-h period following the final injection. Anyhow, it is clear that disorganization of the glycocalyx is an early event in gentamicin intoxication as already within 24 h the first effects could be detected.

Once inside the cell, gentamicin could interfere with the biosynthesis (e.g., glycosylation) of membrane glycoconjugates and/or with their insertion into the plasma membrane either directly or through an interaction with polyphosphoinositides [Schacht, 1986]. Aberrant glycosylation of these membrane glycoconjugates, possibly in combination with conformational changes within the plasma membrane, may impair the function of the glycocalyx and cause derangement of selective membrane permeability (e.g., ATPase activity, specific ion channels, hexose transport). This could eventually result in severe cell volume changes and cell shape modifications caused by alterations of the cytoskeleton [cf., Trump *et al.*, 1983].

Although no stereocilia fusion could be detected in our preparations, in a

later stage of intoxication complete loss of membrane glycoconjugates could very well account for the deterioration of the stereociliary cross-links and subsequent stereocilia fusion [Flock *et al.*, 1977; Pickles *et al.*, 1987].

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UPTAKE AND INTRALYSOSOMAL ACCUMULATION OF GENTAMICIN IN THE COCHLEA. AN IMMUNOELECTRON MICROSCOPICAL STUDY

CHAPTER 6

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UPTAKE AND INTRALYSOSOMAL ACCUMULATION OF GENTAMICIN IN THE COCHLEA. AN IMMUNOELECTRON MICROSCOPICAL STUDY

Since the introduction of aminoglycosides it has been known that chronic exposure to these antibiotics results in degeneration of the cochlear hair cells. However, the exact cellular mechanism underlying aminoglycoside intoxication of hair cells is still subject to conjecture [for reviews see Lim, 1986; Rybak, 1986; Schacht, 1986; Griffin, 1988].

In recent years, it has been demonstrated that aminoglycosides interact with the plasma membrane of the hair cells and reversibly block the ion-selective channels in the apical membranes involved in mechano-electrical transduction [Sokabe *et al.*, 1982; Hudspeth and Kroese, 1983; Tachibana *et al.*, 1984; Kroese *et al.*, 1989]. However, it is not conceivable that blockage of the transduction channels in itself could provoke hair cell degeneration.

Moreover, there is considerable evidence that aminoglycosides are accumulated within the hair cells. Pharmacokinetical studies as well as *in vitro* binding experiments indicate that aminoglycoside binding takes place at the plasma membrane, and at an intracellular pool [Schacht and Van De Water, 1986; Williams *et al.*, 1987; Tran Ba Huy and Deffrennes, 1988]. This is supported by autoradiographical and immunocytochemical studies investigating the intracochlear distribution of aminoglycosides [Von Ilberg *et al.*, 1971; Portmann *et al.*, 1974; Hayashida *et al.*, 1985; Veldman *et al.*, 1985, 1987; Yamane *et al.*, 1988; Hayashida, 1989].

In view of these findings, Lim [1986] postulated that the cellular mechanisms involved in aminoglycoside cochleotoxicity include two events. The initial event is the reversible blockage of the transduction channels, whereas a secondary event comprises internalization of the drug followed by hair cell degeneration, presumably by disturbance of cellular metabolism.

In order to determine the intracellular target of the aminoglycosides, Von Ilberg *et al.* [1971] and Portmann *et al.* [1974] investigated the intracochlear distribution of tritiated dihydrostreptomycin. They found radioactivity dispersed throughout the cytoplasm of the hair cells which they assumed to be due to specific binding of the drug to the ribosomes. Similar results were

obtained with other aminoglycosides using immunofluorescence techniques [Hayashida *et al.*, 1985; Yamane *et al.*, 1988; Hayashida, 1989].
In contrast, Veldman *et al.* [1985, 1987], using an immunoperoxidase technique, observed that specific labelling for gentamicin is present as finely granular deposits which are restricted to the infracuticular region of the outer hair cells. The fact that lysosomes and multivesicular bodies predominate in this region strongly suggests that aminoglycosides are accumulated within the lysosomal compartment of the hair cells [cf., Lim, 1986]. To test the validity of this hypothesis, we have studied the ultrastructural distribution of gentamicin in the cochlea by means of immunoelectron microscopy.

MATERIAL AND METHODS

Immunoreagents
Polyclonal rabbit antiserum to gentamicin was prepared as described previously by Veldman *et al.* [1987], with the following modification: conjugation of gentamicin sulphate (Schering Corp., Kenilworth, USA) to ovalbumin (grade V; Sigma Chemical Comp., St. Louis, USA) was preceded by diazotization of the drug with nitrous acid.
For immunolabelling of resin-embedded tissues the neat, unpurified antiserum was used since insufficient signal was obtained after incubation with either purified IgG fractions or affinity-purified antibodies [De Groot, unpublished results].
Protein A-gold (9 nm) conjugates were prepared according to Slot and Geuze [1984]. Protein A was obtained from Pharmacia (Uppsala, Sweden).

Gentamicin administration
Healthy, female albino guinea pigs (strain GpHi65 *Himalayan*, weighing 250-300 g) received gentamicin (Gentogram^{R}, 100 mg/kg) by daily intraperitoneal injections either for 1 day (n=3), 5 days (n=3), 10 days (n=3) or 15 days (n=3). The animals were sacrificed 24 h after the final injection of the drug. Additionally, three animals were not treated with gentamicin and served as controls.
The animals were anaesthetized by intraperitoneal injection of sodium pentobarbitone (Nembutal^{R}, 60 mg/kg). After decapitation, the cochleas and the kidneys were removed. The kidneys served as positive controls.

Fixation and tissue preparation

Cochleas were immediately fixed by intralabyrinthine perfusion with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and immersed in the same fixative at 4°C for 2 h. Subsequently, cochleas were rinsed in 0.1 M sodium cacodylate buffer, pH 7.4 (2x15 min), decalcified in 10% EDTA.2Na (pH 7.4) under constant agitation in a decalcifying device (LTI, Bilthoven, The Netherlands) for 4-5 days and rinsed in the same buffer (2x15 min), all at room temperature.

Small pieces of renal cortex were fixed by immersion in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 2 h followed by two rinses in 0.1 M sodium cacodylate buffer (pH 7.4), 15 min each.

Post-fixation with OsO_4 was omitted, because of its known deleterious effects on antigenicity.

Embedding and sectioning

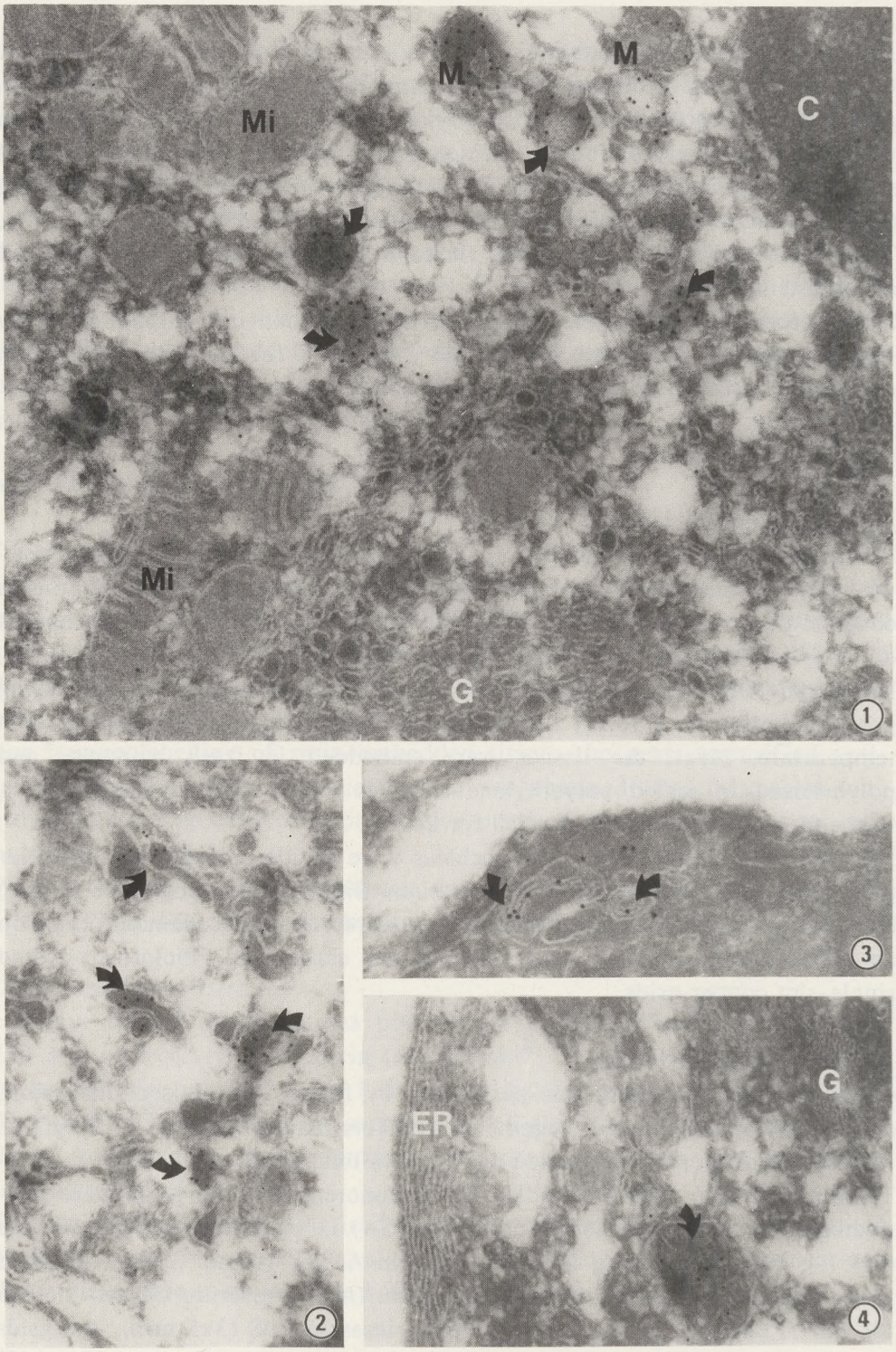
Infiltration with and embedding in LR *White* resin were as described elsewhere [Newman and Jasani, 1984; Timms, 1986]. The cochleas and the pieces of renal cortex were partially dehydrated at room temperature in 2 changes of 70% ethanol, 45 min each. Tissues were then infiltrated with pure, hard-grade LR *White* resin for 1 h followed by an overnight change (using gentle agitation on a rotary device) and a final change for 1 h, all at room temperature. Next, the tissues were embedded in fresh monomer and polymerized in sealed polyethylene capsules (Veco Verkoopmaatschappij, Eerbeek, The Netherlands) at 55°C for 24 h.

For purposes of orientation, the cochleas were divided along a midmodiolar plane and re-embedded. Ultrathin sections (light-gold interference colour) of cochlear quarter turns and renal cortex were cut with a diamond knife on an LKB Ultrotome *Nova* and collected on carbonized, Pioloform-coated single-slot copper grids.

Immunogold cytochemistry

On-grid immunolabelling was performed by floating grids, sections down, on droplets of the immunoreagents and buffers placed on strips of Parafilm. All incubation steps were carried out at room temperature.

To minimize non-specific binding of immunoreagents, the grids were pretreated with 5% bovine serum albumin (BSA) in phosphate-buffered saline (pH 7.4; PBS), containing 10 μg/ml protein A, for 10 min. Next, the grids were incubated with the primary antiserum (neat antiserum diluted 1:60 in 1% BSA/PBS) for 30 min. After several rinses in PBS (3x5 min), the grids were incubated with protein A-gold (9 nm) conjugate (diluted 1:20 in 1%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BSA/PBS) for 30 min followed by successive washes in PBS (4x5 min) and distilled water (2x5 min). Controls in which the primary antiserum was omitted were routinely included in all experiments.
The sections were contrast-stained with a mixture of methyl cellulose and uranyl acetate [Griffiths *et al.*, 1984], air-dried, and examined in either a Philips EM 201c or a JEOL 1200CX transmission electron microscope operating at 80 kV.

RESULTS

Specific labelling for gentamicin in the cochlea was observed after 5-15 days of gentamicin administration; none was detected 24 h after a single dose.
Labelling in the cochlea was restricted to the organ of Corti; other cochlear tissues such as the stria vascularis, Reissner's membrane and the spiral ganglion did not demonstrate any labelling. In addition, no obvious differences in immunoreactivity between the basal and apical turns were detected.
In the outer hair cells labelling was found in granules resembling multivesicular bodies and lysosomes (Fig. 1) situated particularly in the infracuticular region, but also in small vesicles and tubules in the infra- and

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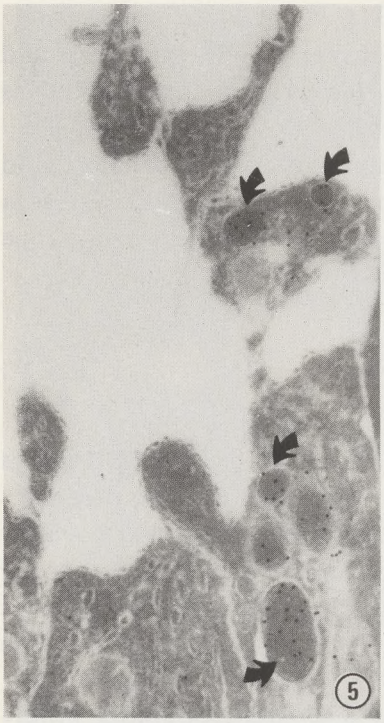
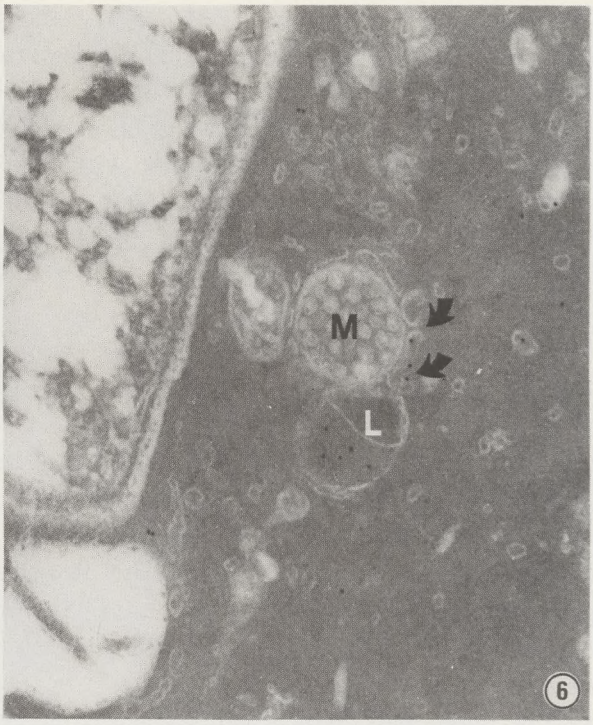
Figure 1. In the outer hair cell (5 days of gentamicin) specific labelling for gentamicin is present on granules resembling lysosomes (arrows) and multivesicular bodies M located strictly in the infracuticular region. The cuticular plate C, mitochondria Mi and Golgi complex G are free of labelling (*x55,000*).

Figure 2. Labelling in outer hair cells is also present in small vesicles and tubules (arrows) which are dispersed throughout the cytoplasm (*x42,000*).

Figure 3. Inner hair cell, 15 days of gentamicin administration. Labelling is present on membrane profiles (arrows) which may represent cisternae of the endoplasmic reticulum (*x63,000*).

Figure 4. Outer hair cell, 15 days of gentamicin administration. Whereas the multivesicular body (arrow) is specifically labelled, the endoplasmic reticulum ER and Golgi complex G are devoid of labelling (*x42,000*).

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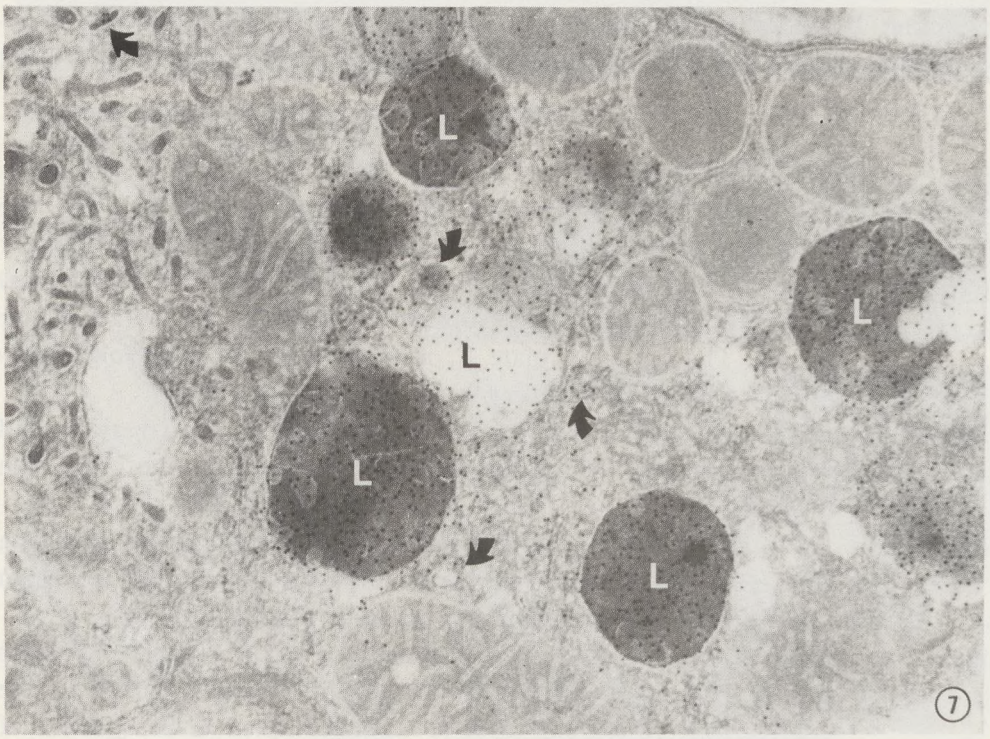
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supranuclear regions (Fig. 2). The inner hair cells demonstrated less gold-labelled granules. Occasionally, gold particles could be seen on profiles of the endoplasmic reticulum (Fig. 3). No specific labelling was present on the mitochondria, the nucleus and the Golgi complex (Figs. 1 and 4).
The Deiters' cells, Hensen's cells and the tympanal cells of the basilar membrane also demonstrated labelling. This was confined to small vesicles, multivesicular bodies and lysosomes (Figs. 5 and 6).
In the renal cortex, labelling was restricted to the proximal tubule cells and could be observed already within 24 h. Substantial labelling was found in the lysosomes, although gold particles were also present on endocytotic vesicles and tubules (Fig. 7). Glomeruli and distal tubule cells were devoid of gold particles. A similar distribution pattern was seen after 5-15 days.
Control sections in which the primary antiserum had been omitted as well as sections of tissues taken from non-treated animals demonstrated no specific labelling.

DISCUSSION

The immunoelectron microscopical data presented in this paper show that gentamicin is accumulated within lysosomes and multivesicular bodies in the hair cells. These results are in close agreement with earlier light microscopical observations [Veldman *et al.*, 1985, 1987] and, moreover, substantiate the hypothesis that aminoglycosides are accumulated within the lysosomal compartment [cf., Lim, 1986]. An analogous finding is that, in the kidney, aminoglycosides are accumulated within the lysosomes of the proximal tubule cells [Just *et al.*, 1977; Kaloyanides and Pastoriza-Munoz, 1980;

Figure 5. In the tympanal cells of the basilar membrane (5 days of gentamicin) labelling (arrows) is demonstrated by lysosome-like granules (*x42,000*).

Figure 6. Deiters' cell (5 days of gentamicin) demonstrating labelling which is present on a lysosome L and in small vesicles (arrows), but is absent on the adjacent multivesicular body (*x 42,000*).

Figure 7. Kidney, proximal tubule cell. Within 24 hours labelling is seen in endocytotic vesicles and tubules (arrows) and lysosomes L (*x42,000*).

Silverblatt, 1982; De Groot *et al.*, this study].

In theory, the presence of gentamicin within the lysosomes may be accounted for by either: (1) permeation of the drug from the cytosol into the lysosomes, (2) autophagocytosis, or (3) endocytosis.

The labelling for gentamicin, present on small vesicles and tubules, suggests the involvement of an endocytotic pathway in the uptake of the drug. This is consistent with the finding that aminoglycoside uptake is an active, energy-dependent process [Takada *et al.*, 1985; Schacht and Van De Water, 1986; Williams *et al.*, 1987]. Moreover, autoradiographical studies demonstrate that the mechanism of renal uptake of aminoglycosides is endocytosis rather than a form of permeation [Just *et al.*, 1977; Kaloyanides and Pastoriza-Munoz, 1980; Silverblatt, 1982]. General evidence for an endocytotic uptake of macromolecules is provided by tracer studies using horseradish peroxidase - a well-recognized marker of fluid-phase endocytosis. Perilymphatically applied horseradish peroxidase is internalized by coated vesicles in the infranuclear region of the hair cells and subsequently transferred to multi-vesicular bodies and lysosomes in the infracuticular region [Siegel and Brownell, 1986; Leake and Snyder, 1987]. Von Ilberg *et al.* [1971] and Portmann *et al.* [1974] found tritiated dihydrostreptomycin dispersed throughout the cytoplasm of the hair cells. They assumed this to be due to specific binding of the drug to ribosomes. In view of our findings, however, it is more likely that this labelling is due to the presence of the drug within endocytotic vesicles.

Whether accumulation of aminoglycosides within the lysosomes is directly responsible for the toxic effects exerted onto the hair cells is still a matter of discussion. Aminoglycosides may act upon the lysosomes by altering their contents, resulting in a lysosomal phospholipidosis and impairment of the normal lysosomal degradation process [cf., Kaloyanides, 1980; Morrin and Fillastre, 1982]. Alternatively, intralysosomal accumulation of amino-glycosides may promote labilization of lysosomes - e.g, by modifying membrane structure [Forge *et al.*, 1989] - with subsequent release of their contents into the cytosol provoking cell degeneration [cf., Lim, 1986].

However, intralysosomal accumulation of the drug can not account for the early ultrastructural changes observed in hair cells during aminoglycoside intoxication. Previously, De Groot and Veldman [1988] observed dis-organization of the hair cell glycocalyx already within 24 hours after a single dose of gentamicin, which they assumed to be due to an aberrant synthesis (e.g., glycosylation) of membrane glycoconjugates. In this study, the endoplasmic reticulum only rarely demonstrated specific labelling for gentamicin, whereas the Golgi complex was always devoid of labelling.

Therefore we suppose the drug to be present in amounts too low to be detected by our method, but high enough to interfere with (glyco)protein synthesis or otherwise induce functional impairment of the endoplasmic reticulum and/or the Golgi complex [cf., Lim, 1986].
In the inner hair cells substantially less labelling was observed than in the outer hair cells. This coincides with lower numbers of multivesicular bodies and lysosomes observed in the inner hair cells during aminoglycoside intoxication. This observation might be explained by the fact that the outer hair cells, in contrast to the inner hair cells, are directly exposed to cortilymph (i.e., Nuel's space) which is, together with perilymph, considered to be the main route of access for the aminoglycosides to the organ of Corti [Tammela and Tjälve, 1986; Tran Ba Huy *et al.*, 1986; Yamane *et al.*, 1988].
In Fig. 8 (see p. 104) we propose a model for the uptake and intracellular trafficking of gentamicin in the (outer) hair cells based upon the data obtained in this study.

Interestingly, labelling for gentamicin was not restricted to the hair cells, but is also present in the accessary epithelia of the organ of Corti such as the tympanal cells of the basilar membrane, the Deiters' cells and the Hensen's cells [cf., Hayashida *et al.*, 1985; Yamane *et al.*, 1988]. This is further evidence that internalization - and subsequent accumulation - of aminoglycosides is not a specific feature of the hair cells, the more so as these drugs are internalized unreservedly by a great variety of cells such as the proximal tubule cells in the kidney [Just *et al.*, 1977; Kaloyanides and Pastoriza-Munoz, 1980; Silverblatt, 1982], macrophages [Bonventre and Imhoff, 1970; Yamane *et al.*, 1987] and fibroblasts [Aubert-Tulkens *et al.*, 1979].
In contrast to the organ of Corti, labelling for gentamicin was not present in Reissner's membrane, neither in the epithelial nor in the mesothelial cells. This is the more remarkable as the epithelial cells of Reissner's membrane are involved in the transport of macromolecules from the perilymph to the endolymph - and *vice versa* [for a review see Duvall and Klinkner, 1983]. This can be explained by assuming transcytosis of the drug, without intralysosomal accumulation, comparable to the tranport mechanism in endothelial cells. Furthermore, gentamicin is not accumulated within the stria vascularis, but it has been demonstrated that aminoglycosides have a detrimental effect on strial glycocalyx cytochemistry [Forge, 1981; De Groot *et al.*, 1988] and interfere with strial membrane-ATPase activity [Iinuma *et al.*, 1967].
Unlike Hayashida [1989], we could not detect any differences in immuno-

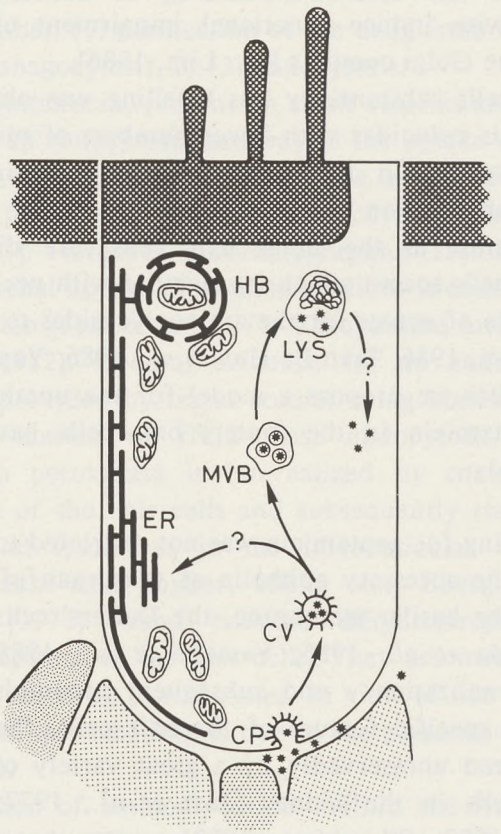

Figure 8. Hypothetical model for the uptake and intracellular trafficking of gentamicin in the (outer) hair cell.
Gentamicin (spiked spheres) binds to the negatively charged groups of the perilymphatic glycocalyx. Internalization of the drug is initiated at coated pits CP at the basal membranes. The internalized drug is transferred to multivesicular bodies MVB by coated vesicles CV and finally accumulates within the lysosomes LYS. Subsequent degeneration of the outer hair cell may be the consequence of gentamicin leaking from the lysosomes into the cytosol. In addition, gentamicin is delivered to the Golgi complex and the endoplasmic reticulum ER where it interferes with protein synthesis, and induces formation of Hensen's bodies HB.

reactivity between the basal and apical turns. Similarly, De Groot and Veldman [1988] could not find any differences between the basal and apical turns with regard to disorganization of the glycocalyx during gentamicin intoxication. Our findings are in line with a study of Tran Ba Huy and Deffrennes [1988] who determined tissue drug concentrations and concluded that the typical basal-apical progression of cochleotoxic degeneration can not be accounted for by a preferential uptake of the drug by the basal turn.

In conclusion, the present study clearly demonstrates that aminoglycosides are accumulated within the lysosomal compartment of the hair cells and accessary epithelia of the organ of Corti. It strongly suggests that the drug is internalized by endocytosis and affects the endoplasmic reticulum as a primary target. Further study, however, is required to confirm this hypothesis. Therefore we have initiated studies to investigate the intracellular disposition of gentamicin using more sophisticated methods such as immunolabelling of ultrathin cryosections.

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SUMMARY AND CONCLUSIONS

The exact mechanism of aminoglycoside action on cochlear hair cells is still obscure. In particular the subcellular events taking place in the early stages of intoxication lack proper ultrastructural support. In this thesis we have focused on these aspects of gentamicin intoxication in order to determine the intracellular target(s) of the drug.

In *Chapter 2* the influence of different fixation methods and of various primary fixatives on the ultrastructural preservation of guinea pig cochlear tissues was investigated. Tri-aldehyde primary fixation resulted, in contrast to other formulae investigated, in an excellent, uniform preservation of all cochlear tissues without obvious fixation artefacts. Using tri-aldehyde primary fixation, no differences in fixation quality were observed between cochleas fixed by intravascular perfusion and cochleas fixed by intra-labyrinthine perfusion.
The influence of $OsO_4/K_4Ru(CN)_6$ and $OsO_4/K_4Fe(CN)_6$ post-fixation was also tested. Cochlear tissues post-fixed with $OsO_4/K_4Ru(CN)_6$ or $OsO_4/K_4Fe(CN)_6$ exhibited more cellular detail (i.e., membrane and glycogen contrast) as compared to tissues post-fixed with OsO_4 alone. Tri-aldehyde primary fixation followed by $OsO_4/K_4Ru(CN)_6$ or $OsO_4/K_4Fe(CN)_6$ post-fixation therefore is recommended as a routine procedure for optimal preservation of cochlear tissues.

In *Chapter 3* the ultrastructural changes in the hair cells during the early stages of gentamicin intoxication were investigated after tri-aldehyde primary fixation and $OsO_4/K_4Ru(CN)_6$ post-fixation. There was no preference for the degeneration of any individual outer hair cell (OHC_1 *versus* OHC_2 *versus* OHC_3), nor for the basal turns as compared to the upper turns. Degeneration of the inner hair cells was not observed which confirms that outer hair cells are more vulnerable to the drug than inner hair cells.
Ultrastructurally, the earliest identifiable changes were an increase in lysosomes, proliferation of the endoplasmic reticulum, and formation of Hensen's bodies. This was followed by dilatation of the endoplasmic reticulum and the nuclear envelope, giving rise to extensive cytoplasmic vacuolation. These results demonstrate that gentamicin-induced changes in the hair cells primarily involve the cell's synthetic apparatus. All further intracellular changes occur at a much later stage and therefore are to be considered secondary events.

In *Chapter 4* the contrast-enhancing properties of $K_4Ru(CN)_6$ and related compounds were investigated. It was observed that addition of either $K_4Ru(CN)_6$, $K_4Fe(CN)_6$ or $K_3Fe(CN)_6$ to aqueous OsO_4 solutions not only imparts a high electron density to glycogen and membranes, but also selectively enhances contrast of the glycocalyx. In addition, contrast-enhancement is dependent on the fixative used.
Contrast-staining was limited to the glycocalyx directly exposed to the scala media, i.e., the endolymphatic glycocalyx. Contrast of the glycocalyx lining the membranes facing Nuel's space and the scalae vestibuli and tympani (i.e., the perilymphatic glycocalyx) was not significantly enhanced. The effect of gentamicin on the glycocalyx was also studied. After 5 days of gentamicin administration the glycocalyx of the hair cells and supporting cells was not affected. The glycocalyx lining the stria vascularis, however, demonstrated signs of disorganization. This might reflect a functional impairment of the blood-endolymph barrier which, in itself, could result in a prolonged exposure of the cochlear tissues to the drug.

In *Chapter 5* the early effects of gentamicin administration on the glycocalyx in the cochlea were further investigated using the cationic probes colloidal thorium and cationized ferritin. Gentamicin administration resulted in a diminished thorium reactivity of both the endolymphatic and perilymphatic glycocalyx of the hair cells after 1 day, and complete abolishment after 5 days. Cationized ferritin reactivity of the endolymphatic and perilymphatic glycocalyx was not significantly influenced. It is suggested that gentamicin induces a loss of glycoconjugates in the hair cells, presumably by interfering with their synthesis.

In *Chapter 6* the ultrastructural distribution of gentamicin in the cochlea was investigated immunocytochemically. Labelling in the cochlea was restricted to the organ of Corti, in particular to the outer and inner hair cells, the Deiters' cells, Hensen's cells and the tympanal cells of the basilar membrane. Other cochlear tissues did not demonstrate any labelling.
At the cellular level, gentamicin was found in multivesicular bodies, lysosomes and small vesicles. A model is proposed in which it is hypothesized that gentamicin is internalized by coated vesicles and is transferred to the lysosomal compartment as well as to the endoplasmic reticulum and Golgi complex.

SAMENVATTING

Gentamicine is een breed-spectrum antibioticum, dat in de klinische praktijk veelvuldig toegepast wordt. Echter, de klinische toepasbaarheid wordt enigermate beperkt door het optreden van neveneffecten zoals bacteriële resistentie en orgaan-specifieke toxiciteit.
De toxische werking van gentamicine blijft beperkt tot de nieren, het evenwichtsorgaan en het gehoororgaan (of: cochlea). De cochleaire aandoening wordt gekenmerkt door een gedeeltelijke of een complete uitval van het gehoor, welke permanent van aard is. Histopathologische onderzoekingen hebben uitgewezen, dat dit gehoorsverlies is te wijten aan beschadiging van de zintuigcellen (of: haarcellen) in de cochlea. Het precieze mechanisme van de toxische werking van gentamicine op de cochleaire haarcellen is nog steeds onbegrepen. In dit proefschrift zijn de subcellulaire veranderingen in de haarcellen na chronische toediening van gentamicine onderzocht, teneinde het intracellulaire aangrijpingspunt vast te stellen.

Voor elektronenmicroscopisch onderzoek van weefsels is een optimale weefselpreservatie noodzakelijk, met name om pathologische veranderingen betrouwbaar te kunnen beoordelen. Een dergelijke optimale preservatie van van de cochlea is tot nu toe een belangrijk technisch probleem gebleken.
In *Hoofdstuk 2* werd de invloed van zowel primaire fixatie als postfixatie onderzocht. Primaire fixatie van cochlea's vond plaats door intralabyrinthaire- of intravasculaire perfusie met diverse aldehydencombinaties.
In tegenstelling tot de gebruikelijke fixatieven bleek primaire fixatie met een tri-aldehyden fixatief te resulteren in een optimale en uniforme preservatie van alle epithelia in de cochlea; zowel na intralabyrinthaire- als na intravasculaire perfusie.
Tevens werd de invloed van postfixatie met behulp van $OsO_4/K_4Ru(CN)_6$ en $OsO_4/K_4Fe(CN)_6$ onderzocht. Postfixatie met deze mengsels resulteerde in een aanzienlijk hoger weefselcontrast dan met de gebruikelijke OsO_4 postfixatie. Door deze gecombineerde fixatiemethode wordt het aantal artefacten gereduceerd tot een minimum, terwijl een contrast-rijk preparaat verkregen wordt voor transmissie elektronenmicroscopie.

In *Hoofdstuk 3* werden de ultrastructurele veranderingen, die optreden in de haarcellen tijdens een vroeg stadium van gentamicine-intoxicatie, bestudeerd. Degeneratieve veranderingen werden slechts waargenomen in de buitenste haarcellen; de binnenste haarcellen vertoonden een normale morfologie. In tegenstelling tot de literatuur konden in dit stadium geen

onderlinge verschillen worden waargenomen tussen de buitenste haarcellen (OHC_1, OHC_2 en OHC_3), noch tussen de basale, middelste en apicale windingen.
Vroege degeneratieve veranderingen in de buitenste haarcellen bleken te zijn een verhoging van het aantal secundaire lysosomen, een proliferatie van het endoplasmatisch reticulum en de vorming van zogenaamde 'Hensen's bodies'. Vervolgens trad dilatatie van het endoplasmatisch reticulum en de kernenveloppe op, hetgeen resulteerde in een ernstige mate van vacuolisatie van het cytoplasma. Afwijkingen aan mitochondriën en stereociliën, zoals eerder beschreven in de literatuur, treden pas in een later stadium op en zijn dientengevolge secundair aan voornoemde veranderingen.

In *Hoofdstuk 4* werd het contrast-verhogend vermogen van $K_4Ru(CN)_6$ en aanverwante verbindingen - zoals $K_4Fe(CN)_6$ en $K_3Fe(CN)_6$ - onderzocht. Het bleek dat toevoeging van dergelijke verbindingen aan waterige OsO_4 oplossingen resulteert in een verhoogd contrast van membranen, glycogeen en de glycocalyx. In de cochlea werd een selectieve aankleuring van de glycocalyx, die grenst aan de scala media waargenomen. De glycocalyx, die het perilymfatische compartiment omgeeft, bleek niet aantoonbaar met deze methode.
Na behandeling met gentamicine bleek de glycocalyx aanwezig op de haarcellen en steuncellen van het orgaan van Corti niet aangedaan, terwijl de glycocalyx van de stria vascularis wel veranderingen vertoonde. Aangezien de glycocalyx van de stria vascularis onderdeel uitmaakt van de bloed-endolymfe barrière, kan deze structurele verandering een verminderde klaring van gentamicine tot gevolg hebben waardoor de haarcellen langer blootgesteld worden aan het gentamicine.

In *Hoofdstuk 5* werd het effect van gentamicine op de glycocalyx uitgebreider onderzocht met behulp van de verbindingen colloïdaal thorium en gekationiseerd ferritine. Reeds na 1 dag was de aankleuring van de glycocalyx van de haarcellen met colloïdaal thorium beduidend minder vergeleken met de controles. Na 5 dagen werd geen reactiviteit van de glycocalyx meer waargenomen. De glycocalyx van de stria vascularis en het membraan van Reissner waren niet aangedaan. Met behulp van gekationiseerd ferritine werden geen verschillen waargenomen. Het is waarschijnlijk dat gentamicine een verlies van glycoconjugaten veroorzaakt, mogelijk door een storing van de intracellulaire aanmaak van deze verbindingen.

In *Hoofdstuk 6* werd de subcellulaire verdeling van gentamicine in de

cochlea onderzocht met behulp van de proteïne A-goud methode. Gentamicine bleek uitsluitend aanwezig te zijn in de cellen van het orgaan van Corti, met name de haarcellen, de Deiterse cellen, de cellen van Hensen en de tympanale bekleding van het basilaire membraan. De overige epithelia en cellen in de cochlea vertoonden geen reactiviteit. Op subcellulair niveau werd gentamicine aangetroffen in zogenaamde 'multivesicular bodies', lysosomen en in endocytose-blaasjes. Een hypothetisch model gebaseerd op de resultaten verkregen in dit proefschrift wordt beschreven. In dit model wordt gesteld, dat gentamicine actief door de cel wordt opgenomen middels endocytose en dat het gestapeld wordt in de lysosomen. Tegelijkertijd wordt het getransporteerd naar het endoplasmatisch reticulum en het Golgi-complex waar het de eiwit-synthese beïnvloedt en mogelijk de vorming van 'Hensen's bodies' induceert.

PUBLICATIONS RELATED TO THIS THESIS

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CURRICULUM VITAE

John de Groot werd geboren op 25 december 1956 te Utrecht. Na het behalen van het H.A.V.O. diploma aan de Sint Gregorius Scholengemeenschap te Utrecht, werd in september 1974 aangevangen met de studie Biologie (M.O. dag-opleiding) aan de Rijksuniversiteit te Utrecht.
De M.O. akte werd behaald op 29 juni 1981. Op dezelfde dag werden tevens afgelegd het kandidaatsexamen B_1 en het doctoraalexamen Biologie, met als hoofdvak Scheikundige Dierfysiologie en als bijvakken Algemene Plantkunde en Moleculaire Genetica.

Gedurende de periode van maart 1981 tot en met november 1981 was hij werkzaam op de afdeling Elektronenmicroscopie (hoofd: dr. L.H.P.M. Rademakers) van het Pathologisch Instituut der Rijksuniversiteit te Utrecht.
Van december 1981 tot en met mei 1982 was hij onbezoldigd werkzaam op het Laboratorium voor Histofysiologie en Experimentele Pathologie (hoofd: dr. J.E. Veldman) van de vakgroep Keel-, Neus- en Oorheelkunde (hoofd: prof. dr. E.H. Huizing).
Sinds juni 1982 is hij werkzaam op hetzelfde laboratorium als klinisch-wetenschappelijk medewerker; van juni 1982 tot en met december 1988 met een 50%-aanstelling - gedurende de periode maart 1985 tot en met december 1988 financieel aangevuld vanuit de Stichting O.R.L.U. - en sinds januari 1989 met een volledige aanstelling.
In deze hoedanigheid verricht hij onderzoek naar de cochleotoxiciteit van farmaca en is hij verantwoordelijk voor de ontwikkeling van elektronenmicroscopische technieken ten behoeve van het binnenoor-onderzoek alsmede de begeleiding van het celbiologisch onderzoek verricht door promovendi en arts-assistenten.
Van augustus 1984 tot en met februari 1986 was hij bibliothecaris *ad interim* van de vakgroep Keel-, Neus- en Oorheelkunde. Sinds maart 1986 vertegenwoordigt hij de vakgroep binnen de gebruikerscommissie van de Medische Bibliotheek Utrecht; sinds oktober 1986 is hij tevens lid van de facultaire bibliotheekcommissie van de Medische Faculteit Utrecht.

Naast zijn werk als celbioloog gaat zijn belangstelling vooral uit naar het Westeuropese beeldverhaal, de grafische kunst van Moebius, de muziek van Philip Glass en de krijgsgeschiedkunde van de Tweede Wereldoorlog.

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