Microtubule Assembly in the Cochlea of the Mouse.

by

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Declaration for the Degree of Master of Science.

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ABSTRACT.

Pillar cells of the organ of Corti of the mouse were examined after fixation and embedding for transmission electron microscopy. These specialised epithelial cells have transcellular microtubule arrays which are parallel to the longitudinal axes of the cells.

Array microtubules have diameters of about 28 nm and are probably composed of 15 protofilaments. These microtubules have, in inner pillar cells, nucleating sites which are associated with the lateral plasmalemma. Microtubules of the inner pillar cell array start to elongate during the second day post-partum. Assembly of microtubules of outer pillar cells is temporally distinct. This assembly does not appear to start until about the third day post-partum.

Array assembly in inner pillar cells occurs at the same stages *in vitro* as *in vivo* but with a reduction in the overall number of microtubules present. These have the same diameter as those which elongate *in vivo* and also exhibit similar end-on anchorage to the lateral plasmalemma.

After culturing the organ of Corti *in vitro*, the microtubule array is similar in structure to that which assembles *in situ*: the arrays have a tubular configuration. This is produced by the annular configuration of lateral plasmalemma-associated microtubule nucleating sites. This contrasts with the array configuration in outer pillar cells. Nucleation sites in these cells are situated in a small apical turret-like process which produces the rod-like configuration of the microtubule array.

In both inner and outer pillar cells, microtubules which have small diameters of about 21 nm, are associated with the centrosome. These microtubules are randomly oriented at the apical region of each cell.

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Introduction.

General Introductory Remarks.

Many supporting cells of the organ of Corti contain large transcellular arrays of microtubules. The largest microtubule arrays present in any mammalian cell are contained within the pillar cells of the organ of Corti. However, there is no indication of when these incredibly large bundles of microtubules begin to assemble.

This thesis therefore investigates when assembly of each of these massive transcellular arrays within pillar cells of the mouse organ of Corti commences and when assembly is complete. Once assembly is complete, the number of microtubules present within the array is analysed. Also investigated are the sites of nucleation of array microtubules and the number of protofilaments which constitute each microtubule.

Organs of Corti can be cultured *in vitro* without any alteration to the overall morphology. Hence, the effects of the culture procedure on the assembly of the microtubule array are investigated.

Hearing Research and the Mouse Cochlea.

The study of the development of human hearing poses severe problems for cell biologists. Specimens are only likely to be obtained from elderly persons or aborted fetuses. These specimens are liable to be pathologically altered, or, to have developmental abnormalities respectively. In addition to these problems, the ossification of the petrous temporal bone around the human cochlea, makes dissection, without major damage, almost impossible. As a result, it is desirable to make use of animal models to observe the developmental process in genetically normal individuals. Hence, in this study, genetically normal mice were utilised.

However, why use the mouse as a non-human mammalian model? Mice have 2 major advantages over many mammals :

i) they are born at a relatively immature stage and much cochlear development takes place post-natally,

ii) there are a number of mouse mutant strains with genetically induced hearing abnormalities which have been discovered and maintained.

The development of the mouse cochlea, at birth, is equivalent to the human fetus of 15 weeks gestation (Kikuchi and Hilding, 1965). It is not until 2 weeks after birth that the mouse cochlea is fully developed but gross development in mice is almost complete by birth (Kikuchi and Hilding, 1965). Part of the maturation process is the development of fluid filled spaces (the spaces of Nuel) between different cell types. The tunnel of Corti begins to open basally, between the inner and outer pillar cells, about 6 days post partum (Walsh and McGee, 1986) and is complete by the tenth day. Hearing does not occur unless the pillar cells part to form the tunnel of Corti (Larsell *et al.*, 1944).

In certain mouse mutants with hearing abnormalities, development appears normal at birth but degeneration occurs thereafter. In the deafness mutant, there is a delay in the formation of the tunnel of Corti and the fluid filled Nuel spaces (Bock and Steel, 1983; Steel and Bock, 1985). Delay also occurs in the formation of the Nuel spaces in the mutant Shaker-1 (Kikuchi and Hilding, 1965) while in the Ames waltzer, the Nuel spaces fail to open (Osako and Hilding, 1971). In the mutant W/Wv mouse, the organ of Corti eventually collapses: this is attributed to loss of supporting cell function (Schrott and Spoendlin, 1987).

Histology of the Organ of Corti.

The organ of Corti is the portion of sensory epithelium of the cochlea where sensory transduction concerned with hearing occurs. It is composed of sensory cells surrounded by a complex network of supporting cells (Engstrom and Ades, 1973). These supporting cells are divided into two main types:

i) pillar cells,

ii) Deiters' cells.

The pillar cells provide the main support within the organ of Corti (Iurato, 1967). In mice, as in other mammals, the pillar cells of the organ of Corti contain bundles (arrays) of microtubules. The arrays of these specialised cells lie perpendicular to the apical surface of the organ of Corti and run in the longitudinal axis of each cell.

The pillar cells act as part of the supporting structure for the hair cells (the sensory cells) of the organ (Fig. 1). Pillar cells are important in the development of hearing; a tunnel develops between the two cells. The failure of this structure to develop is reported to prevent auditory sensation (Larsell et al., 1944).

Both pillar cells are attached to the basilar membrane; their basal portions contain a number of microtubules and the nucleus is located basally (Engstrom and Ades, (1973). Inner and outer pillar cells are closely apposed to each other in the mouse at birth: there is no tunnel of Corti (Sobkowicz *et al.*, 1984). Microtubules are absent, in the pillar cells of the rat at birth, but they gradually increase in number during development until 2000-2500 per cell are present in the young animal (Iurato, 1967). Similarly, in the young guinea pig, around 2400 microtubules are present in the pillar cells (Angelborg and Engstrom, 1972). The microtubules are oriented so that their direction corresponds to the lines of stress imparted upon the cell (Iurato, 1967). The microtubules, in association with microfilaments increase the rigidity of pillar

Figure 1.

Schematic diagram showing the histology of the mature organ of Corti.

Hair cells (H) are supported by Deiters' cells (D). Inner (IP) and outer (OP) pillar cells are situated on either side of the tunnel of Corti (T). Nuclei are labelled N.

Not to scale.

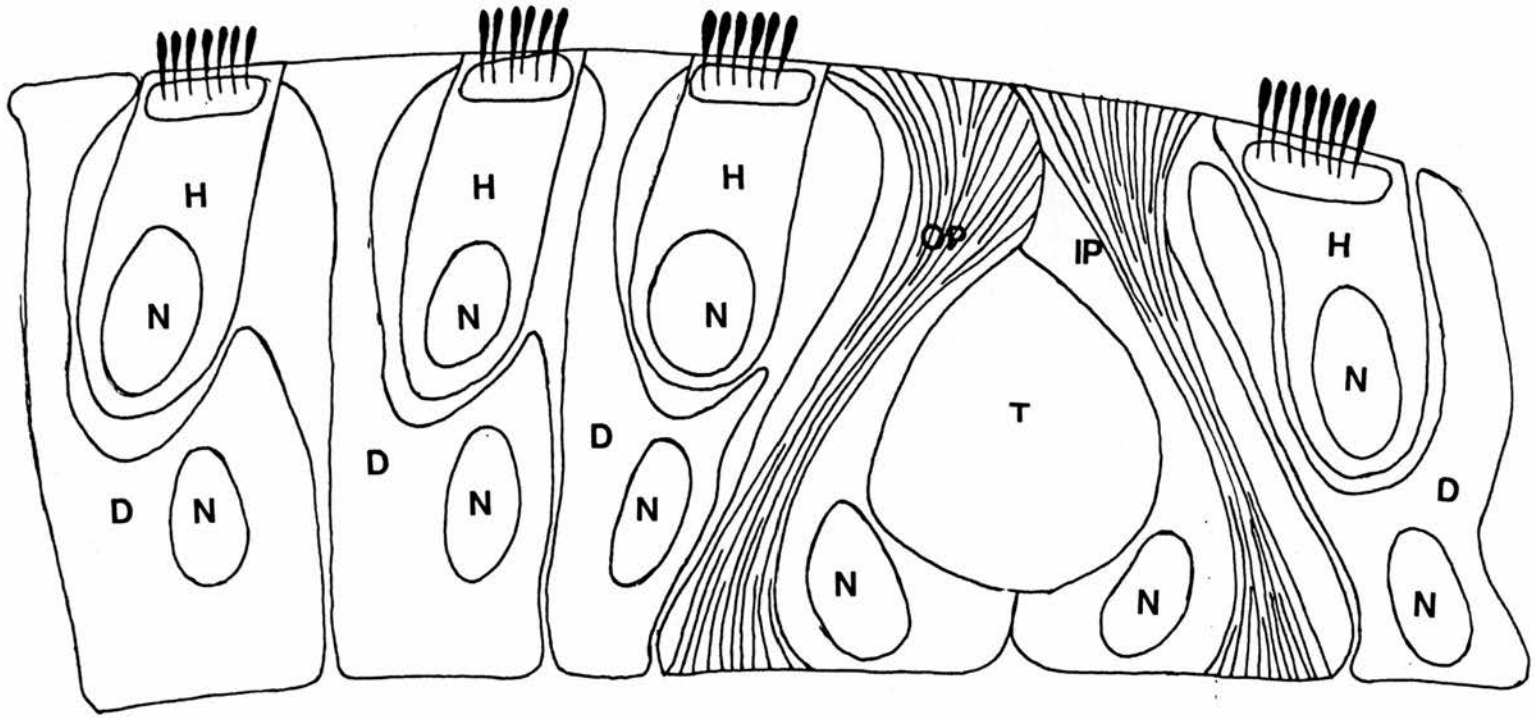


Fig. 1

cells (Iurato, 1967). This rigidity of pillar cells is further enhanced by the intimate way in which the supporting cells interdigitate with one another.

In each pillar cell, these large arrays of microtubules have a precise configuration to increase the rigidity of each cell. Within the chinchilla organ of Corti, the inner pillar cell contains a transcellular microtubule array which appears cylindrical in form (Slepecky and Chamberlain, 1983). However, in the outer pillar of the same animal, the microtubule array forms a solid rod-like structure (Slepecky and Chamberlain, 1983).

Deiters' cells provide a direct supportive role for hair cells of the organ of Corti. Their cell bodies lie basally in the organ with their phalangeal processes interdigitating between the hair cells. Each outer hair cell 'sits' in a concavity produced by the phalangeal processes of a number of Deiters' cells. The Deiters' cell phalangeal processes are reported to contain microtubule bundles. They were not however studied in this investigation.

Hair cell cuticular plates which are composed mainly of actin (Furness *et al.*, 1989 - in press) and surrounded by a ring of microtubules act to increase the mechanical strength of the hair cells (Furness and Hackney, 1988; Hackney and Furness, 1989). These hair cell structures, in conjunction with tight junctions which bind pillar cells, Deiters' cells and hair cells together (Beagley, 1965; Bagger-Sjoback *et al.*, 1988) and microtubules of the supporting cells aid the rigidity of the organ of Corti..

Functions of Pillar Cells.

Pillar cells are part of the supporting structure of the organ of Corti (Engstrom and Ades, 1973). In addition, the tops of pillar cells are attached to both inner and outer hair cells by substantial cell junctions (Beagley, 1965; Bagger-Sjoback *et al.*, 1988). Hair cells do not contact the basilar membrane; they are associated with it *via* the pillar and Deiters' cells (Fig. 1). It has been

argued that pillar cells may help to increase the rigidity of the basilar membrane through actin/microtubule interactions (Slepecky and Chamberlain, 1983). Slepecky and Chamberlain (1983) suggested that this may result in alterations to the displacement of hair cell stereocilia against the tectorial membrane in response to modified vibrations of the basilar membrane. Such interactions may be promoted by acoustic stimulation, thereby modulating the excitation of hair cells. Alternatively, pillar cells may, through cross-linkage of microtubules and association with actin, become 'relaxed', thus also modulating the excitation of hair cells in response to acoustic stimulation.

Microtubules and their Microtubule Organising Centres.

Microtubules are dynamic structures which have great functional diversity. They play a role in such diverse activities as the development and maintainance of cell shape (Tucker, 1979), increasing the rigidity of organ of Corti pillar cells (Iurato, 1967), intracellular transport within axons (Allen *et al.*, 1985) and spindle elongation and movement of chromosomes during cell division (Inoue and Sato, 1967).

Microtubules are capable of being rapidly assembled and disassembled. They are usually nucleated at centrosomes in most animal cell types. They can be spatially arranged and later rearranged to accomodate a change in the structure of the cell which contains them (Brinkley, 1985). Microtubules can have a precise spatial position in certain arrays which is often determined by their nucleating sites (Tucker, 1984).

Most metazoan tissue cells possess one main microtubule organising centre (MTOC) - the juxtanuclear centrosomal MTOC. This centrosomal MTOC consists of the centriole and its surrounding dense pericentriolar material

(PCM). Centrioles and PCM are two functionally distinct components of the centrosome (Gould and Borisy, 1977). The PCM firmly anchors the microtubules and these radiate from this dense material through the surrounding cytoplasm.

When microtubules assemble they require a nucleating site on which to initiate the polymerisation process. Importantly, microtubules are nucleated by the PCM but not by the centriole (Gould and Borisy, 1977). Microtubules are incapable of spontaneous self-assembly in living cells (Gould and Borisy, 1977).

Unlike the centriole (Calarco-Gillam *et al.*, 1983), pericentriolar material has no distinct morphology. PCM can exist, disperse and reorganise during the cell cycle independently of the centriole within mouse embryos (Calarco-Gillam *et al.*, 1983). Also, some cells can convert centralised, focussed, microtubule organising centres into dispersed forms (Brinkley, 1985).

All microtubules have an inherent polarity - this is a consequence of the polarised arrangement of their tubulin dimers. During microtubule elongation, subunit addition occurs from one end only: the end distal to the MTOC (McIntosh, 1983; Tucker, 1984). Most microtubules have one end contained within the pericentriolar material - not attached to the centriole itself. This suggests that it is the PCM which includes the nucleating elements. McIntosh (1983) also suggests that the centrosome is capable of specifying the number of microtubules which can elongate from it i.e. there is a specific and saturable number of sites.

Nucleating elements have been described as a cap-like structure which prevents addition of tubulin dimers at the 'minus' end of microtubules (Tucker, 1984). Therefore, an MTOC defines not only the site of nucleation but also microtubule polarity.

Microtubule polymerisation occurs in 2 phases. Initiation followed rapidly by elongation. This is rapid elongation is due to the energetics of the system as previously described. As soon as a microtubule is nucleated, at an MTOC, it will tend to elongate (McIntosh, 1983).

Two possible control mechanisms for microtubule assembly have been suggested by Osborn and Weber (1976). The first is a positive control mechanism which regulates microtubule assembly in a directed manner and the organising structure is where the microtubules originate from. The second mechanism is that of negative control. This forbids microtubule assembly outwith this pathway, and prevents unwanted, unoriented microtubule assembly. Thus, it suggests that there may be a limited number of microtubule nucleation sites which can become saturated. Once this occurs, no further assembly of microtubules can occur.

Throughout the cell division cycle, the centrosome contributes to the reorganisation of the cytoskeleton (McIntosh, 1983). However, there is increasing evidence to suggest that microtubule nucleation, in some metazoan cell types, can occur at sites not associated with centriole-containing centrosomes. This has been reported for such diverse cell types as differentiating human myoblasts (Tassin *et al.*, 1985), differentiating rat odontoblasts (Sasano and Kagayama, 1987), Madin-Darby canine kidney cells (Bre *et al.*, 1987) and *Drosophila* wing epidermal cells (Mogensen and Tucker, 1987).

During the differentiation of such cells, alterations in the deployments of their microtubule nucleating sites apparently occur. For example, in the rat odontoblast, during differentiation, the arrangement of microtubules gradually becomes poorly related to the pericentriolar area (Sasano and Kagayama, 1987). However, in *Drosophila* wing epidermal cells, a switch occurs from centriole-associated nucleating sites to plasma membrane-associated sites.

This occurs when these cells are no longer undergoing cell division (Tucker *et al.*, 1986). Also, importantly, the microtubules of these transcellular arrays assemble from the apex to the basal region of the cell only. Thus, within *Drosophila* wing epidermal cells there are only apical nucleation sites (Mogensen and Tucker, 1987; Mogensen *et al.*, 1989).

Kinetics and Stability of Microtubules.

Assembly of microtubules begins at nucleating sites situated at the MTOC (Brinkley, 1985). Subsequent elongation of microtubules mainly occurs by the addition of tubulin to the free end of each microtubule (the plus end). Under certain conditions microtubules exhibit dynamic instability. Disassembly of microtubules occurs through the loss of tubulin subunits from the plus end of each microtubule. Under conditions of dynamic instability, individual microtubules are either slowly elongating or rapidly shrinking. They seem to alternate between these two states in a random fashion (Kirschner, 1990).

Two types of dynamic instability have been proposed. The first, catastrophic instability occurs when microtubules begin disassembly and progresses until complete disassembly of the microtubules has occurred. The second is tempered instability; each microtubule rapidly switches from the assembling state to the disassembling state and vice versa. However, in this state complete disassembly rarely occurs (Sammack and Borisy, 1988).

The mechanism of tempered instability can efficiently accelerate the pace of microtubule turnover without rearranging an entire microtubule array. This model of tempered instability may be, as Sammack and Borisy (1988) have suggested, an important one for understanding control of cell motility and cell morphogenesis.

When dynamic instability is operating, newly formed microtubules can only persist if both ends are protected from depolymerisation (Alberts *et al.*,

1989). Capping can occur at an MTOC and free ends to produce stable microtubules (Schulze and Kirschner, 1986; Kirschner, 1990). Minus ends are protected from depolymerisation by MTOCs: nucleation sites can be considered as permanently stabilised GTP-bound ends (Kirschner and Mitchison, 1986). Plus ends of microtubules may be captured by specific cell membrane-associated proteins which control the stability of these ends (Kirschner, 1990).

There is evidence that during tubulin polymerisation, GTP-bound tubulin dimer binds to the microtubule. GTP hydrolysis occurs only after dimer polymerisation. Thus, GTP-bound dimers form a cap at the plus end of each microtubule. GTP-bound dimers do not dissociate as readily as GDP-bound dimers: if the GTP-bound tubulin cap is lost, rapid dissociation of GDP-bound dimers will occur and rapid shortening of a microtubule results (Kirschner, 1990).

When depolymerisation commences, GDP-bound dimers are continuously exposed at the plus end of each microtubule (Mitchison and Kirschner, 1984b). Each microtubule will rapidly shorten and may disassemble completely leaving an unoccupied nucleation site (Mitchison and Kirschner, 1984a). This is termed catastrophic instability.

Microtubules which have formed in the correct orientation and location can become stabilised by modification of the tubulin dimers. Acetylation and detyrosination of tubulin provides sites for the binding of microtubule-associated proteins which stabilise the microtubule against disassembly (Kirschner, 1990).

Why are Pillar Cells Interesting?

Large numbers of pillar cells are present within mammalian organs of Corti. In the human organ there are approximately 3500 inner hair cells

(Kimura, 1975). Since inner hair cells are in a one-to-one relationship with pillar cells, there will be approximately 3500 of each type of pillar cell within the human organ of Corti. In mice the organ of Corti is smaller. Therefore, probably between one thousand and two thousand of each of the two types of pillar cells are present.

Pillar cells in some mammals have massive transcellular arrays composed of up to 2500 microtubules (Iurato, 1967; Angelborg and Engstrom, 1972). These cells have a "head inclusion body" apically and cement-like material basally which form fixtures for the transcellular array (Engstrom and Ades, 1973). Nothing is known about the role of centrosomes in relation to the assembly of microtubule arrays within these cells. How could centrosomes nucleate the assembly of microtubule arrays that are attached to the cell surface at both ends? Generation of an aster-like array such as that which is nucleated by centrosomes in most animal cells (Mitchison and Kirschner, 1984a) could not be directly appropriate without substantial remodelling. Perhaps they are similar to non-centriole containing plasma membrane-associated nucleating sites such as those employed to initiate assembly of transcellular microtubule bundles in *Drosophila* wing epidermal cells (Mogensen and Tucker, 1987).

Pillar cell microtubule arrays within the mouse organ of Corti have not previously been described. Thus, there is no description of when these massive transcellular microtubule arrays begin and end their development.

Importantly, the incomplete assembly of the transcellular microtubule arrays causes the failure of the formation of the tunnel of Corti and Nuel spaces (Gabrion *et al.*, 1984). The failure of the formation of these structures is believed to result in hearing abnormalities (Kikuchi and Hilding, 1965; Osako and Hilding, 1971; Bock and Steel, 1983; Steel *et al.*, 1983; Steel and Bock, 1985). Hence, an understanding of microtubular pillar formation may aid in the

understanding of some of the mechanisms involved in certain hearing abnormalities.

Microtubule Diameters.

Microtubules which are nucleated by the centrosome have diameters of about 24 nm (Tucker *et al.*, 1986). However, microtubules with other diameters have been reported. For example, microtubules with diameters of about 30 nm have been reported in the transcellular arrays of *Drosophila* wing epidermal cells (Mogensen and Tucker, 1987) and the pillar cell transcellular arrays in the guinea pig (Saito and Hama, 1982). Pillar cell microtubules, in the organ of Corti of the rat, have diameters of 28 nm (Engstrom and Ades, 1973). Interestingly, nucleation of such large diameter in Drosophila microtubules takes place at non-centrosomal sites (Mogensen and Tucker, 1987).

Microtubule Protofilament Numbers.

Microtubules are composed of dimeric tubulin subunits which are aligned linearly along the microtubule (Tilney *et al.*, 1975) to form protofilaments. The grooves between the protofilaments can be stained with tannic acid. When this procedure is carried out, the subunits remain unstained i.e. it is a form of negative staining. This produces the appearance of electron-lucent globules surrounded by electron dense haloes. However, to visualise the protofilaments, the microtubules must be sectioned perpendicular to their longitudinal axes. If they are not sectioned in this plane, any slight tilt will obscure protofilament number.

The number of protofilaments within microtubules is highly variable. In *Paramecium*, the micronuclear mitotic spindle at early stages of elongation contains microtubules composed of 13 protofilaments with diameters of about 24 nm. Later, the spindle is largely composed of microtubules with 15-16

protofilaments and which have diameters of between 27 and 32 nm (Tucker *et al.*, 1985). In the nematode *Caenorhabditis elegans*, most cells have microtubules which are composed of 11 protofilaments but touch receptors have microtubules with 15 protofilaments (Chalfie and Thomson, 1982).

In pillar cells of the guinea pig organ of Corti, microtubules are composed of 15 protofilaments. These have diameters of about 33nm (Saito and Hama, 1982). However, guinea pig hair cell microtubules have diameters of 26 nm (Furness *et al.*, 1989 - in press) and these workers suggest that these microtubules are composed of 13 protofilaments (Steyger *et al.*, 1989).

In epidermal cells of *Blattella* (Nagano and Suzuki, 1975), in wing epidermal cells of *Drosophila* (Mogensen and Tucker, 1987) and thoracic epidermal muscle attachment cells of *Drosophila* (Paton, 1988) microtubules of transcellular arrays are composed of 15 protofilaments. The diameters of all these microtubules are 30 nm.

Virtually nothing is known about the molecular basis for the differences in protofilament number. However, interestingly, in touch receptor neurons of *Caenorhabditis elegans* (Savage *et al.*, 1989), the *mec-7* gene codes for a ß-tubulin (which is almost identical to vertebrate ß-tubulin). This tubulin results in 15 protofilament microtubules; individuals with a mutation in this gene produce a tubulin which results in microtubules composed of 11 protofilaments.

Actin Filaments in the Mammalian Organ of Corti.

Actin filaments have been described in numerous cell types of the cochleae of various mammals (Flock *et al.*, 1982; Slepecky and Chamberlain, 1983; Thornell and Anniko, 1987, Furness *et al.*, 1989 - in press). In the human cochlea, actin is present in the outer hair cells (Thornell and Anniko, 1987) and, it has been suggested, that this is present as part of the contractile

system which becomes active after electrical stimulation. This contractility, after acoustic stimulation, may increase the rigidity of the outer hair cells and, therefore, make the cells less susceptible to further stimulation. Actin filaments are also present within the supporting cells of the organ of Corti of the chinchilla (Slepecky and Chamberlain, 1983) guinea pig pillar cells (Angelborg and Engstrom, 1972) and rat pillar cells (Engstrom and Ades, 1973).

A similar contractile response has been described in bats (Henson *et al.*, 1985). Anchoring cells between the spiral ligament and the otic capsule have parallel arrays of intracellular filaments. These arrays contain actin, myosin and tropomyosin. It is suggested that these cells create and maintain tension on the spiral ligament-basilar membrane complex thereby altering the mechanical properties of the basilar membrane.

Actin filaments are also present within 'pillar-like' transcellular microtubule arrays (Mogensen and Tucker, 1988). These authors describe 6 nm actin filaments which are situated in dense intertubular material in the transalar microtubule arrays of *Drosophila* wing epidermal cells. The actin filaments, by decoration with myosin sub-fragment S-1, are suggested to all have the same polarity within each cell i.e. they were apparently nucleated apically near the plasmalemma-associated microtubule nucleation sites. However, actin filaments in the chinchilla organ of Corti have opposite polarities (Slepecky and Chamberlain, 1983).

The actin/microtubule complex of *Drosophila* wing epidermis does not seem to have an active contractile role (Mogensen and Tucker, 1988). It has been suggested (Johnson and Milner, 1987) that this actin/microtubule complex is present to act as a rigid support. This maintains basal cell extensions and hence, allows the maintenance of the haemocoelic spaces during the final stages of wing morphogenesis.

In Vitro Culture of the Organ of Corti.

Sobkowicz *et al.* (1975), have devised a culture procedure for explants of cochlear tissue from newborn mice. After culturing *in vitro*, the differentiation of outer hair cells was found to be similar to that of the intact animal. But, the cells retained some undifferentiated features such as kinocilia and their associated basal bodies (Sobkowicz *et al.*, 1975).

Russell and Richardson (1987), with neonate mouse organ of Corti explants maintained for up to 5 days *in vitro*, have reported that hair cells retain their morphological integrity during the culture period. However, they also report the retention of certain embryological features such as kinocilia along with their associated basal bodies and additional microvilli on the apical surface of the cells.

Summary of the Aims of this Investigation.

One of the main aims of this investigation has been to ascertain when assembly of the massive transcellular arrays of microtubules of pillar cells of the mouse organ of Corti begins. All previous work on pillar cell microtubule arrays has focussed solely on the mature transcellular array.

Equally important, and a central aspect of this work, have been the questions of where the microtubule organising centre is situated within each of the two pillar cells, where the assembly starts from and the direction of elongation of the microtubules. An indication of the direction of elongation will provide evidence as to where the MTOC is situated within each pillar cell. Is it situated apically, basally or in both of these regions?

Within each of the two types of pillar cells in the chinchilla organ of Corti, the transcellular microtubule arrays have different configurations (Slepecky and Chamberlain, 1983). Are there similar configurations of

microtubule bundles present in mouse pillar cells and is this configuration related to the configuration of the MTOC in each cell type?

The microtubules which constitute the transcellular arrays of rat pillar cells are reported to have diameters of 28 nm (Engstrom and Ades, 1973) and in guinea pig pillar cells, array microtubules are composed of 15 protofilaments (Saito and Hama, 1982). Part of this investigation has been to determine the diameter of microtubules of the assembling microtubule array and to find how many protofilaments these microtubules contain. Do these array microtubules have diameters of 28 nm? If so, are they composed of 15 protofilaments?

Actin filaments are reported in supporting cells of the chinchilla organ of Corti (Slepecky and Chamberlain, 1983). Are actin filaments associated with array microtubules present within pillar cells of the mouse organ of Corti?

Russell and Richardson (1987) have cultured, for up to 5 days, neonate mouse organ of Corti for neurophysiological studies. This procedure, they state, does not alter the overall morphology of the tissue. An experimental procedure described in this investigation has been the culturing of mouse organ of Corti using Russell and Richardson's technique and assessing if assembly of the microtubule array occurs *in vitro*. If assembly does occur, then how normal is this assembly compared to the *in vivo* material? Russell and Richardson (1987) also report that hair cells, after culturing, retain some embryological features such as kinocilia. Are similar embryological features retained in cultured pillar cells?

Materials and Methods.

i) Isolation of the Organ of Corti.

The cochleae of new born mice (Swiss CD1) were removed in dissecting medium (Hanks Balanced Salts Solution (HBSS) (Gibco) and Hepes Buffered Salts (Gibco) (50 ml HBSS : 0.5 ml Hepes). The animals were previously killed by decapitation; the cochleae were obtained by cutting the head in the sagittal plane. After the brain was removed, the cartilaginous capsule of the cochlea was carefully dissected from the cranium and placed in fresh dissecting medium.

The cartilaginous capsule surrounding the cochlea was gently removed to reveal the $2^1/_2$- $2^3/_4$ turns of the cochlea. The organ of Corti was dissected from Reissners membrane and the stria vascularis using electrolytically sharpened tungsten needles.

The organs of Corti were isolated and further divided into apical and basal coils.

The criterion used for aging newborn mice was based on the day on which they were born; this was designated as Day 0.

ii) Organ Culture.

The tissue was cultured in Maximow depression slide assemblies using a slight modification (Russell and Richardson, 1987) of the method originally described by Bornstein (1973) and later used by Sobkowicz *et al.* (1975) for the growth of mouse cochlear tissue. Mouse pups were killed by cervical dislocation and surface sterilised for 10 minutes in 80% ethanol. The heads were removed and dissected as previously described (see section i) in Hepes Buffered Salts and HBSS. The tissue was further dissected into apical and basal coils. Tissue pieces were then explanted onto rat-tail collagen coated 25mm diameter round Thermanox Tissue Culture Discs (Miles Scientific). The tissue was oriented so that the apical surfaces of the cells of the organ of Corti were facing upwards. Before being sealed in Maximow slide assemblies and

cultured in the lying drop position for a maximum of 3 days at 37^0C, they were 'fed' with $1^1/_2$ drops of complete medium.

Complete medium consisted of 8 parts of Eagle's minimum essential medium with Earle's salts, 1 part heat inactivated horse serum and 1 part mouse embryo extract. This was additionally buffered with 10mM Hepes Buffered Salts pH 7.2. Mouse embryo extract was prepared as described by Russell and Richardson (1987).

Rat tail collagen was solubilised as described by Bornstein (1958) with the omission of the dialysis step. Hydrated collagen gels were prepared on Thermanox Tissue Culture Discs by exposing a spread drop of acid soluble collagen to ammonia vapours for 15 minutes. Polymerised gels were washed with water and stored in HBSS containing 0.1% horse serum until use.

Before fixation of cultured material, the specimens were washed in rinsing buffer (phosphate buffer, 0.05M, pH 7.6; 2% sucrose) briefly to remove any traces of the serum from the culture medium. Fixation and embedding was carried out as described in section v).

iii) Light Microscopy.

Bright field and Nomarski differential interference contrast microscopy were carried out using a Zeiss Universal Microscope. A form of Nomarski D.I.C. optics were used to identify the positions of hair cells and pillar cells after embedding of the organs of Corti in the resin blocks; this was employed for orientation purposes.

Thick (1 μm) sections of the organ of Corti were stained using methylene blue (1 %) dissolved in aqueous borax (1%) solution.

iv) Visualisation of Microtubule Protofilaments.

The organs of Corti of 6 day old mouse pups were dissected as described previously (i).

The tissue was permeabilised with a detergent-containing extraction solution for 10 minutes at 37^0C with 0.125% or 0.25% Triton X-165 in 0.5M PME (0.5M Pipes, pH 6.9; 0.1mM EDTA; 0.1mM $MgCl_2$), 0.0625% deoxycholate and 0.025% sodium dodecylsulphate. The organ of Corti was then fixed in 2.5% glutaraldehyde in 0.1M PME (0.1M Pipes, pH 6.9, 1mM EDTA, 1mM $MgCl_2$) containing 2% tannic acid for 30 minutes.

After en block staining with 1% aqueous uranyl acetate overnight at 40^0C in the dark, the material was embedded in the water soluble melamine resin Nanoplast (BioRad, Polaron Equipment Ltd, Watford) (MME 7002 and B52 mixed in the ratio of 50:1) at 40^0C for 48 hours (in a desicator) and left to harden at 60^0C for 48 hours.

This protocol was a modification of one devised by Dr Mette Mogensen, University of St. Andrews, for use with *Drosophila* wing epidermis (Mogensen and Tucker, 1988).

v) Fixation for Electron Microscopy.

After isolation, the organs of Corti were fixed in 2.5% gluteraldehyde solution (Emscope) in phosphate buffer (0.05M, pH 7.6) for a minimum of 30 minutes and post-fixed in 1% osmium tetroxide (Emscope) in the same buffer for 30 minutes. The fixed tissue was then dehydrated in a graded series of ethanol, transferred to 100% propylene oxide. To allow adequate penetration of resin, tissue was passed through a graded series of propylene oxide/Araldite resin mixtures before being flat embedded in Araldite epoxy resin (Emscope).

vi) Transmission Electron Microscopy.

Electron microscopy was carried out using a Philips 301 electron microscope operated at 60kV. Micrographs were taken using Ilford Technical (Electron Microscope) Film which was developed in Kodak D-19. Prints were

prepared using Ilford Ilfospeed and Ilford Multigrade photographic paper and developed in Ilford Contrast FF and Ilford Multigrade respectively.

Glass knifes (made on a LKB Knifemaker, LKB, Stockholm) were used to cut both thick and ultra-thin sections on a Reichert Om-U3 ultramicrotome. A diamond knife (RMC, Tucson, Arizona) was also used to cut ultra-thin sections (silver and grey interference colours) when sequences of serial sections were required.

Longitudinal sections were cut perpendicular to the surface of the Araldite block.

Copper grids were coated with a solution of 1% pioloform in chloroform, allowed to dry and coated with carbon.

Ultra-thin sections were mounted on coated copper grids. The sections were then stained with 2% uranyl acetate in 70% ethanol for 20 minutes followed by lead citrate (Reynolds, 1963) for 5 minutes.

vii) Protocol for Obtaining Transverse Sections Of Cells in the Epithelium of the Organ of Corti.

Araldite plaques containing the embedded organs of Corti, when viewed by Nomarski D.I.C. optics, showed the positions of the cell types within the epithelium. This technique did not, however, show the orientation of the pillar cells or their microtubule bundles. Hence, it was necessary to cut specimens in a plane perpendicular to the surface of the organ of Corti to obtain longitudinal sections of the cells.

To obtain transverse sections, the block was removed from the original stub and remounted at 90^0 to the original plane of sectioning. Sections could then be cut in a plane at right angles to the longitudinal axes of the pillar cells. If the bundle was not in perfect transverse section, the angle of the face relative to the knife could be altered. Sectioning was carried out from above

the surface of the hair cells; the stereocilia on the surface of the hair cells being used as a marker when nearing the surface of the organ of Corti.

Nanoplast melamine resin is extrememly brittle. The stub was not mounted and remounted according to the above procedure. The positions of the pillar cells relative to the hair cells, with their distinctive stereociliary bundles, were obtained by Nomarski D.I.C. optics. The block was mounted on a stub, trimmed so that only the pillar cells and the hair cells remained and sectioning commenced from above the apical surface. A diamond knife (as described in vi)) was used throughout this procedure and electron microscopy carried out as previously described in vi).

viii) Protofilament Enhancement of Cross-sectioned Profiles of Microtubules.

Prints of individual microtubule profiles, after detergent and tannic acid treatment (as described in section iv)), were rephotographed on 35mm film. The photographic rotational reinforcement method of Markham *et al.* (1963) was used for 13, 14, 15 and 16 fold rotations.

Chapter 1.

Initial Stages in the Assembly of Transcellular Microtubule Bundles in Inner Pillar Cells.

Introduction.

The main objectives of the investigation dealt with in this chapter were as follows :

i) When in the immediate post-natal period does the microtubule bundle begin to assemble?

ii) Is the centrosome associated with this microtubule array during its assembly?

iii) Do the microtubules of the inner pillar cell have diameters of about 30 nm diameter and composed of more than 13 protofilaments? Or, do they have "conventional" diameters of 24 nm and 13 protofilaments?

iv) How many microtubules assemble to construct a transcellular bundle?

v) Where does nucleation of microtubule assembly take place?

vi) Do the microtubules elongate from one, or both, ends of the pillar cells?

Results.

General Histology of the Organ of Corti.

The pillar cells of the organ of Corti are easily identified when sections in the longitudinal axis of the pillar cells are stained with methylene blue and viewed by light microscopy. They are found at a depression in the apical surface of the organ of Corti; lying between the inner hair cell row and the first row of outer hair cells (OHC-1). Hair cells have a distinct spherical appearance with stereocilia on their apical surface. These stereocilia are rod-like protrusions which attach, by the stereociliary rootlet, to a dense cuticular plate on the cell surface.

The pillar cells of the organ of Corti are approximately 25 μm long. The nuclei lie basally in the cell close to the basement membrane. This is a distinct

feature of these cells; Kolliker's and Deiters' cells have a pseudostratified appearance. The hair cells are supported by processes of Deiters' cells as well as by pillar cells. This region can also be recognised because it is situated above a blood vessel. This vessel lies in close proximity to the pillar cells just below the basement membrane.

Projecting from the apical surfaces of epithelial cells of the organ of Corti are numerous microvilli. The stereocilia of the hair cells are specialisations of these microvilli. They are useful identifying features for hair cells. They are graded in length; short on the inner hair cells, reaching their maximum length on the third row of outer hair cells. When viewed from above, the bundle of stereocilia have a characteristic V-shape. The tip of the 'V' of the inner hair cell points to the position of the inner pillar cell.

Organs of Corti were at selected stages up to a point 6 days post-partum. At this stage, the ossification of the cartilaginous capsule has progressed substantially; after this stage considerable damage may be caused to the organ during dissection.

Day 0.

Little, if any, assembly of the transcellular array has occurred. Very few microtubules are present in the inner pillar cell and they are not evenly aligned throughout the cell (Figs. 3 and 4). Glycogen granules are distributed evenly throughout the cytoplasm. Mitochondria are not aligned at any particular orientation; many are rounded in shape (c.f. Day 6). The nucleus is present basally in the cell where it remains throughout development.

Day 1.

At this stage, numerous microtubules are present in each inner pillar cell. Apically in the cell, a centrosome is present: some microtubules appear to

be associated with this and radiate from it (see Fig. 5). These microtubules have diameters of about 21 nm.

Below this apical region there is another population of microtubules. These are aligned and presumably represent an initial stage in the formation of a transcellular bundle parallel to the longitudinal axis of a cell (Fig. 5). No microtubule cross-sectional profiles are observed within the transcellular bundle (Fig. 5) i.e. microtubules are only aligned in the longitudinal axis of the cell.

At this stage of development, electron dense aggregations are present in the region where the sub-apical annulus is later located. Glycogen particles are observed above the sub-apical annulus but no mitochondria are present within this region.

Dense pericentriolar material is seen surrounding the centriole (Figs. 5 and 6). However, there is lateral to this, an annular-like structure against the plasmalemma at the sides of the cell. Microtubules of the assembling putative transcellular array are associated with this annulus.

Day 3.

General Morphology of Inner Pillar Cells.

Inner pillar cells are tall columnar cells with a cylindrical shape. These cells all have a circular profile at all cross-sectional levels.

Apically, a centrosome is present with microtubules radiating from it (Fig. 9). The microtubules which are associated with the centriole in the sub-apical region do not cross into the region containing the assembling transcellular bundle.

The microtubules of the transcellular array have progressed in their assembly compared with Day 1 (Fig. 7). Microtubules project basally, from sites on the lateral plasmalemma, near the apical surface of the cell. These

sites are composed of dense material and form an annular configuration within the sub-apical region (Fig. 7). This dense material is seen as a sub-apical peg-like process onto which a group of microtubules attach (Fig. 8).

The array microtubules are closely packed together along the lateral 'sides' of the cell giving the array a tubular configuration. Apically, microtubules are present in large numbers (see Table 1, Fig. 10a). At lower levels in the cell, the tubular array structure is still present but the microtubule numbers have decreased (Figs. 10b and 10c). The microtubules are grouped in small bundles within the array. Basally, few microtubule profiles are observed. Those microtubules which are present are diffusely scattered throughout the cytoplasm but retain their orientation parallel to the longitudinal axis of the cell (Fig. 10c).

Microtubules with Different Diameters and their Sites of Nucleation.

Microtubules in the population which radiates from the centrosome have diameters of about 21 nm. However, microtubules of the assembling transcellular array have diameters of about 28 nm. Between array microtubules, dense intertubular material is present. This material contains filaments which are about 6 nm in diameter.

Day 6.

General Morphology of Inner Pillar Cells.

A substantial bundle of microtubules has developed by this stage. They are not associated with a centrosome, but are attached to a dense sub-apical structure - the sub-apical annulus.

In the intervening region between the sub-apical annulus and the apical cell surface, the cytoplasm is less dense; no large organelles are present but

aggregations of dense glycogen particles occur. Few, if any, microtubules are present in this apical region (Fig. 11)

At 6 days old, the microtubule array almost entirely fills the upper region of the cell below the sub-apical annulus (Figs. 11 and 12). The microtubules of the array are closely packed and well aligned. They have diameters of about 28 nm. Microtubules of the array do not associate with any structure apically other than the sub-apical annulus. This annulus is composed of diffuse electron dense material which is similar in appearance to the pericentriolar material (Fig. 12). The microtubules do not traverse this structure into the apical region of the cell (Fig. 13).

Microtubule numbers are high just below the sub-apical annulus (see Table 1, Fig. 14). The number of microtubule profiles decreases at lower levels in each cell (see Table 1, Figs. 15a-c). Beneath the annular microtubule nucleating/capture site, the microtubule array has taken on a tubular configuration. The array retains this configuration throughout the cell length. At lower levels, the microtubule array is composed of small fascicles of microtubules (Figs. 15b and 15c). Within these small bundles, dense intertubular material is present which contains 6 nm diameter filaments. These filaments are identical in appearance and positioning to those shown in Fig. 21 in 1 Day old tissues cultured for 2 days *in vitro*.

Few organelles are present apically in the cell. Any mitochondria which are present, are observed lower in the cell. They have their longitudinal axes parallel to the alignment of the microtubule array.

Analysis of Microtubule Protofilament Number.

Cross-sections of microtubules which are fixed in the presence of tannic acid after detergent treatment reveal the presence of protofilaments which make up the microtubule.

Two concentrations of detergent were used to facilitate the entry of tannic acid into the organs since the optimum concentration was not known. Organs extracted with the lower concentration were examined first. It transpired that the tissue was more than adequately extracted, hence, the higher detergent concentration was not examined. The tissue used in this part of the investigation was treated with 0.125% Triton X-165 detergent (see Materials and Methods, section iv)).

Detergent extraction of this material has resulted in loss of much of the cellular organisation and cytoplasmic contents. Few cells could be recognised; the pillar cell positions could only be determined by their relative positions to the relatively unextracted hair cell cuticular plate. The effects of this treatment were rather drastic, the material was excessively extracted with only a small percentage of microtubules of the transcellular bundle unextracted. The microtubules which were left were present in small aggregations.

Protofilaments could be detected in cross-sectional profiles of microtubules. It was fairly clear that such microtubules possessed more than 13 protofilaments but in certain regions around the microtubule it was impossible to clearly distinguish individual protofilaments all the way round the profile for the unequivocal count of protofilaments. They would probably have been more distinct if the degree of detergent extraction had been slightly less. The negative staining of the microtubule cross-sectional profiles, with tannic acid, was not adequate (Fig. 16a). This prevented the unequivocal count of the number of protofilaments present around the cross-sectional profile. A few microtubules were present for which the protofilament number could be assessed. Hence, rotational photographic reinforcement analysis (Markham *et al.*, 1963) was utilised to obtain a clearer indication of the number of protofilaments present.

Fifteen rotations of cross-sectional profiles through $(360/15)^{o}$ about their centres gave strong reinforcement (Fig. 16d). Thirteen rotations through $(360/13)^{o}$ (Fig. 16b), 14 rotations through $(360/14)^{o}$ (Fig. 16c) and $(360/16)^{o}$ (Fig. 16e) did not provide such substantial reinforcement.

Table 1.

This table shows the numbers of microtubules decreasing at lower levels within each of the two types of pillar cells. Few microtubules were present in outer pillar cells before Day 3.

Table 1.

	Just below annulus.	5 µm below annulus.	10 µm below annulus.
Inner pillar cell.			
Day 1	236		
Day 3	2654	2119	1558
Day 6	2241	2090	1733
1 Day *in situ* followed by culturing for 2 Days.	1254	1073	845
Outer pillar cell			
Day 3	186	91	45
Day 6	844	833	588

Figure 2.

Schematic diagram showing the transcellular microtubule array (m) in an inner pillar cell of the mouse organ of Corti.

Sub-apically, microtubules attach to lateral plasmalemma-associated sites (S). Microtubules decrease in number at lower levels. The nucleus (N) is positioned basally.

Not to scale.

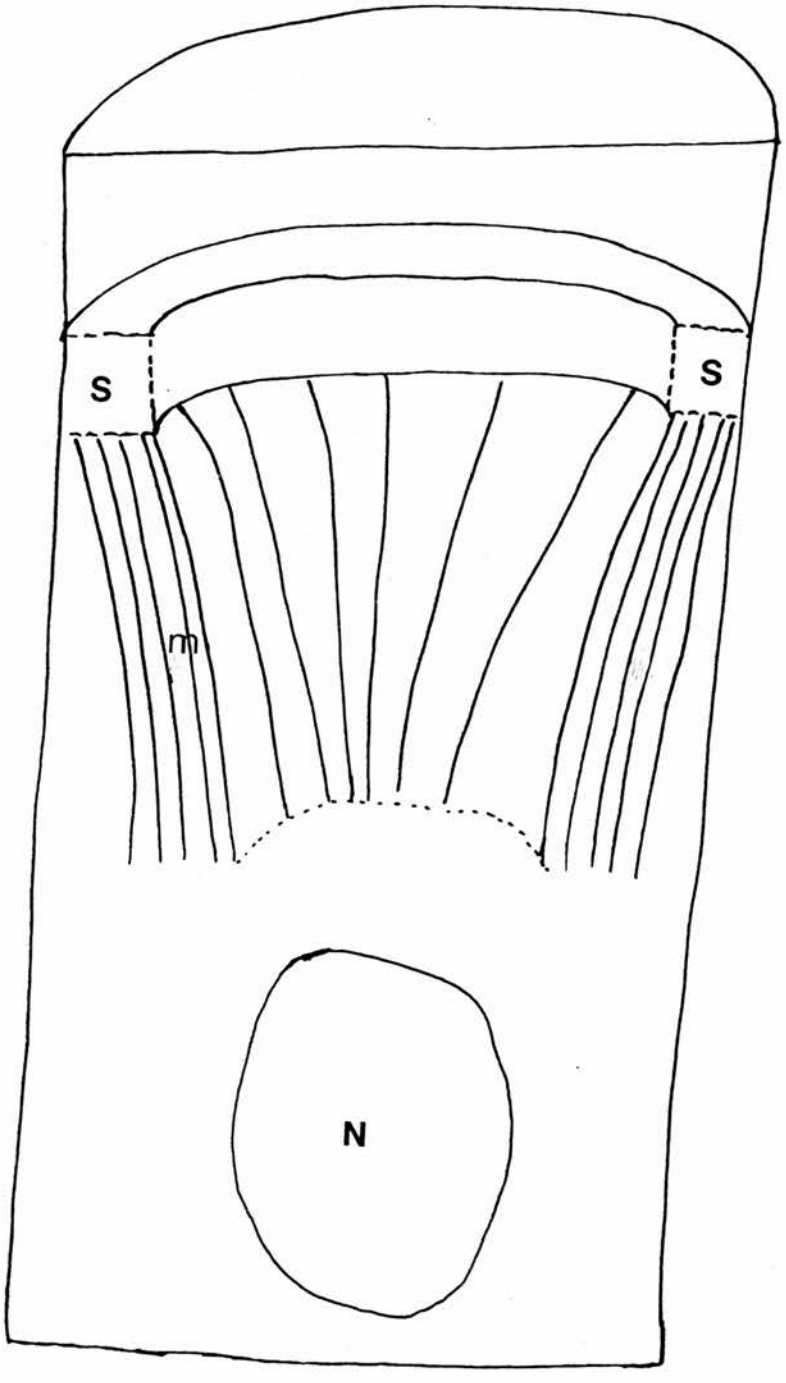


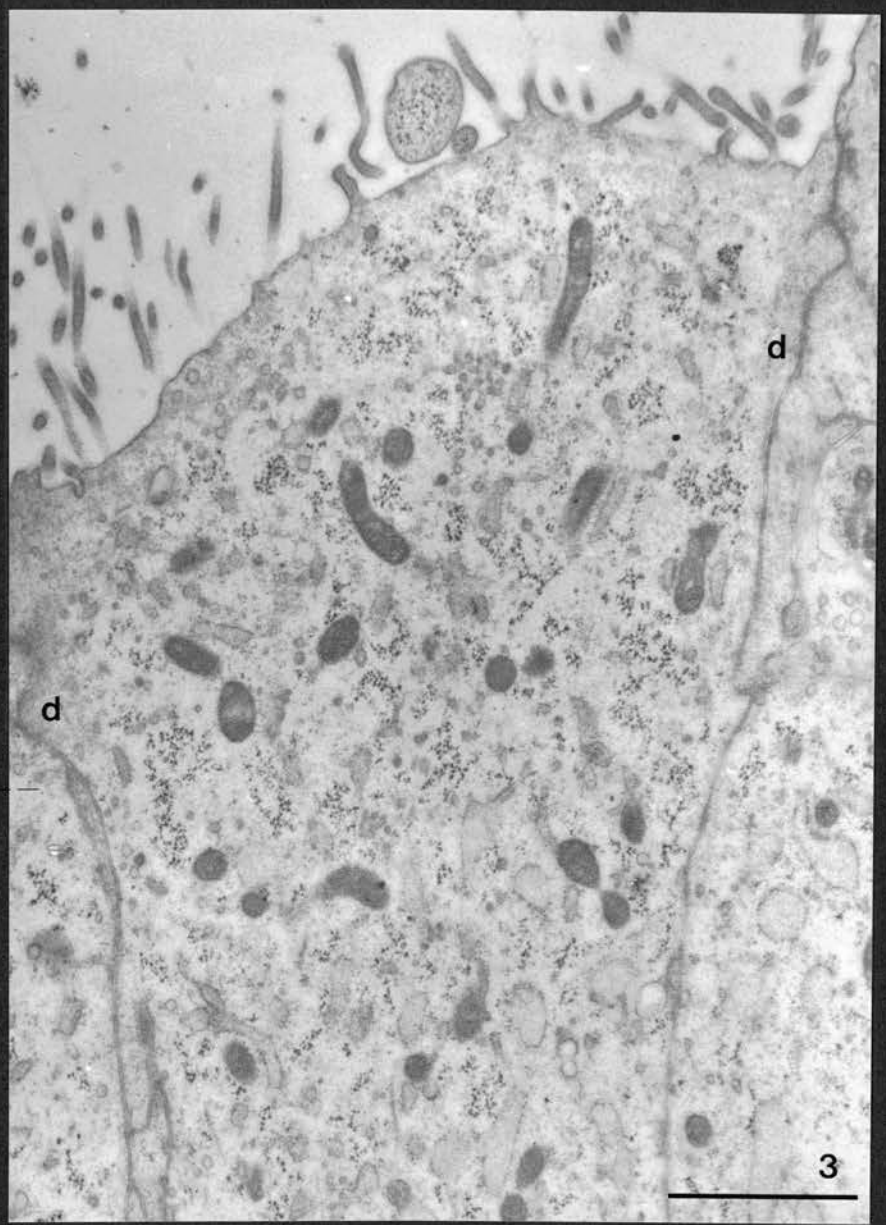
Fig. 2

Figure 5.

Inner pillar cell Day 1.

A few microtubules (a) are associated with the centriole (c) surrounded by its dense pericentriolar material. Microtubules (m) of the assembling array are aligning in the longitudinal axis of the cell.

Bar - 0.1 μm.


d
d
3

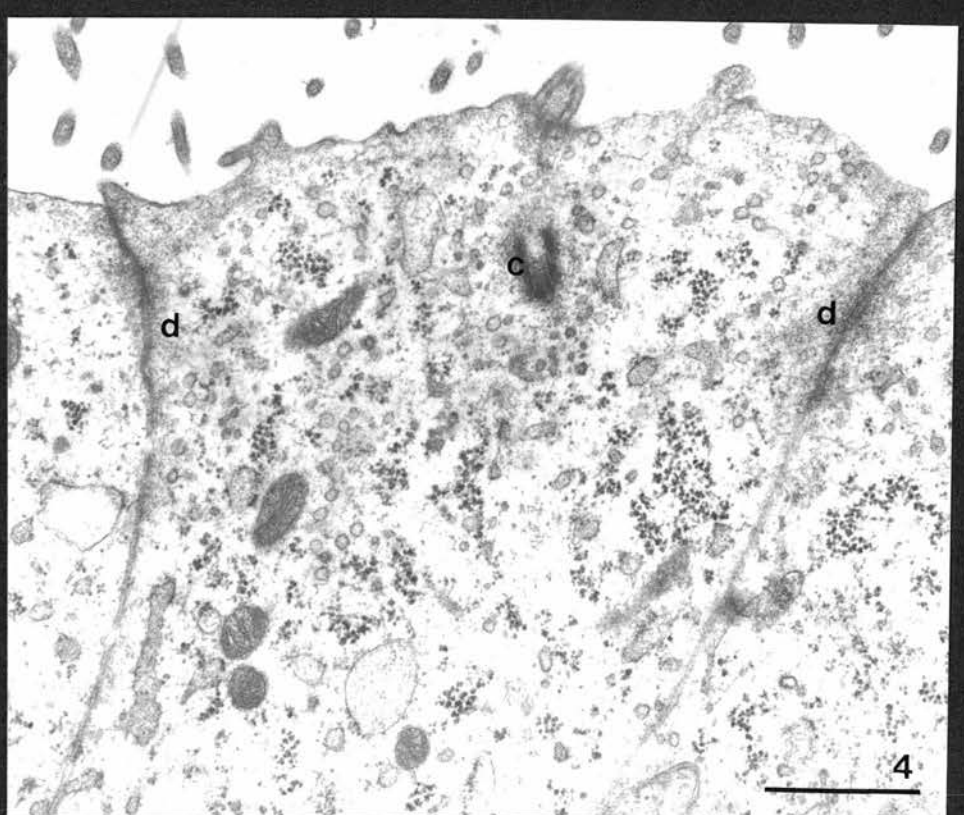

c
d
d
4

Figure 3.

Inner pillar cell Day 0.

No microtubules of the developing transcellular array are observed lower in the cell. Dense granular material (d) is associated with the lateral plasmalemma sub-apically.

Bar - 1 μm.

Figure 4.

Inner pillar cell Day 0.

The centriole (c), surrounded by electron dense pericentriolar material (PCM), is observed sub-apically. No microtubules appear associated with this structure. Material (d) associated with the lateral plasmalemma sub-apically has similar density and appearance to PCM. No microtubules are associated with this material laterally.

Bar - 1 μm.

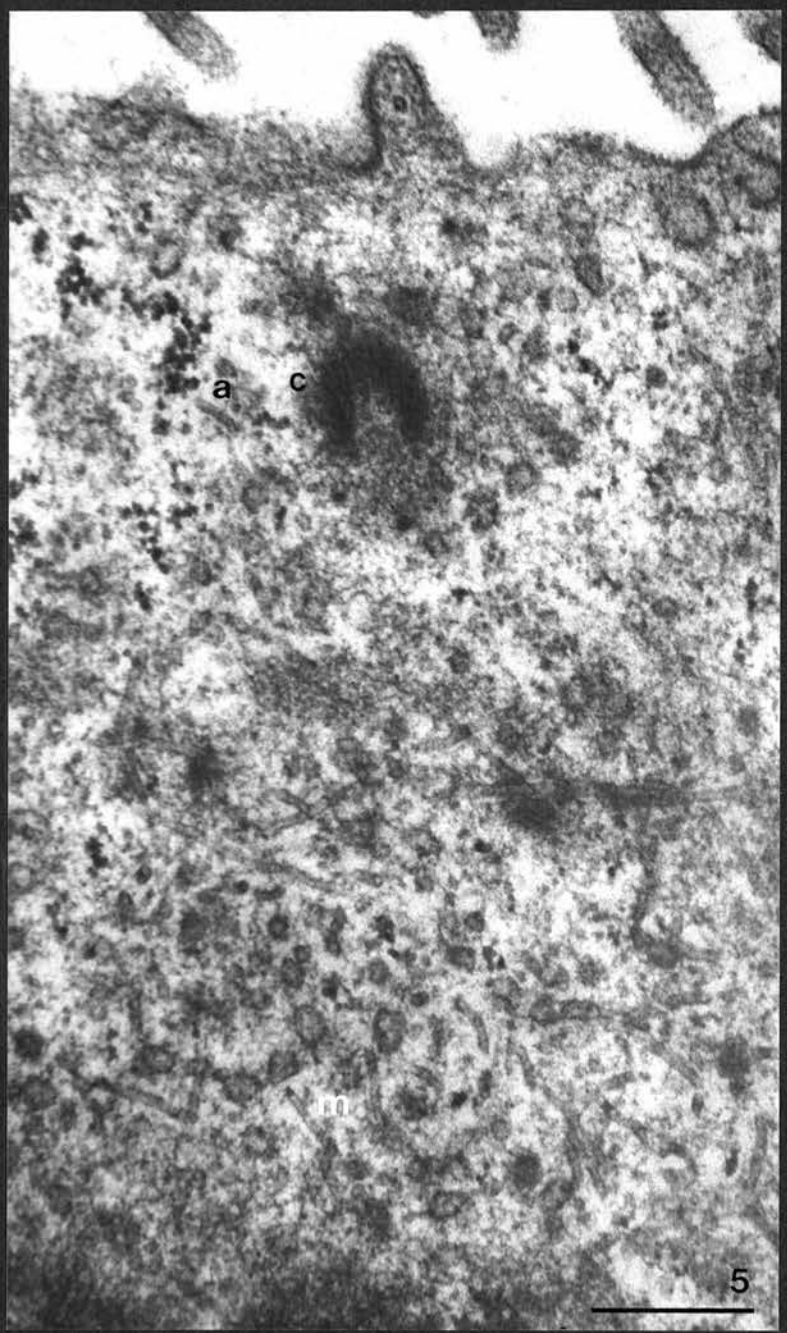

a
c
5

Figure 6.

Inner pillar cell Day 1.

Microtubules (a) associated with the centriole have diameters of about 21 nm. Microtubules (m) of the assembling transcellular array are aligned in the longitudinal axis of the cell. A band of PCM-like material passes across the cell sub-apically (arrow).

Bar - 0.5 μm.

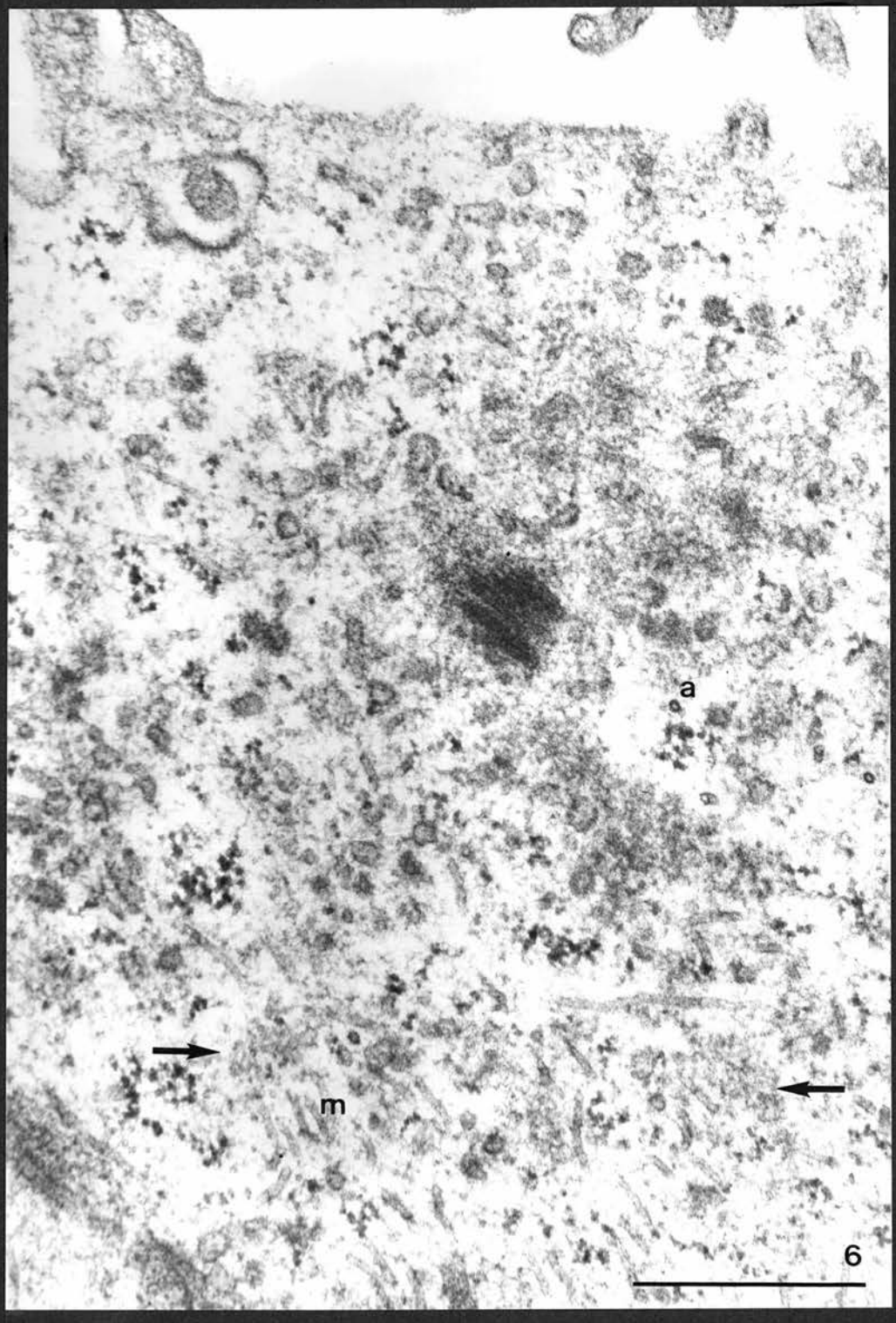

a
m
6

Figure 7.

Inner pillar cell Day 3.

A few microtubules (a) are associated with the centriole (c) and basal body (b). Microtubules (m) of the transcellular array attach to sub-apical sites associated with the lateral plasmalemma (d). Most of these microtubules lie parallel to the lateral plasmalemma.

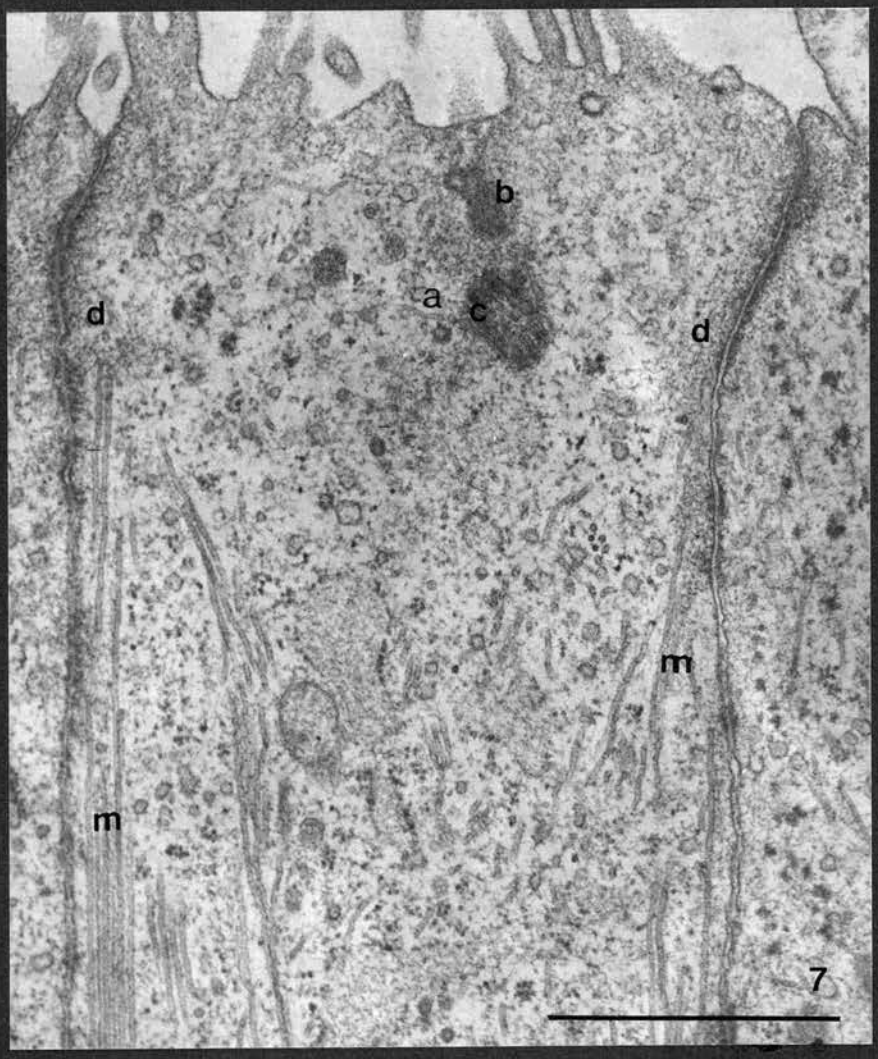
Bar - 1 μm.

Figure 8.

Inner pillar cell Day 3.

Microtubules (m) attach to sites associated with the lateral plasmalemma (d).

Bar - 0.2 μm.


b
a
c
d
d
m
m
7

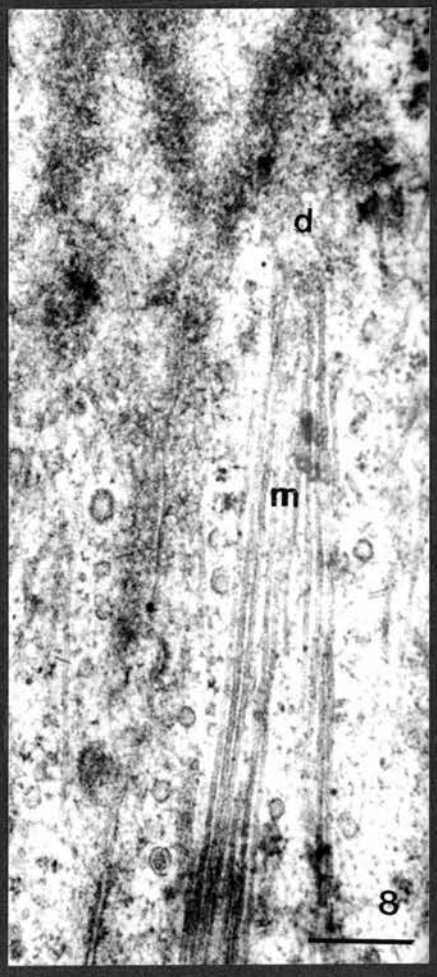

d
m
8

Fig. 9.

Inner pillar cell Day 3.

Few microtubules (arrow) associate with the centriole (c) and basal body (b). Transcellular array microtubules (m) attach at sub-apical sites (s). The microtubule array has a tubular configuration.

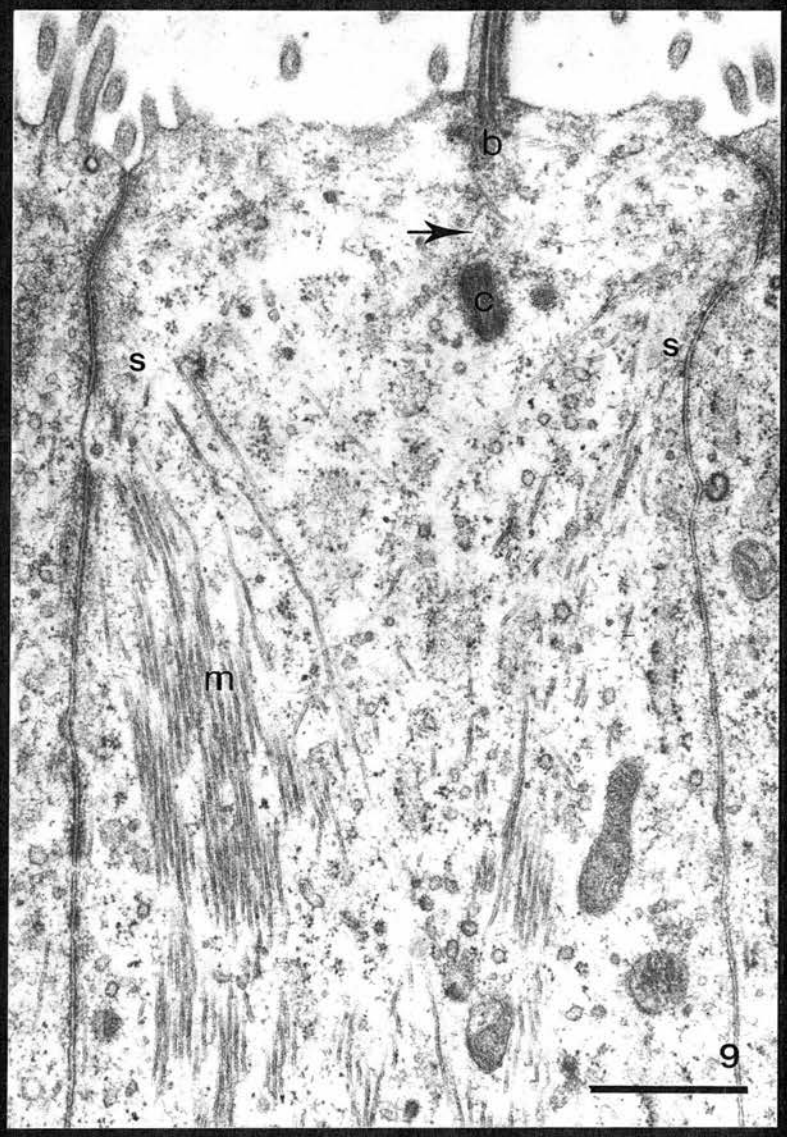
Bar - 1 μm.

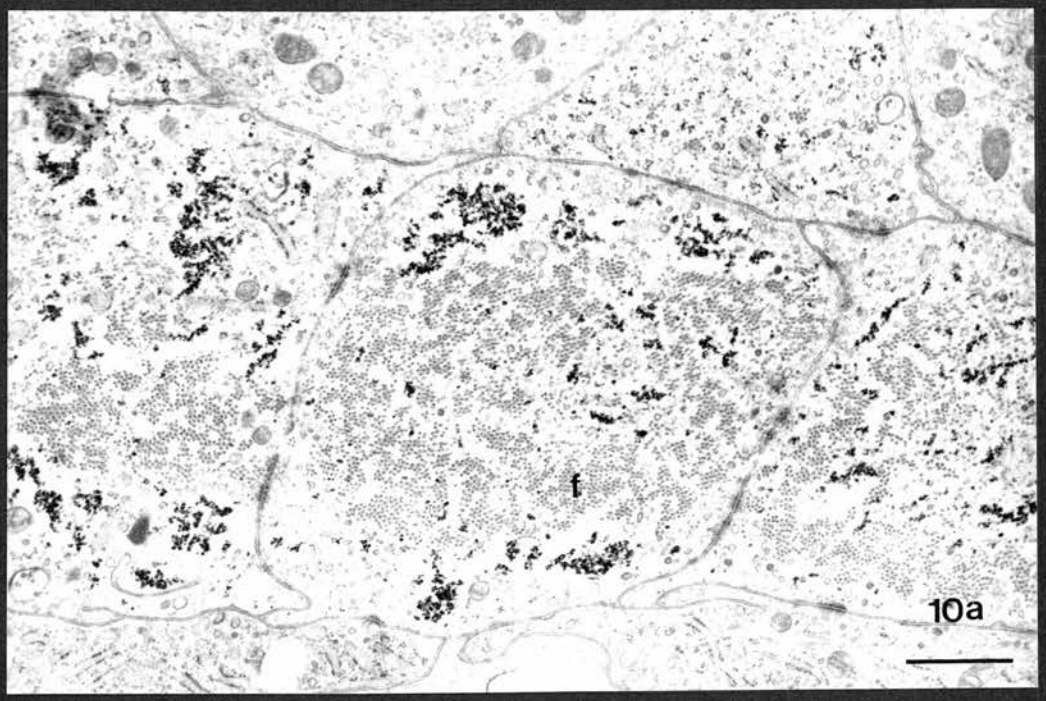
Figure 10a-c.

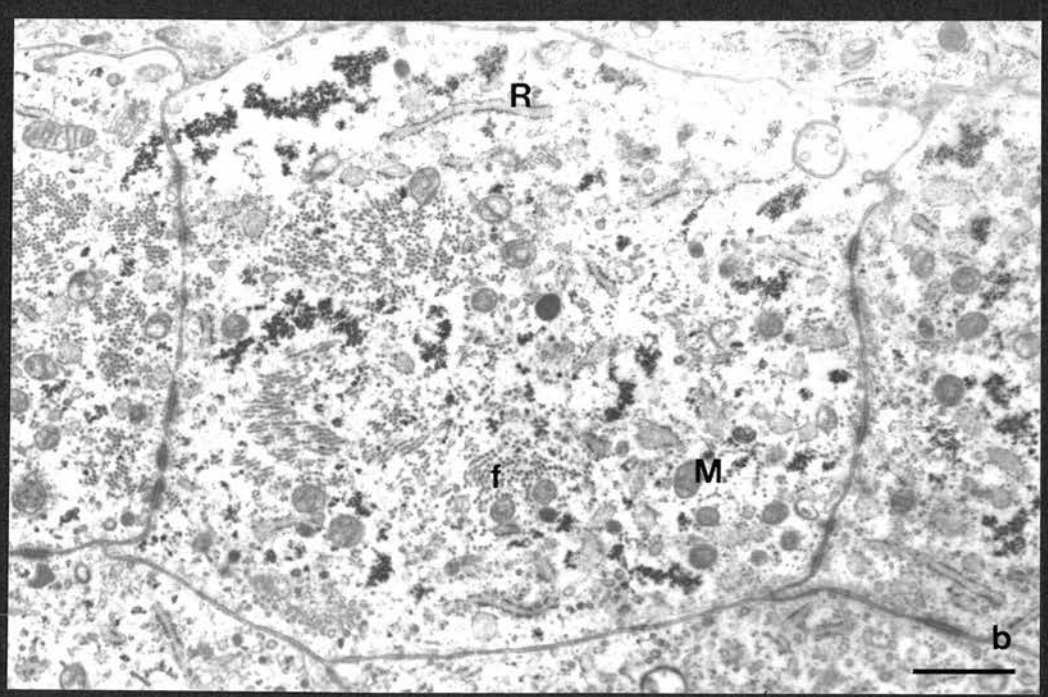
Sequence through the inner pillar cell at Day 3.

Numbers of microtubules are high in the apical region (10a) and decrease basally (10b, 10c). Apically there are large fascicles (f) of microtubules but these become smaller (f) and more diffuse at lower levels. More organelles (G - Golgi bodies, R - rough endoplasmic retculum, M - mitochondrion) are present at lower levels.

Bar - 1 μm.


b
c
s
s
m
9


f
10a


R
f
M
b

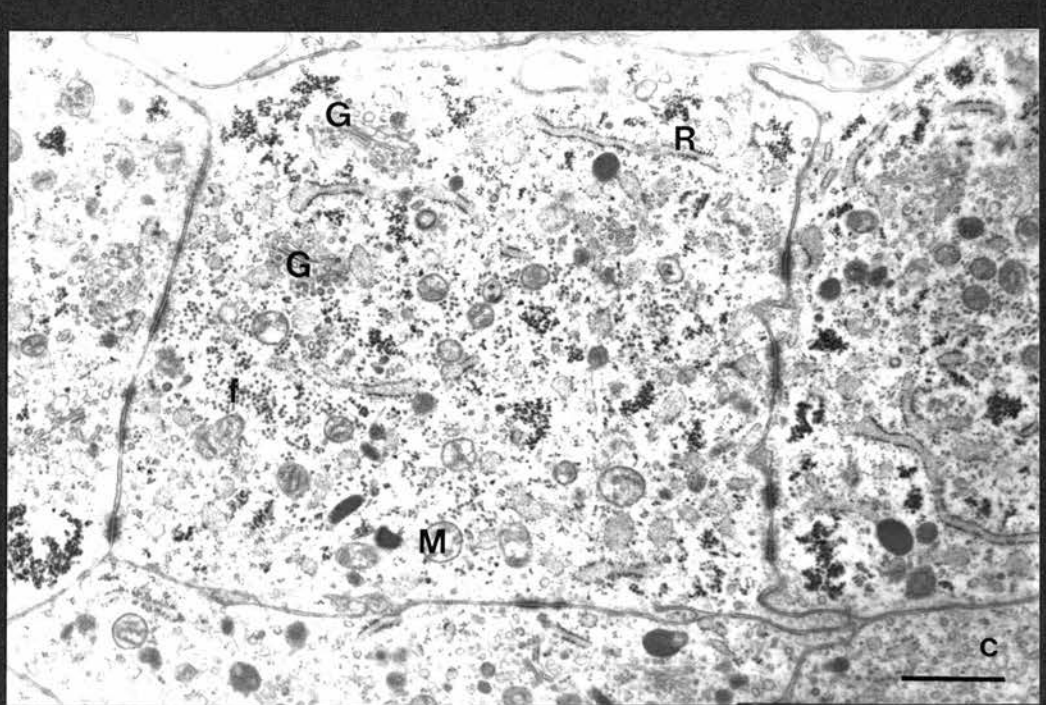

G
R
G
f
M
c

Fig. 11.

Inner pillar cell Day 6.

Microtubules of the assembling transcellular array attach to sub-apical sites. When viewed in cross-section, these sites appear to have a laminar configuration(S).

This section grazes through two cells; M labels cell membrane between the two cells.

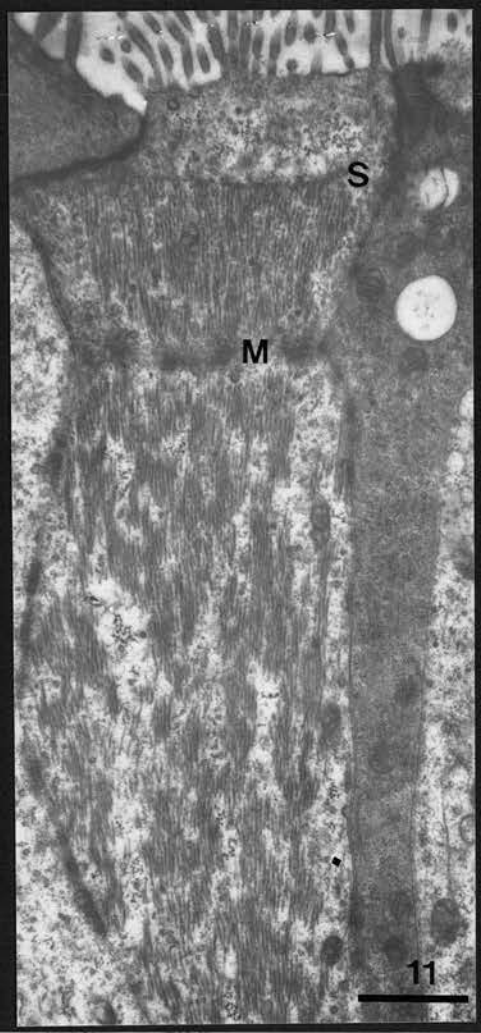
Bar - 1 μm.

Fig. 12.

Inner pillar cell Day 6.

Sub-apical sites (S) have a granular appearance. All microtubules of the assembling array are nucleated at these sites.

Bar - 1 μm.


S
M
11

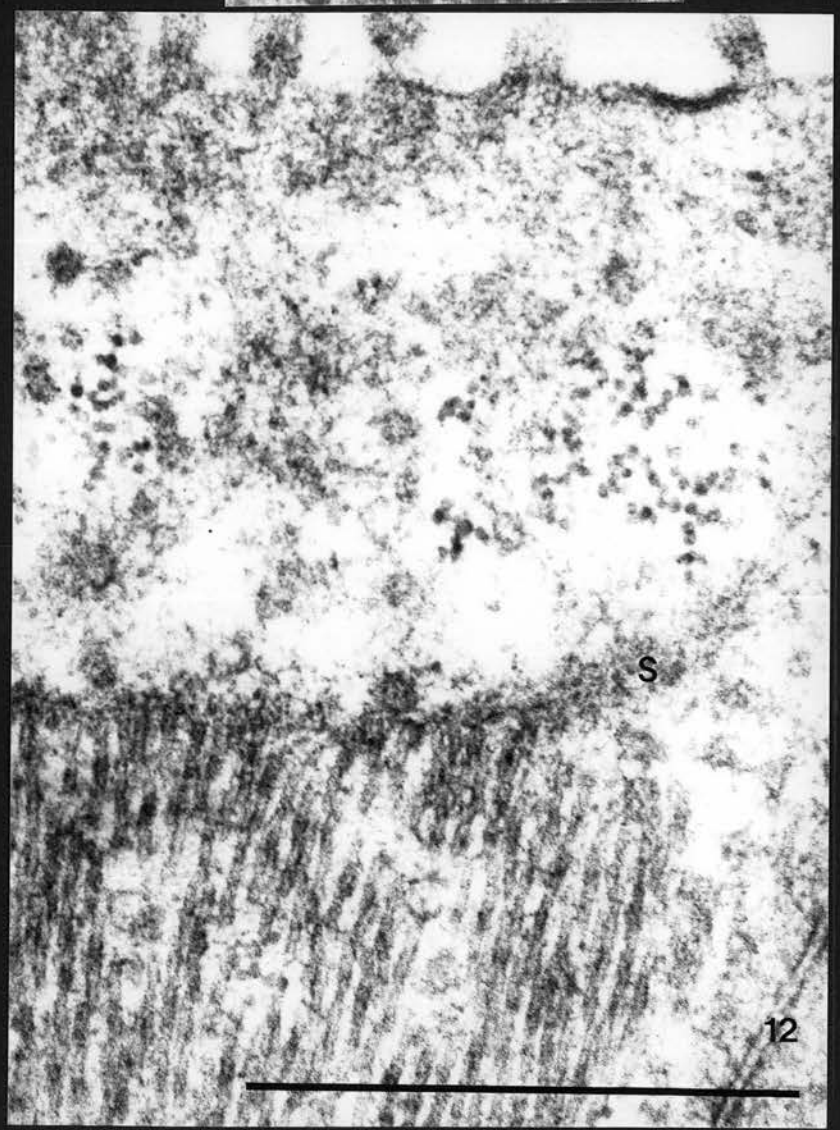

S
12

Figure 13.

Inner pillar cell Day 6.

Few microtubules (arrow) are associated with the centriole (c) and its PCM. Electron dense material (d) similar in density to PCM is in close proximity to the lateral plasmalemma. A small group of array microtubules (m) is associated with this.

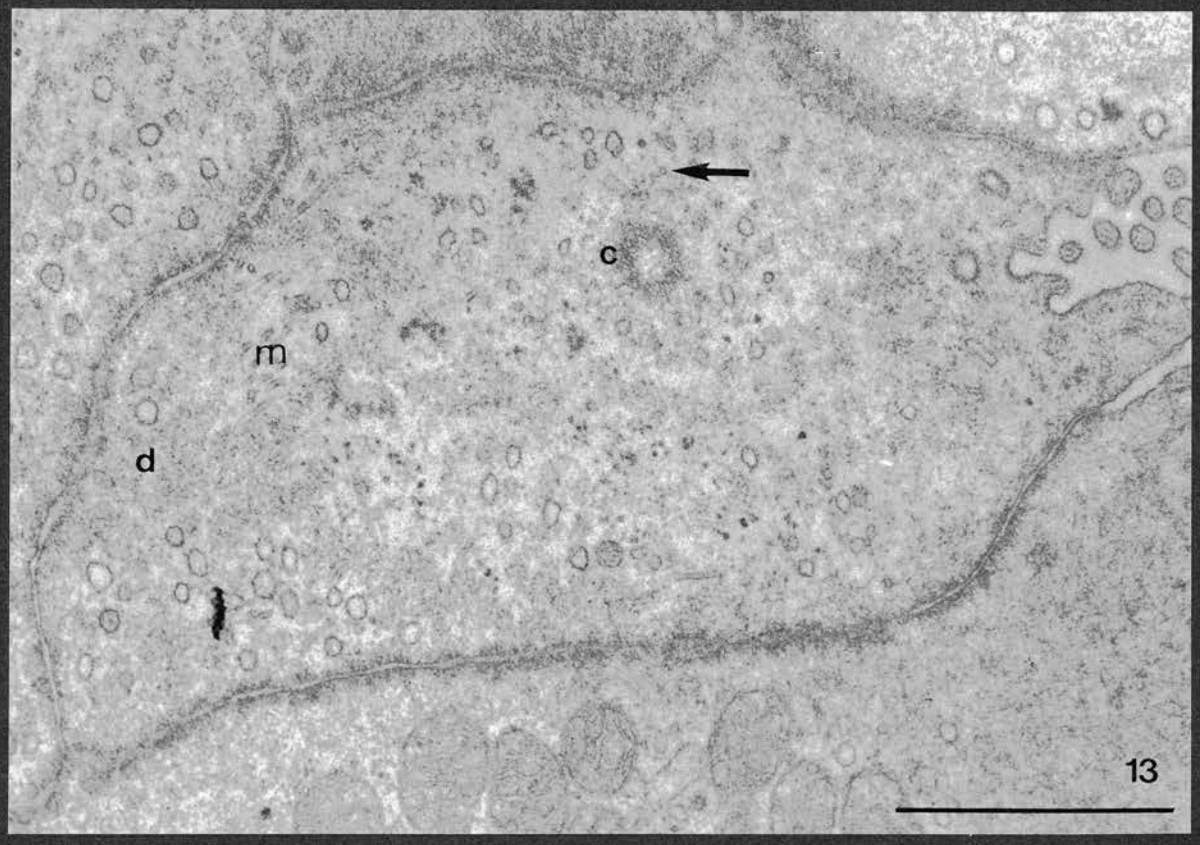
Bar - 1 μm.

Figure 14.

Inner pillar cell Day 6.

Microtubules are tightly packed but small fascicles (f) can be distinguished.

Bar - 1 μm.


c
m
d
13

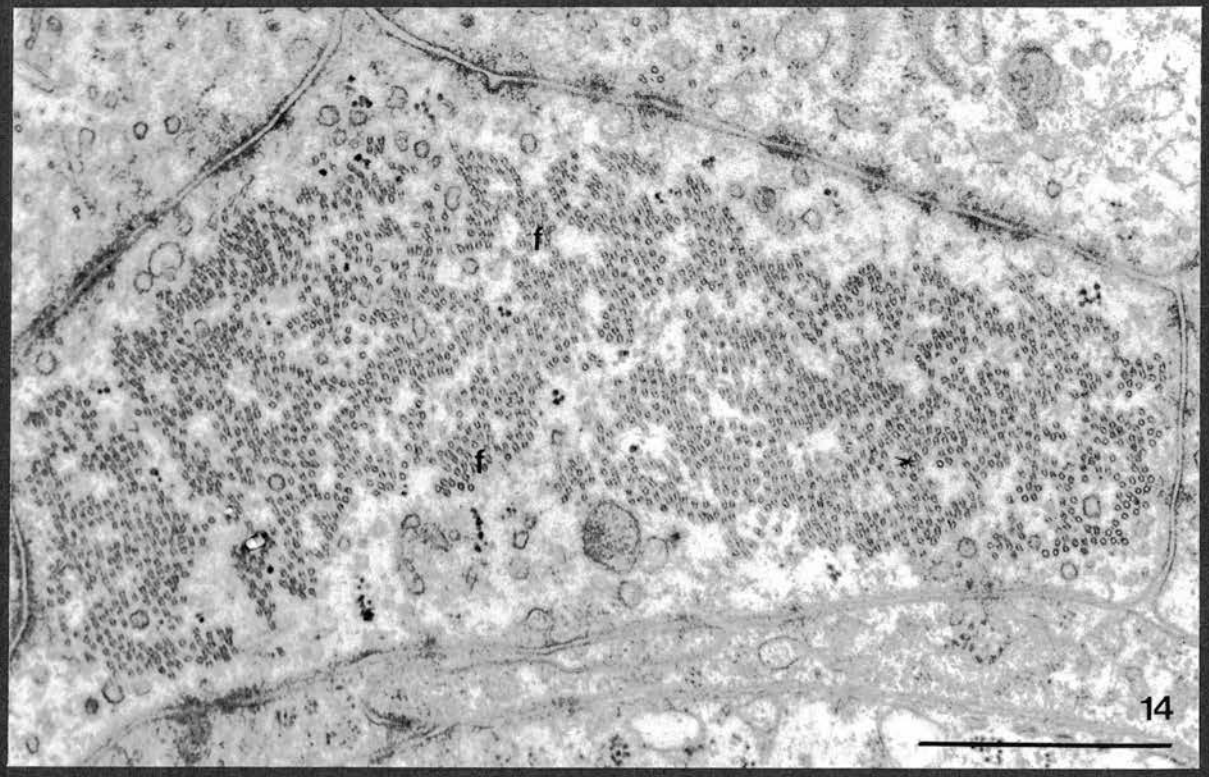
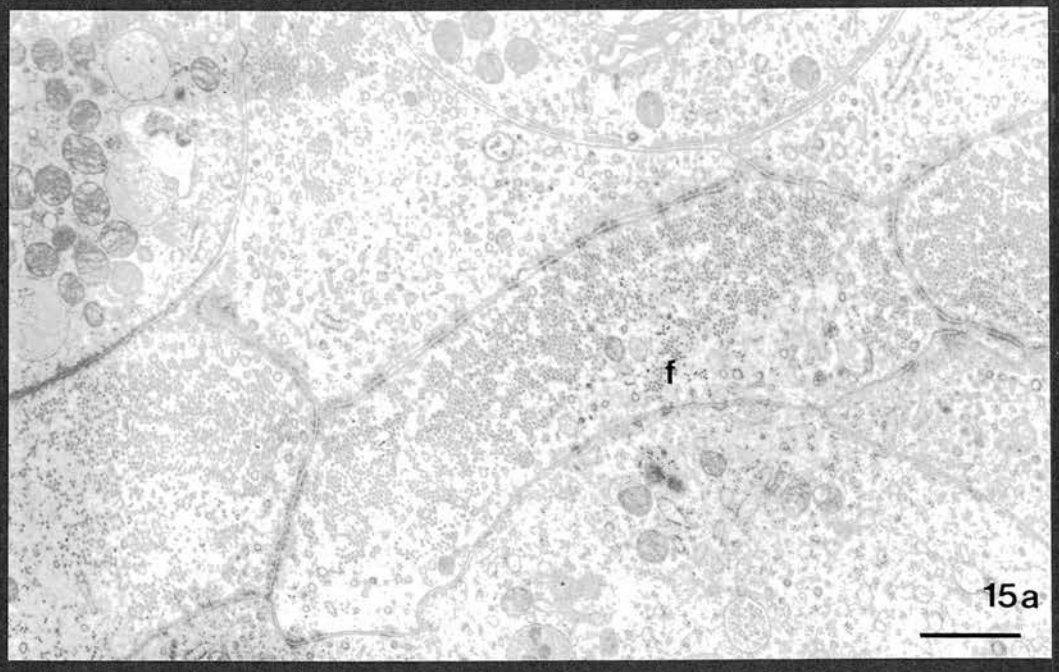

f
f
14

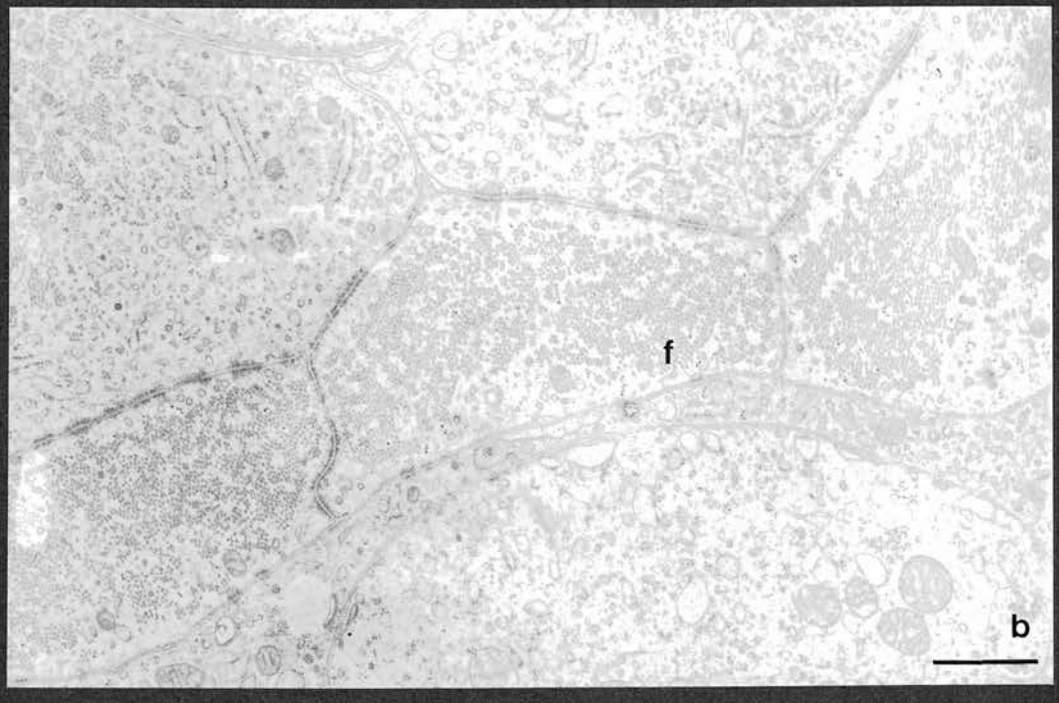
Figure 15a-c.

Sequence through inner pillar cell at Day 6.

Microtubule numbers are high in the apical region (a) but decrease at lower levels (b and c). Microtubules are arranged in fascicles (f).

Bar - 1 μm.


f
15a


f
b

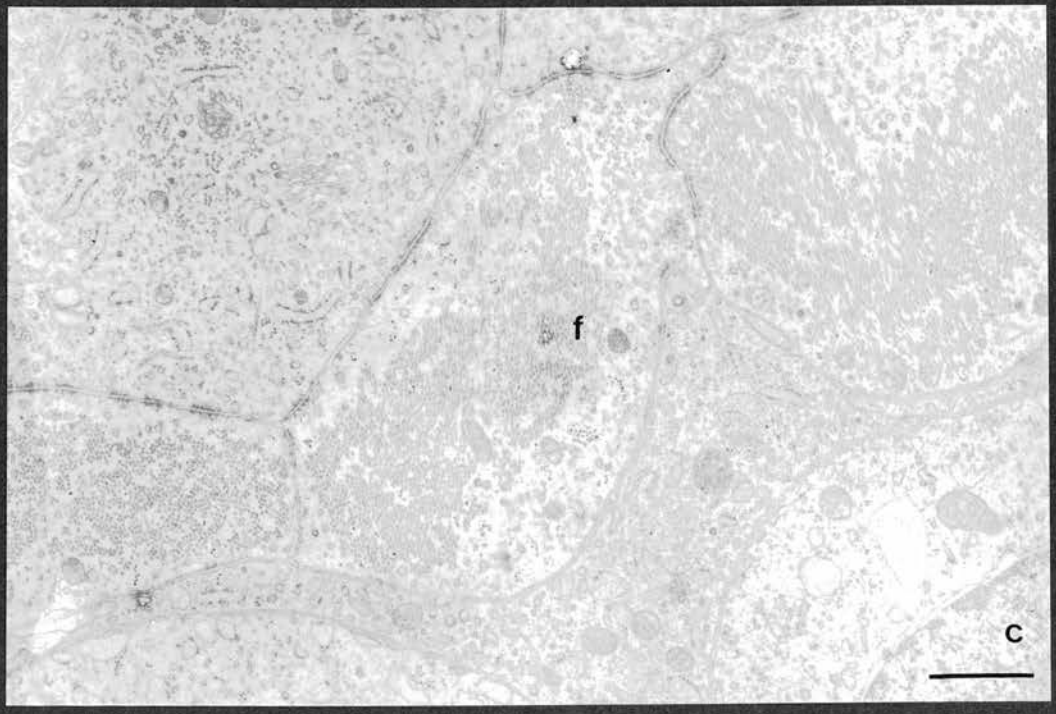
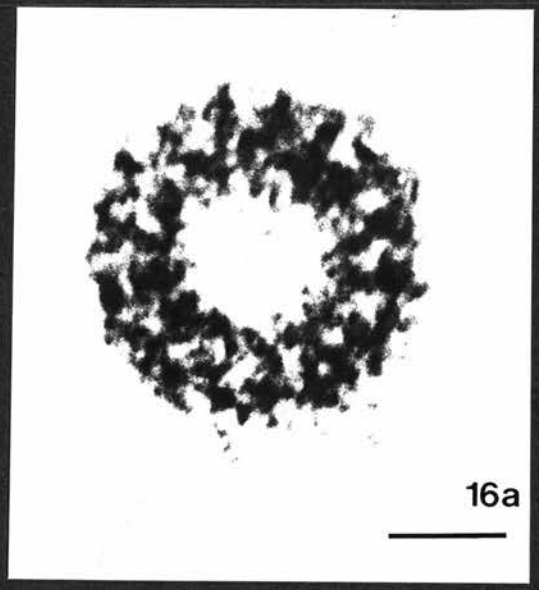

f
c

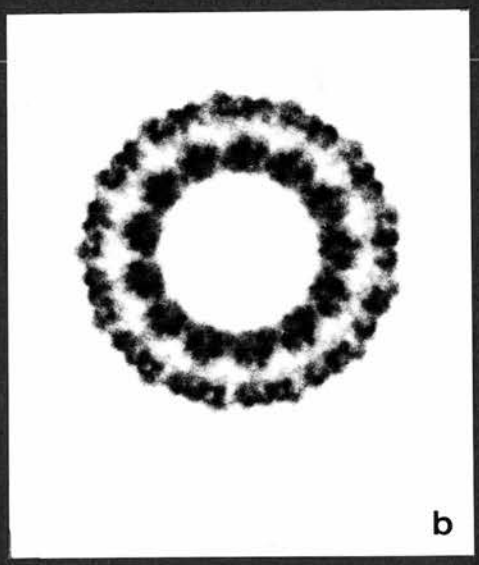
Figure 16a-e.

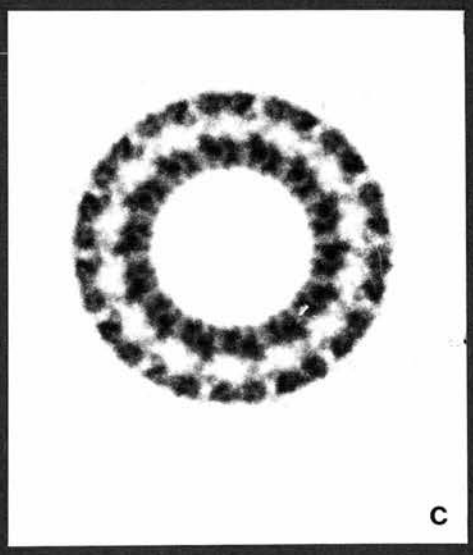
a. Unenhanced print of a microtubule after detergent and tannic acid treatment. Only parts of the microtubule show protofilaments present.

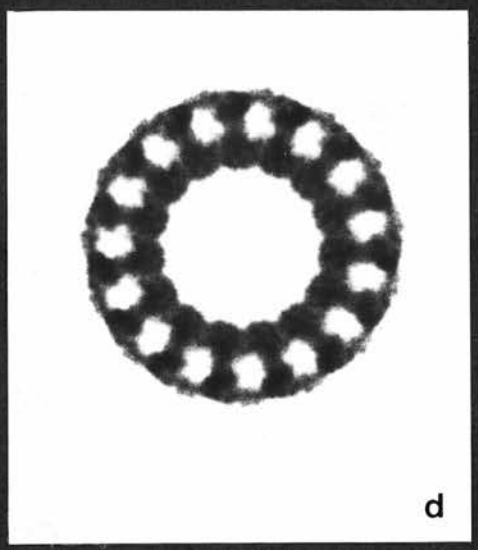
b-e. Rotations of 13, 14, 15 and 16 fold respectively. This data suggests that the microtubules are composed of more than 13 protofilaments - probably 15.

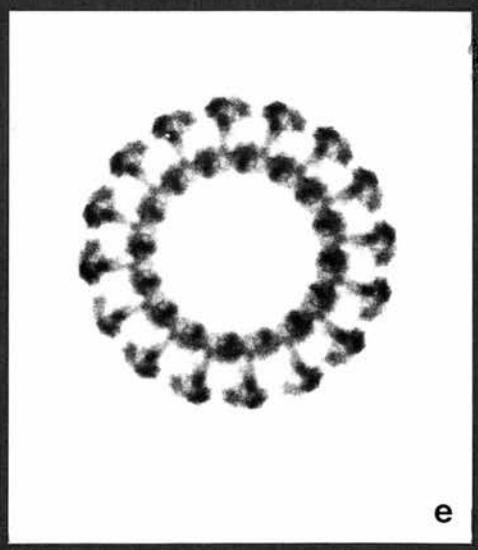
Bar - 10 nm.


16a


b


c


d


e

Discussion.

Apical Form of Inner Pillar Cells.

The apical region of each inner pillar cell has a circular profile which is similar to the profiles present at lower levels of each cell. The entire apical profile emerges at the apical surface of the organ of Corti. This is in contrast to that observed and discussed in Chapter 3 on outer pillar cells.

Structure of the Microtubule Array and its MTOC.

The microtubule arrays of the pillar cells of the mouse organ of Corti have not been previously described. The data presented above suggests that two populations of microtubules exist within the inner pillar cell.

The first microtubule population is observed in close proximity to the centrosome. These microtubules have diameters of about 21 nm and are probably composed of 13 protofilaments. They appear to be centrosomally nucleated and radiate from this structure.

The second population, which have diameters of about 28 nm, form the array during the first 6 days post-partum, but they do not appear to be associated with the centrosome. The microtubules of the array appear to elongate from nucleation sites associated with the lateral plasmalemma. These nucleation sites are situated, sub-apically, in an annular-like configuration around the cell's lateral sides (Fig. 2). The nucleation sites are composed of a diffuse electron dense material similar in composition to PCM. Does this suggest that PCM-like material diffuses from the centrosome towards the lateral membrane? Or, alternatively, are the lateral sites already present, but a switch in control of nucleation occurs?

From the results obtained above, the numbers of microtubule profiles decrease at lower cross-sectional planes in the cell. This suggests that

microtubules are nucleated only at the apical region of the cell. Microtubules do not appear to elongate from the basal region.

Similar microtubule diameters have been found by Angelborg and Engstrom (1972) working on the guinea pig organ of Corti. This group demonstrated thin 'microfilaments' and thicker 'tubular filaments' which are arranged in a characteristic pattern. They describe tonofilaments with a diameter of about 28 nm and a wall thickness of about 6 nm, i.e. microtubules. Thin microfilaments which were also observed have diameters of about 6 nm, i.e. actin. The microtubules of the inner pillar cell in the mouse have the same dimensions.

The array structure is similar to that found in the transcellular microtubule arrays of the *Drosophila* wing (Mogensen and Tucker, 1987). The pillar cell bundles are similar in structure and have microtubule diameters greater than 24 nm. This would suggest that array microtubules, in inner pillar cells, are not nucleated by centrosomes in the normal manner: centrosomal microtubules normally radiate in all directions from the centrosome. There may be a redistribution of centrosomal material around the lateral plasmalemma which results in the distinct structure of the inner pillar transcellular array.

The microtubules of *Drosophila* transalar arrays have plasma membrane-associated nucleation sites apically, microtubule profiles decrease in number at lower cross-sectional planes within the cell. These microtubules have diameters of about 30 nm. In the inner pillar cell, the number of microtubule profiles also decreases in a similar fashion within the cell. This would suggest that microtubules in inner pillar cells, which decrease in number at lower levels, are also nucleated apically. Also, when comparing the nucleation sites of the inner pillar cells to those of the epidermal wing cells of *Drosophila*, they are very similar. They are both composed of PCM-like

material located at the plasma membrane to which groups of microtubules attach.

Microtubule Stability within the Transcellular Array of Inner Pillar Cells.

From the data presented above, microtubule nucleation appears to occur only within the apical region of inner pillar cells: very few microtubules are present in basal regions. Up to Day 6, the transcellular array microtubules may still be in a labile condition: they have not yet been captured at specific sites on the basal plasmalemma. Thus, array microtubules are possibly in a dynamically unstable state where rearrangement of the microtubule array is occurring as microtubules "search" for the cell bases. Short term fluctuations in the lengths of individual microtubules would not have been detected in this study which simply reveals a net apicobasal elongation of each entire array.

There is no published information regarding the stability of 30 nm diameter microtubules composed of more than 13 protofilaments. However, large 30 nm diameter microtubules in elongating transcellular arrays of *Drosophila* wing epidermis cells are cold and nocodazole stable (M. M. Mogensen - personal communication) and hence, presumably, they are not dynamically unstable. If large diameter microtubules of inner pillar cell transcellular arrays are similarly stable, this may suggest that short term length fluctuations do not occur, or, at most, that tempered instability of these microtubules occurs. This would allow some rearrangement of the transcellular array as the inner pillar cell continues in its development.

Numbers of Protofilaments Present in Array Microtubules.

Microtubules of the array, are shown by the technique of rotational enhancement (Markham *et al.*, 1963), to probably be composed of 15 protofilaments. These microtubules have diameters of about 28 nm. This provides further evidence for a switch in the control of nucleation sites from the centrosome to the lateral plasmalemma.

When centrosomes act as the microtubule nucleation site, the number of protofilaments specified is 13 in microtubules of about 24 nm diameter (Tucker *et al.*, 1986). Tilney *et al.* (1975) found, in a number of different species, that the centrosome specifies 13 protofilaments. Microtubules of the *Drosophila* transalar array which are not nucleated by the centrosome are composed of 15 protofilaments (Mogensen and Tucker, 1987). Evans *et al.* (1985) found that microtubules which were nucleated by a centrosome *in vitro* consistently gave rise to 13 protofilament microtubules while non-centrosomally nucleated microtubules were predominantly composed of 14 or 15 protofilaments. This would strongly suggest that the microtubules of the inner pillar cell, which, by protofilament enhancement, contain 15 protofilaments, are organised by a structure which is not operating in a manner identical to that of centrosomes in animal tissues generally.

In the supporting cells of the guinea pig organ of Corti, Saito and Hama (1982) found that 15 protofilaments were predominantly present although some 16 and 13 protofilament microtubules were also present. Importantly, this group have also stated that the diversity of microtubule diameter and the number of protofilaments does not result from treatment by either detergent or tannic acid.

Does this suggest that the apical, primary, array of microtubules is nucleated by the centrosome and, between Day 0 and Day 1, reorganisation of MTOC material results in a second population of microtubules elongating?

These microtubules elongate from nucleating regions dispersed throughout the apical region of the inner pillar cell i.e. the sub-apical annulus. These sites have features which are similar to the plasma membrane-associated microtubule nucleation sites found by Mogensen and Tucker (1987) in the transalar array of *Drosophila.*

It would seem that centrosomes are present at an early stage in development of inner pillar cells and are lost after the development of the transcellular array. This switch in nucleation sites is slightly altered from that found in *Drosophila* wing epidermal cells (Tucker *et al.*, 1986) and in *Drosophila* thoracic muscle attachment cells (Paton, 1988). Does this suggest that MTOC-like material disperses from the centrosome before loss of the centriole?

Non-centrosomally nucleated microtubules have been reported in some mammalian systems. Sasano and Kagayama (1987) describe, in the undifferentiated rat odontoblast, how microtubules are seen to radiate from the pericentriolar area. In the differentiated odontoblast, the microtubules become poorly related to the centrioles. In developing human striated muscle, Tassin *et al.* (1985), observed that myoblast nuclei align along the centre of the future myofibril. The microtubules then became nucleated on the nuclear surface and aligned to act as a template for the formation of the actin/myosin complex. Is this a similar redistribution of MTOC-like material as found in the inner pillar cells? Or, is there a switch in the control of nucleation sites after the cells become differentiated?

Microfilaments Within the Transcellular Array.

Dense intertubular material is present between microtubules of the transcellular array. Filaments with diameters of about 6 nm are observed within this material.

Dense intertubular material has been found in the transcellular arrays of *Drosophila* (Mogensen and Tucker, 1987). This material is predominantly composed of actin filaments (Mogensen and Tucker, 1988). The dense intertubular material is similar to that found between the microtubules in the inner pillar cell.

Slepecky and Chamberlain (1983) found similar transcellular bundles in the inner pillar cells of the chinchilla organ of Corti. Filaments with diameters of about 6nm were present within the dense intertubular material. This has been confirmed as actin by myosin sub-fragment S1 decoration. It has been suggested that as well as maintaining the shape of the organ of Corti, these supporting cells may alter, by an actin-microtubule interaction (similar to the actin-myosin interaction of muscle), the rigidity and thereby the mechanics of the basilar membrane.

Slepecky and Chamberlain (1983) suggest that the pillar cells, through their supporting function, help maintain the mechanical coupling between the stereocilia of the hair cells and the tectorial membrane. Also, any change in rigidity which alters the mechanics of the basilar membrane will influence the movement of stereocilia against the tectorial membrane. This would modulate the excitation of hair cells in reponse to acoustic stimulation.

Altering the mechanics of the basilar membrane may occur through microtubules maintaining the rigidity of inner pillar cells while actin filaments contribute to contractile force generation (Slepecky and Chamberlain, 1983). Contractile force generation may occur in the region of the basilar cone of each cell: microtubules attach to basilar cones and many mitochondria are located in their vicinity (Angelborg and Engstrom, 1972). It is postulated that under certain conditions, microtubules may undergo some depolymerisation, creating a relaxed state. In this state, an actin-based contractile mechanism could then generate a contractile force (Slepecky and Chamberlain, 1983)

which would have much greater influence on the length and shape of a pillar cell.

If some microtubule depolymerisation occurs, this may suggest the mechanism of tempered instability of microtubules (Sammack and Borisy, 1988) predominates within inner pillar cells. This would create a suitably relaxed condition of inner pillar cells allowing modulation of hair cell excitation in response to acoustic stimulation as proposed by Slepecky and Chamberlain (1983).

Opportunities for Further Analysis.

In further work on this material, it would be advantageous to reduce the detergent concentration (to around 0.06% Triton X-165) in the presence of tannic acid. This would decrease the degree of extraction of microtubules and increase the percentage remaining to permit a larger population to be analysed.

As discussed above, Slepecky and Chamberlain (1983) found that 6 nm filaments were composed of actin in pillar cells of the chinchilla organ of Corti. Are 6 nm filaments composed of actin in mouse pillar cells? Do all 6 nm filaments have the same polarity as in *Drosophila* (Mogensen and Tucker, 1988)?

It would also be beneficial to use the tubulin hook decoration technique described by Euteneur and McIntosh (1980) and subsequently used to determine microtubule polarity in *Drosophila* transalar arrays (Mogensen *et al.*, 1989). This would provide a more substantial indication of the polarity of the

microtubules. Hence, this would indicate, more strongly, whether the nucleation sites are situated solely at the apical region or not.

Importantly, the number of protofilaments which are present in microtubules associated with the centrosome should be analysed. Are they composed of 13 protofilaments as would be expected from previous work on microtubules of about 24 nm diameter?

Transalar microtubule arrays in *Drosophila*, when treated with reagents such as taxol, produce microtubule arrays which are reduced in their protofilament number i.e. predominantly 12 protofilaments (personal communication - Dr M.M. Mogensen). Since these two transcellular systems appear similar, it would be interesting to note if a similar occurrence is observed in the pillar cells of the organ of Corti. Due to the nature of this experimental protocol, culturing of the material *in vitro* is necessary. It was therefore necessary to evaluate whether the organ of Corti, when cultured *in vitro*, undergoes any alteration to the composition of its transcellular microtubule array compared to the normal tissue i.e does the array assemble in a normal fashion?. It was also necessary to ascertain how long the organ of Corti could be maintained in this environment before cellular alterations and tissue deterioration begin to occur. This aspect of the investigation is dealt with in Chapter 2.

The transcellular microtubule array of the inner pillar cell has now been described. However, what is the overall arrangement of microtubules in the outer pillar cell? Do the microtubules elongate solely from the apical region, the basal region or, from both apical and basal regions of the cell? Are the microtubules nucleated primarily by a centrosome and then secondarily, form an array which is nucleated independently of the centrosome? Is there a

similar location of MTOC-like material sub-apically in the outer pillar cell as has been found in the inner pillar cell? These important issues are dealt with in Chapter 3.

Chapter 2.

Microtubule Organisation after *In Vitro* Culture.

Introduction.

The aims of the investigative work reported in this chapter were as follows :

i) Do the inner pillar cells follow a similar pattern of differentiation when the organ of Corti is cultured *in vitro* as that which progresses *in vivo*?

ii) Are the numbers of microtubules which assemble for the transcellular array similar during culture *in vitro* to those which are formed *in vivo*?

iii) How long can the organ of Corti be maintained in *in vitro* cultures before deterioration of cell organisation occurs?

Results.

A) Pillar Cell Organisation Following Culture, *In Vitro* of 1 Day-old Organs for a Further Two Days.

Thick (1 μm) sections stained with methylene blue have a similar appearance to *in vivo* material. The general histology of the organ of Corti is unaltered. The apices of pillar cells which are still located to either side of the groove at the apical surface of the organ, contain substantial bundles of microtubules. Hair cells have retained their relative positions: there is still one row of inner hair cells and three rows of outer hair cells. Pillar cells are still situated between the row of inner hair cells and the first row of outer hair cells. Hair cells retain their spherical shapes and are supported by phalangeal processes of Dieters' cells as described in Chapter 1.

B) Pillar Cell Organisation Following Culture, *In Vitro* of 3 Day-old Organs For a Further Three Days.

Within this material, large numbers of vesicles are present in all cell types of the organ of Corti. The tissue has otherwise, retained its overall morphology as described in above.

Many thick (1 μm) sections were analysed throughout hundreds of micrometres: large numbers of vesicles with an ultrastructural similarity to lysosomes are present throughout the organ of Corti. Any further investigation of this material by electron microscopy was not pursued because of this marked deterioration. Microtubule bundles could not be observed distinctly in methylene blue stained sections.

Microtubule Arrangement of 1 Day-old Organs Further Cultured For 2 Days *In Vitro.*

Within the sub-apical region lies the centriole, which is associated with a cilium and its associated basal body. Many microtubules, which have diameters of about 21 nm, radiate from the pericentriolar material. These microtubules do not, however, associate with the microtubules of the transcellular array. The microtubules of this population lie parallel to the apical surface of the cell and perpendicular to the assembling transcellular array.

Apically in the cell, in the region of the sub-apical annulus, two microtubule populations are visible - the centriole-associated microtubules and microtubules of the transcellular array. These two populations clearly have different orientations (Figs. 18 and 19). Microtubules are tightly packed to form the array while those which are centriole-associated are seen radiating from around the centriole (Fig. 20).

Microtubules are well aligned and form a distinct bundle in each inner pillar cell (Fig. 17). These microtubules do not reach the cell apex: they

terminate just below the apical plasmalemma at sub-apical sites on the lateral membranes of the cell (Figs. 18 and 19). These sites form an annular-like structure around the lateral plasmalemma. The array which is forming lies in a plane parallel to the longitudinal axis of the inner pillar cell.

The transcellular array is very similar to that of 3 days *in vivo*; the microtubules (which have a diameter of about 28 nm) have formed a tubular-like configuration within the cell (Fig. 22a-c). Small fascicles of microtubules are observed within this tubular structure (Figs. 21 and 22). Between the microtubules, dense intertubular material is present and which contains distinctive 6 nm diameter filaments (Fig. 21).

In transverse sections of cultured material, many more organelles are seen apically. Mitochondria are observed between the fascicles of microtubules just below the sub-apical annulus. Throughout the inner pillar cell cytoplasm, lysosome-like organelles are present (Fig. 22a-c). They are large electron dense spherical organelles which are approximately 1 μm in diameter. These lysosome-like organelles are also observed in both types of hair cells and Deiters' cells.

Figure 17.

Longitudinal section through inner pillar cells, 1 Day old and cultured for 2 days *in vitro*.

Microtubules of the transcellular array have formed fascicles (f) but many organelles with an ultrastructural similarity to lysosomes (l) are observed in the cytoplasm.

Bar - 1 μm.

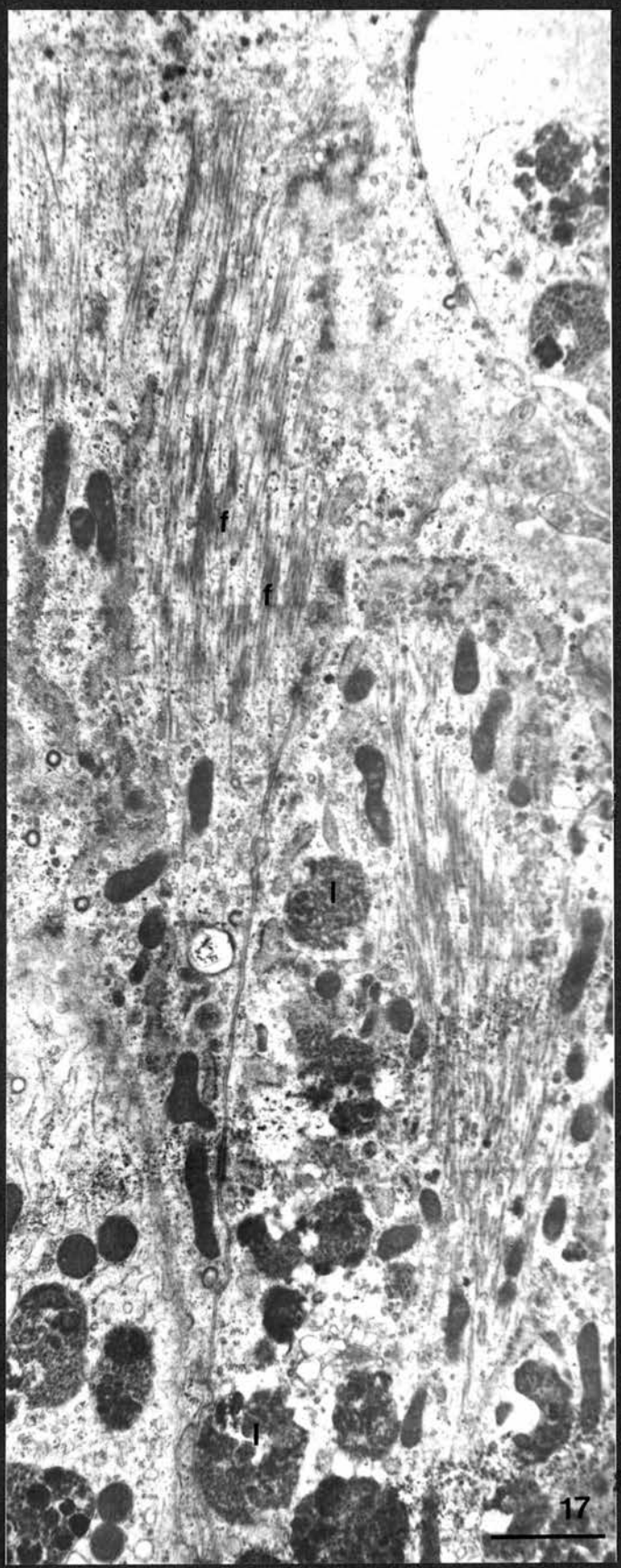

f
f
l
l
17

Fig. 18.

Inner pillar cell of 1 Day old organ cultured for 2 Days *in vitro*.

Microtubules (m) of the assembling transcellular array are aligned in the longitudinal axis of each cell. They do not associate with the centriole (c).

Many more microtubules (a) are associated with the centriole than in 3 Day old inner pillar cells that have not been cultured

Bar - 1 µm.

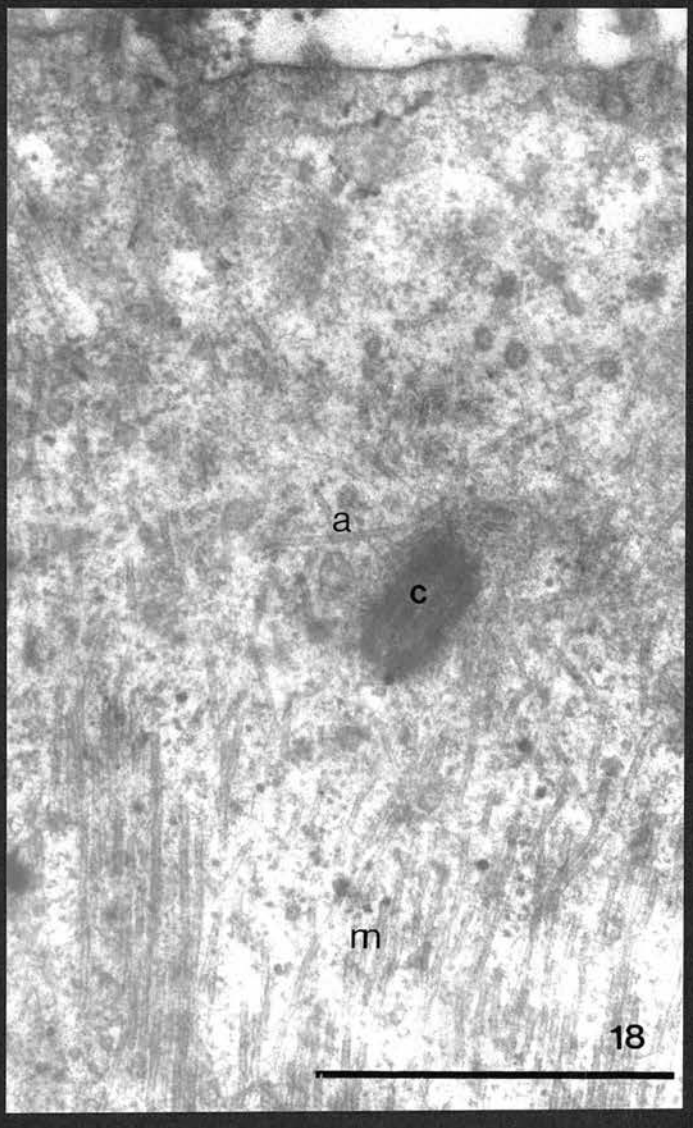

a
c
m
18

Figure 19.

Inner pillar cell of 1 Day old organ cultured for 2 Days *in vitro*.

Transcellular array microtubules (m) are aligned in the longitudinal axis of the cell. These do not associate with the centriole (c), its PCM or the basal body of the cilium (b). Microtubules (a) with a different orientation are associated with the centriole and its PCM.

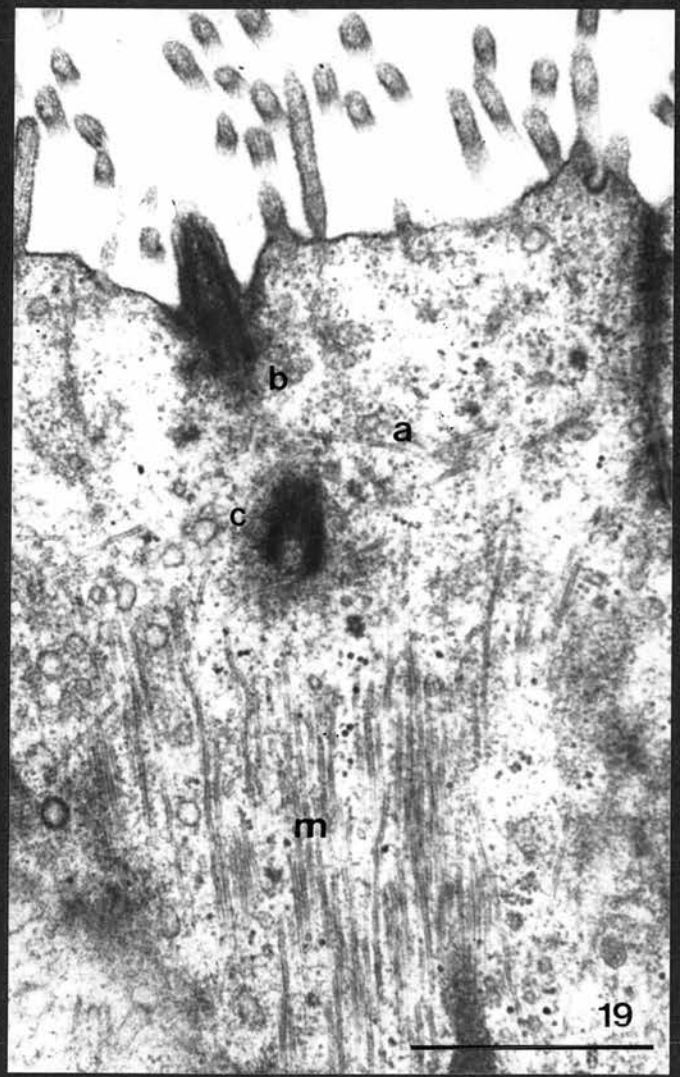
Bar - 1 μm.

Figure 20.

Inner pillar cell of 1 Day old organ cultured for 2 Days *in vitro*.

Microtubules (a) associated with the centriole (c) have diameters of about 21 nm. Dense fibrous material (d) similar in density to PCM is associated with the lateral plasmalemmae.

Bar - 0.5 μm.


b
a
c
m
19

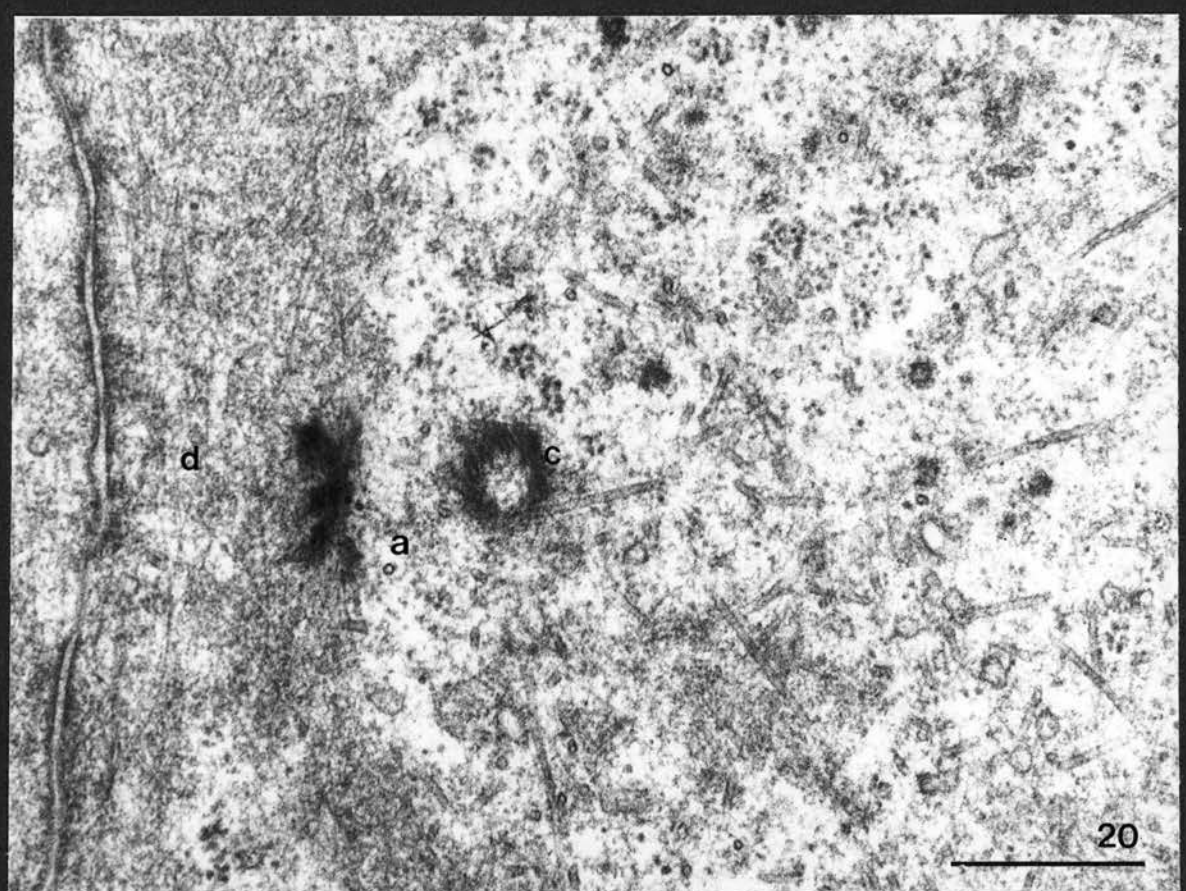

d
c
a
20

Figure 21.

Inner pillar cell of 1 Day old organ cultured for 2 Days *in vitro*.

Microtubules (m) of the transcellular array have diameters of about 28 nm. Within the fascicles of microtubules, dense intertubular material which contains 6 nm diameter filaments (arrow) is present.

Bar - 0.5 μm.

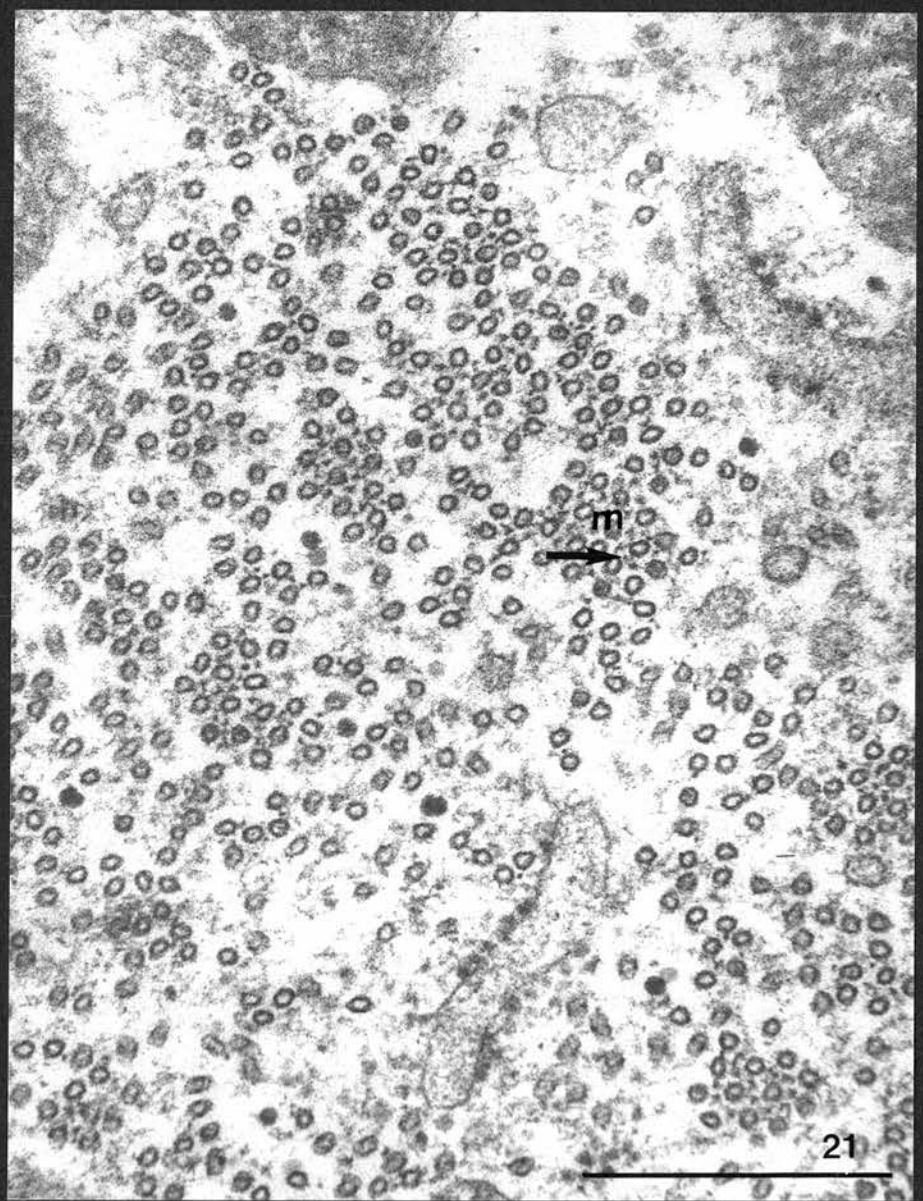

m
21

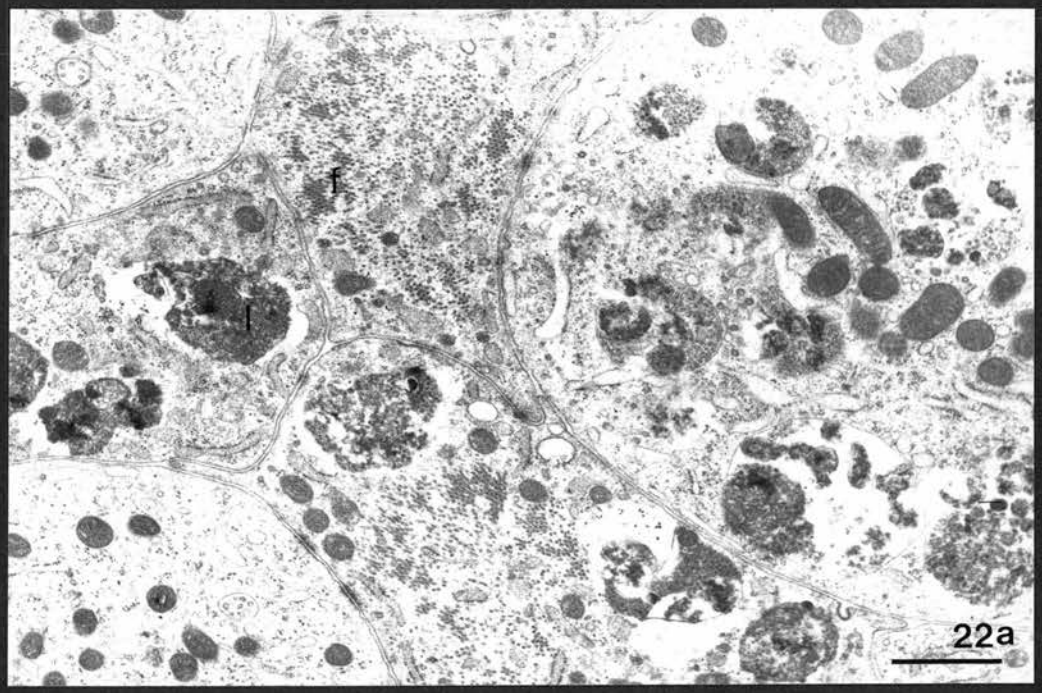
Figure 22a-c.

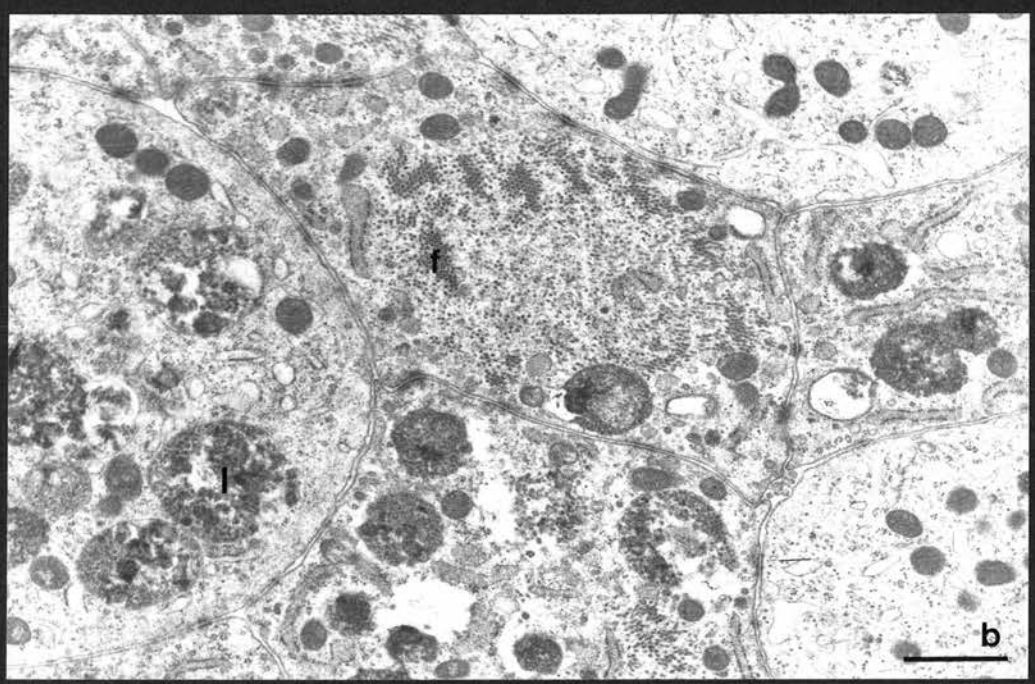
Inner pillar cell of a 1 Day old organ cultured for 2 Days *in vitro*.

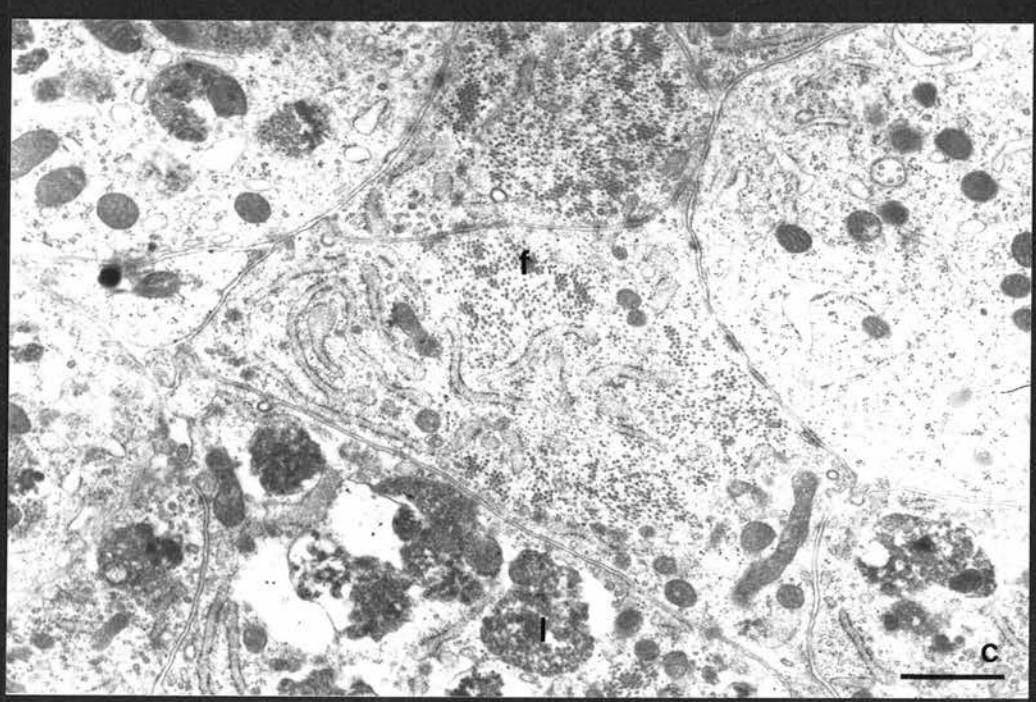
Apically (a), microtubules are tightly packed. This arrangement becomes more 'tubular' lower in the cell (b) but fascicles (f) are still retained. Lower (c), few fascicles remain and microtubules are diffusely scattered.

Lysosome-like organelles (l) are present in surrounding cells and at all levels of sectioning.

Bar - 1 μm.


f
l
22a


f
l
b


f
l
c

Discussion.

General Morphology of the Organ of Corti After Culturing *In Vitro.*

From the data presented above, the *in vitro* culture technique is successful in maintaining overall morphology of the organ of Corti for 2 days if isolated 1 Day after parturition.

However, if the organs, in a more advanced state of differentiation are cultured, *in vitro*, for more than two days, without the addition of fresh complete medium, alterations to the cell structure become apparent. The microtubule bundle cannot be observed in longitudinal sections because of the high concentration of vesicles which have an ultrastructural similarity to lysosomes within the inner pillar cells. It is not clear from these studies whether this marked deterioration is caused by the longer time in culture or, is due to the more advanced state of the organ. However, it would seem reasonable to assume that lysosomes are present as some cellular degeneration has occurred. This is most obviously indicated by the reduced numbers of transcellular microtubules within inner pillar cells after culturing one day old tissues for two days *in vitro* (see Table 1). Further degeneration occurred after culturing for three days *in vitro* : cellular degeneration was so extensive at this stage that transcellular microtubule arrays could not be observed by light microscopy. Histochemical techniques would be required to establish that these organelles are, in fact, lysosomes.

Cytoplasmic organelles, such as mitochondria, are more concentrated near the apices of the cells after culturing. This is in contrast to tissue developed entirely *in vivo*, where mitochondria are predominantly located at more basal levels in the cells.

Structure of the Microtubule Array and its MTOC.

The data presented above indicates that two populations of microtubules are present within the inner pillar cell after culturing *in vitro*. The centriole-associated microtubules have diameters of about 21 nm. The microtubules of the transcellular array are not centriole-associated and have diameters of about 28 nm.

The transcellular array in the inner pillar cell has retained its overall architecture. The numbers of microtubule profiles decrease at lower levels in the cell. This is similar to the findings described in Chapter 1.

Importantly, the assembly of the transcellular array has not been arrested by the culture procedure. The assembling array, after culturing for 2 Days *in vitro*, has continued its development from that observed at Day 1 (see Fig. 6, Chapter 1). Microtubules have a tubular-like arrangement which is similar to that found at Day 3 (Fig. 7, Chapter 1). However, the number of microtubules within the inner pillar cells is substantially less after being cultured *in vitro* compared to those which assemble *in vivo*: the number has been reduced to approximately half (see Table 1).

Microtubules of the transcellular array in cultured organs, like those which elongated in organs which developed *in vivo*, are apparently nucleated at sub-apical sites, in close proximity to the lateral plasmalemma. This diffuse electron dense material has a similar composition and distribution around the lateral plasmalemma to that described in Chapter 1.

Thus, it would appear that the *in vitro* tissue culture protocol has no adverse effects on the apparent change from centriole-associated nucleation sites in the inner pillar cell to those which are plasmalemma-associated. The only effects which were observed are that the assembly of the transcellular array has been inhibited slightly and perhaps, more importantly, many more microtubules remain in the vicinity of the centriole after culturing than at the

equivalent *in vivo* age. This may suggest that the annulus which appears to nucleate the assembling transcellular array microtubules is derived from PCM but this translocation of PCM from its pericentriolar position to the lateral membranes is less complete *in vitro* than *in vivo.*

Russell and Richardson (1987) have reported that hair cells of mouse explant cultures, maintained up to 5 days *in vitro*, retain their morphological integrity. However, they also exhibit some distinctive embryological features such as cilia. The data presented above shows that, after culturing, the pillar cells retained similar features i.e. cilia, a reduced number of microtubules and large numbers of cytoplasmic organelles apically in the cells compared to those samples of material of an equivalent *in vivo* age.

It has been reported that mouse organ of Corti explants can be maintained for up to 27 days *in vitro* (Sobkowicz *et al.*, 1975). Replacement of fresh complete medium occurred at 2-3 day intervals. They report that the highly organised structural pattern of the tissue remains until 2 weeks after explantation.

It would appear from the results presented above, and comparing them with the findings of Russell and Richardson (1987), that in the stated culture conditions, certain embryological features such as cilia, kinocilia and a reduced number of microtubules are retained. Thus, it would appear that the culture system delays normal development. However, by gradually substituting saline for mouse embryo extract, Sobkowicz *et al.* (1975) found that normal development occurs. Thus the presence of embryonic factors within the embryo extract may not allow the normal processes of development to occur.

Further Analysis of the *In Vitro* Culture Technique.

In future work, it may be beneficial to evaluate an effective regime for replacement of complete medium. The results obtained, and from those described by Sobkowicz *et al.* (1975) indicate that the tissue would require the addition of complete medium after 2 days. This may reduce the cellular changes which take place at around 3 days *in vitro* and hence permit long term cultures without the deterioration in cellular organisation which has been described and discussed in this chapter. However, more importantly, it may also be of benefit to reduce the concentration of mouse embryo extract at each stage of medium replacement (as carried out by Sobkowicz *et al.*, 1975). The embryonic factors are reduced in concentration at each replacement stage in a similar manner to their reduction *in vivo*. Would this produce near normal development of the transcellular microtubule array within the inner pillar cells of organs of Corti cultured *in vitro*?

Microtubules of the inner pillar cell array, when cultured *in vitro*, are nucleated sub-apically at the lateral plasmalemma and have diameters of about 28 nm. Array microtubules which developed *in vivo* have similar diameters and are probably composed of 15 protofilaments. In future studies, the protocol for the visualisation of microtubule protofilament number (see Section iv , Materials and Methods) should be utilised. This would confirm that microtubules of the transcellular array which have diameters of greater than 24 nm, are probably composed of more than 13 protofilaments.

It would appear, from the results, that, if required, the organ of Corti could be maintained, in a reasonably healthy state using the *in vitro* culture procedure. Importantly, microtubule nucleation and assembly proceeds, in a near normal manner, while in culture.

This would allow the tissue to be treated with reagents, such as taxol, which alters the pattern of microtubule assembly and MTOC action in some instances. Would this result in microtubules composed of 12 protofilaments as has been found in the transcellular microtubule array of Drosophila wing epidermal cells (personal communication - Dr M.M. Mogensen)?

Other drugs which interfere with and/or inhibit microtubule assembly such as colchicine and nocodazole could also be exploited to assess if informative perturbations could be induced. Similarly, cytochalasin B could be used to disrupt 6 nm diameter actin filaments and assess the effects of disrupted filaments on the microtubule array.

Chapter 3.

Initial Stages in the Assembly of the Microtubule Array of Outer Pillar Cells.

Introduction.

The main objectives of the investigation reported in this chapter were as follows :

i) Is assembly of the microtubule array in an outer pillar cell comparable to assembly of the array in an inner pillar cell?

ii) Does an outer pillar cell have plasma membrane-associated nucleating sites similar to those of an inner pillar cell?

iii) Do microtubules in an outer pillar cell elongate in the same direction as those of an inner pillar cell?

iv) Do microtubules of an outer pillar cell have diameters of about 30 nm, or, do they have "conventional" diameters of about 24 nm?

v) How many microtubules assemble to construct the transcellular bundle in an outer pillar cell?

Results.

General Histology of the Organ of Corti.

The histology has been discussed in Chapter 1 (see pages 22-23). However, the tunnel of Corti between the pillar cells has not opened by Day 6. The spaces of Nuel which are present, in the mature organ, between the first row of outer hair cells and the outer pillar cells have, however, opened.

Day 0.

Within an outer pillar cell, few, if any, microtubules of its transcellular array have assembled by this stage. No centrosomally-associated microtubules are observed within the apical region of an outer pillar cell.

Day 1.

Little, if any, assembly of the transcellular microtubule array has occurred. Only a few microtubules of the array were observed oriented parallel to the longitudinal axes of most of the cells which were examined. Those microtubules which were detected were situated near the lateral plasmalemma adjacent to a first outer hair cell. The region of cytoplasm in an outer pillar cell, near where the inner and outer pillar cell membranes are closely apposed, was apparently devoid of microtubules.

Day 3.

General Morphology of Outer Pillar Cells.

Outer pillar cells of the organ of Corti lie between the row of inner pillar cells and the first row of outer hair cells.

Only a small turret-like process of each cell emerges on the apical surface of the organ of corti (Figs. 24 and 30). It is this turret which appears to be important in the nucleation and assembly of transcellular microtubules for about 5 μm below the turret region, each outer pillar cell has a triangular cross-sectional profile.

At this stage only a few microtubules are present in the apical portions of each outer pillar cell. They are clustered in small fascicles in the lateral ridge. These microtubules are scattered throughout the cytoplasm in this region (Figs. 23 and 24). The numbers of microtubules present within the transcellular array is highest in the apical region of each cell and this decreases progressively at successively lower cross-sectional levels (see Table 1 and Fig. 26a-c). Dense intertubular material containing 6 nm diameter actin filaments is present within the assembling array.

Microtubules with Different Diameters and their Sites of Nucleation.

Apically, an outer pillar cell contains a cilium with its associated basal body and, in close proximity, a centriole. Microtubules with diameters of about 21 nm are associated with the centriole and the basal body of the cilium (Fig. 25).

Importantly, the microtubules of the transcellular array are not associated with the centrosome. These microtubules, which have diameters of about 28 nm, are present in small fascicles which project from sub-apical sites. These sites are composed of dense material. They are predominantly situated on the lateral plasmalemma which is adjacent to the first outer hair cell. This sub-apical site is also observed on the membrane adjacent to the inner pillar cell. However, few microtubules are associated with this latter site.

Day 6.

General Morphology of Outer Pillar Cells.

As has previously been described above, in 3 Day old tissue the outer pillar cell has a triangular cross-sectional shape. A lateral ridge-like process passes between the first outer hair cells and the first-formed portion of the transcellular microtubule array lies within this ridge (Figs. 29a-c, 30 and 31). The region between the array and the cell membrane facing the adjacent inner pillar cell is devoid of microtubules.

Few cytoplasmic organelles are observed apically in the cell but the sub-apical region is packed with microtubules (Fig. 27). Mitochondria, Golgi bodies and rough endoplasmic reticulum are present at lower levels where the microtubules are more widely spaced throughout the cytoplasm.

Microtubules with Different Diameters and their Sites of Nucleation.

During Day 6, the microtubule array almost entirely fills the sub-apical region of an outer pillar cell. Few small fascicles of microtubules are observed (Fig. 27) because the microtubules are so tightly packed throughout the array. The cell is considerably narrower in this region than at lower cross-sectional levels (Fig. 29a-c).

At more basal levels, the transcellular microtubule array is composed of several small fascicles of microtubules (Fig. 28). Dense intertubular material is situated between the microtubules and this includes 6 nm diameter filaments (Fig. 28). This configuration is found at all levels until those just above the nucleus about 15μm below the cell apex. Just above the nucleus the microtubules are relatively widely spaced throughout the cytoplasm, although some small bundles consisting of a few microtubules are present (Fig. 29c). Microtubule numbers in each cell cross-section progressively decrease at successively lower levels along the length of the cell (see Table 1, Fig. 29a-c).

Dense granular material is present in the apical region of each cell; microtubules are associated with this (Fig. 27) and have diameters of about 28 nm. The material is similar in electron density and ultrastructural composition to pericentriolar material (Fig. 27). Microtubules associated with the centriole have a different orientation to that of the microtubules of the assembling longitudinal array. Furthermore, the centriole-associated microtubules are about 21 nm in diameter which contrasts with the 28 nm diameter microtubules of the assembling array. Microtubules present in the first outer hair cell have diameters of about 21 nm. The difference in microtubule diameters between those of an outer pillar cell array and a first outer hair cell can be directly compared in Fig. 28.

Analysis of Microtubule Protofilament Number.

Cross-sectional profiles of microtubules, when fixed, after detergent extraction, in the presence of tannic acid may reveal the presence of protofilaments which make up the microtubules. After this treatment few microtubules remained in outer pillar cells. These were either not cut in good cross-section and/or the negative staining of microtubule profiles with tannic acid was not adequate to clearly reveal protofilaments. Hence, the protofilament numbers were not obtained for these microtubules.

Figure 23.

Outer pillar cell Day 3.

The apical turret region is observed in this section.

A few microtubules (arrow) are associated with the centriole and the basal body. Microtubules (m) of the assembling transcellular array attach at lateral plasmalemma-associated sites (d). Array microtubules form small fascicles (f).

Bar - 1 μm.

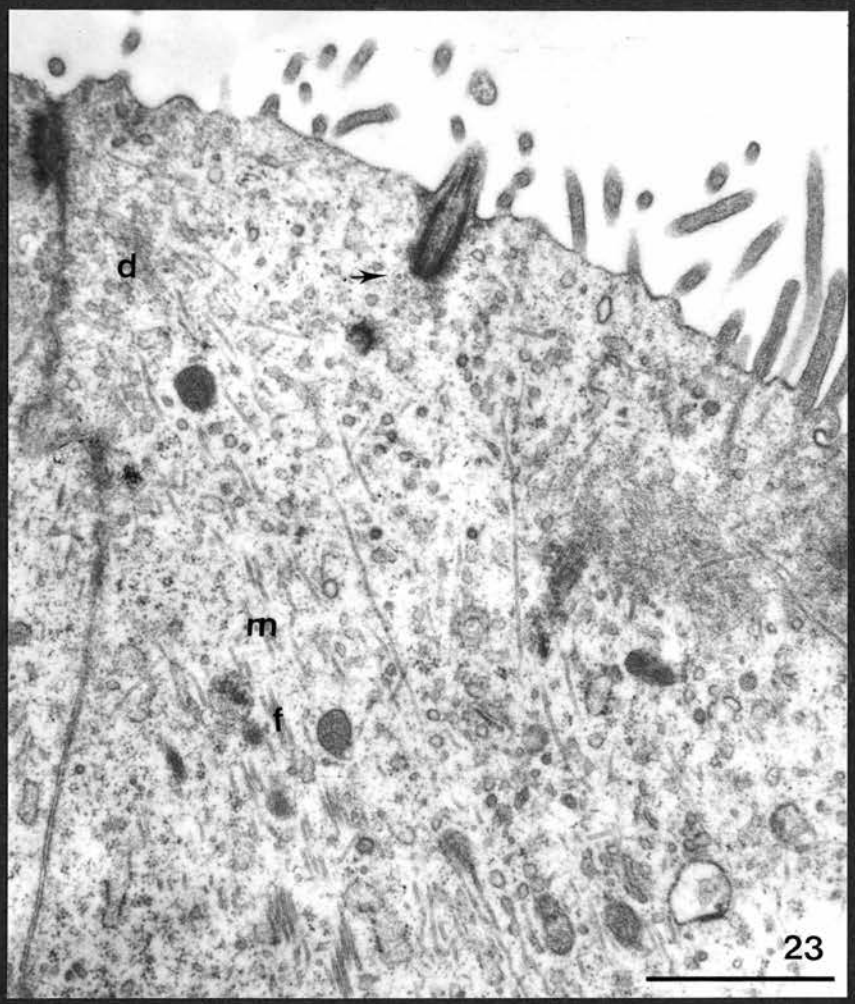
Figure 24.

Outer pillar cell Day 3.

This section is grazing through part of the apical turret (t) and grazing the edge of the transcellular microtubule array.

Dense material (d) associated with the lateral plasmalemma (p) has microtubules (m) in close proximity to it. Microtubules of the array are well aligned.

Bar - 1 μm.


d
m
f
23

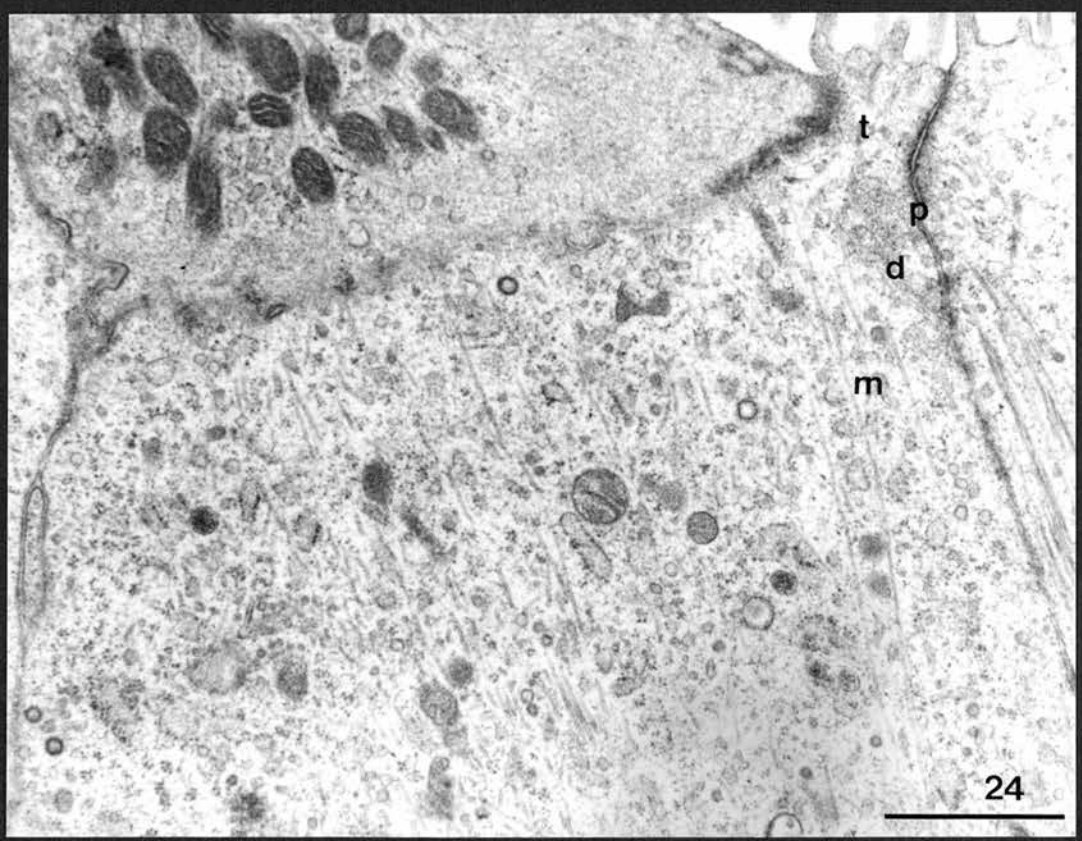

t
p
d
m
24

Figure 25. Outer pillar cell Day 3.

Microtubules (a) of small diameter (about 21 nm) are in close proximity to the centriole (c) and basal body (b).

Microtubules (m) of the developing transcellular array are not nucleated at the centriole (c) or the basal body (b).

Bar - 1 μm.

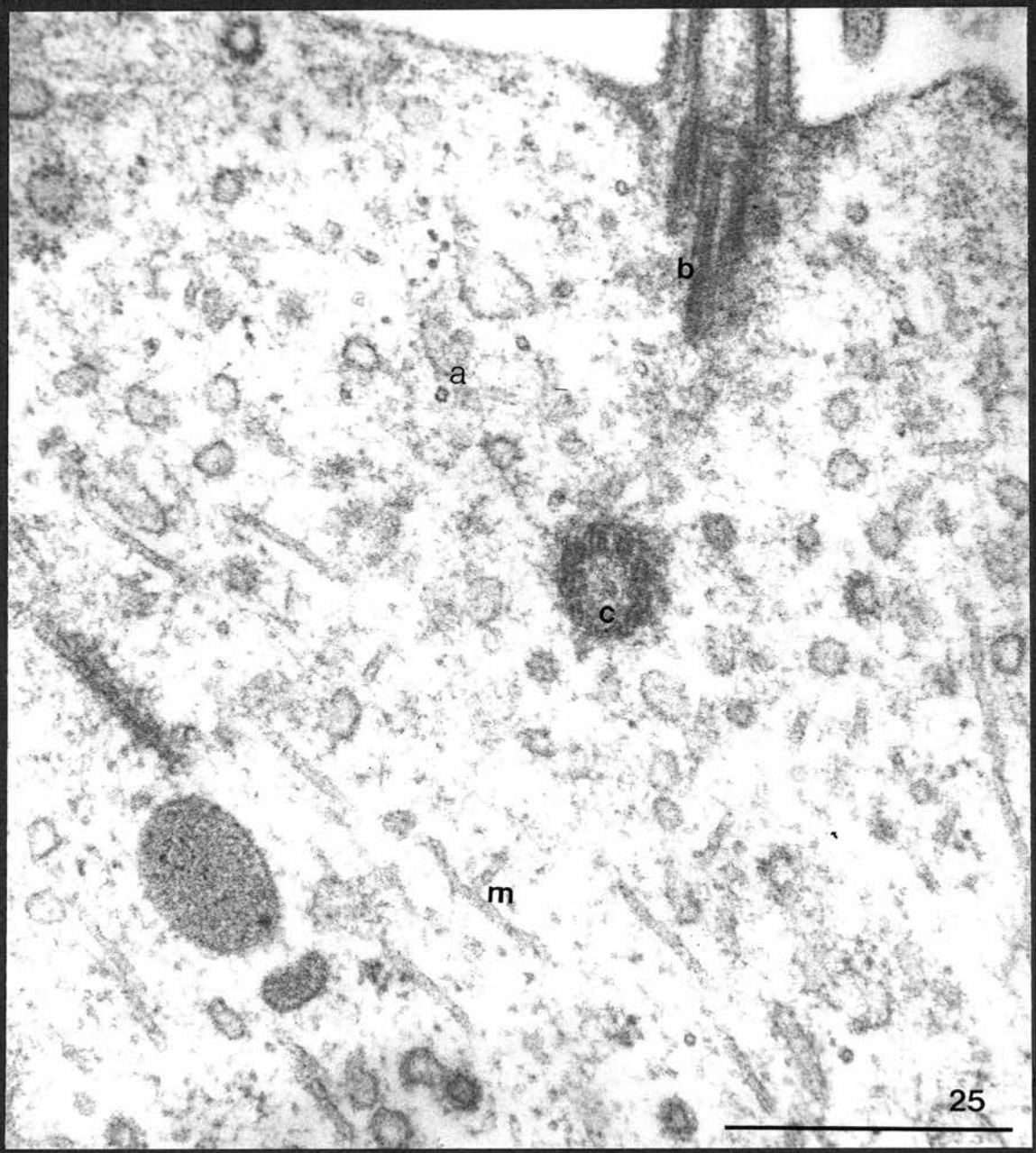

b
a
c
m
25

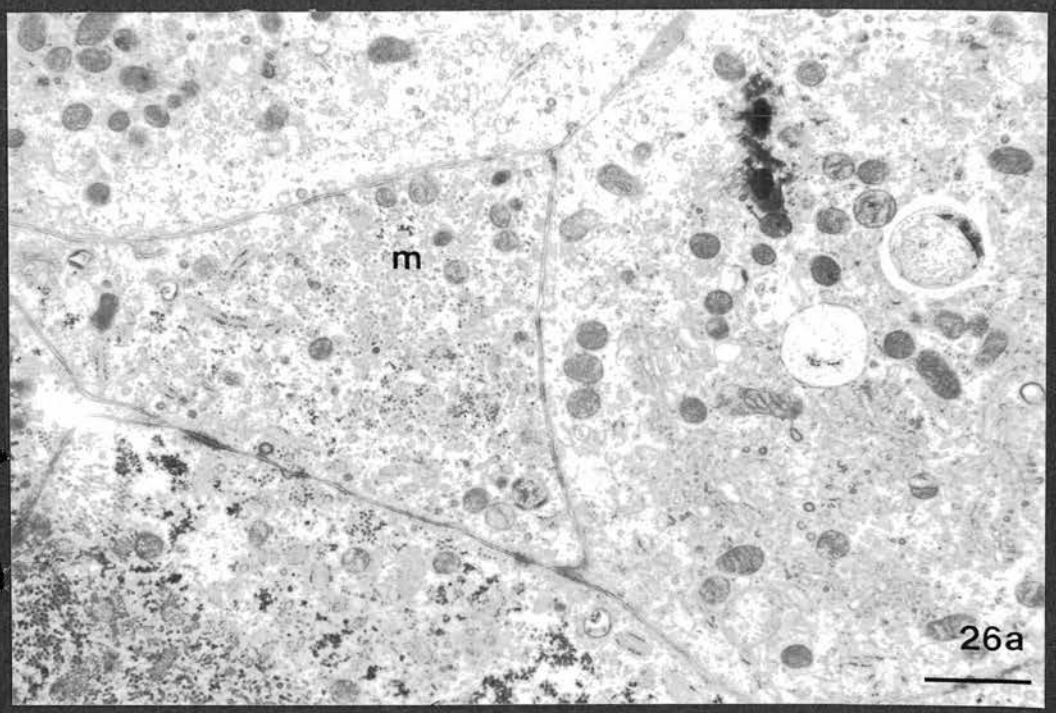
Fig. 26a-c.

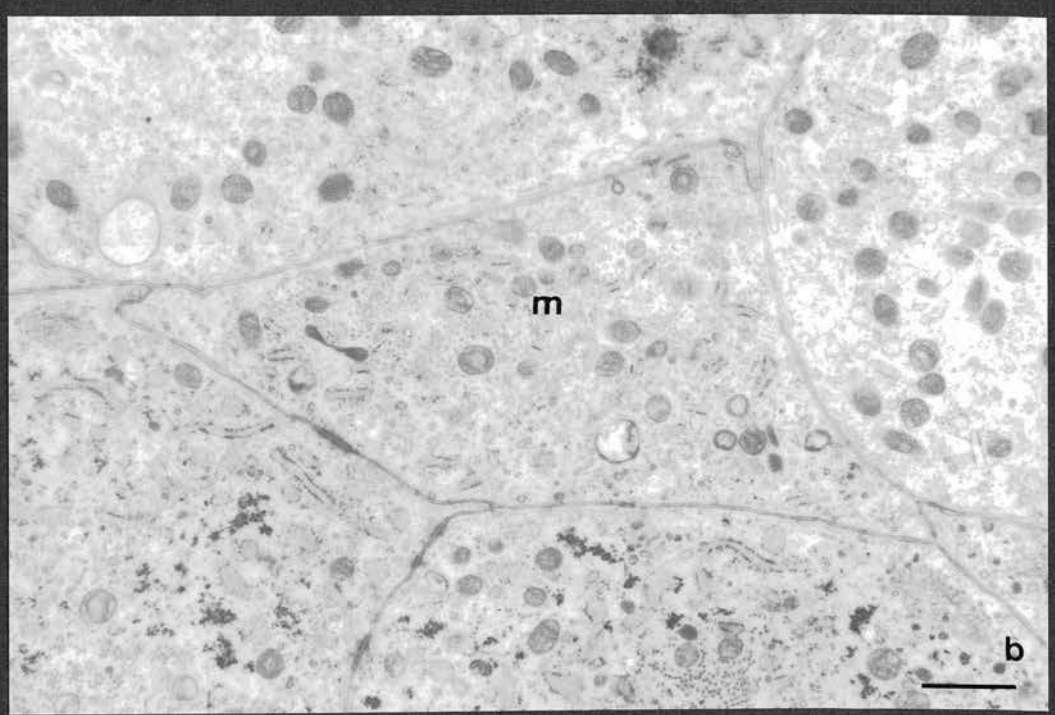
Outer pillar cell Day 3.

Microtubules of the assembling transcellular array (m) are scattered within the outer pillar cell.

Microtubule numbers are more numerous apically in each cell (a) but decrease in number at lower levels (b and c).

Bar - 1 μm.


m
26a


m
b

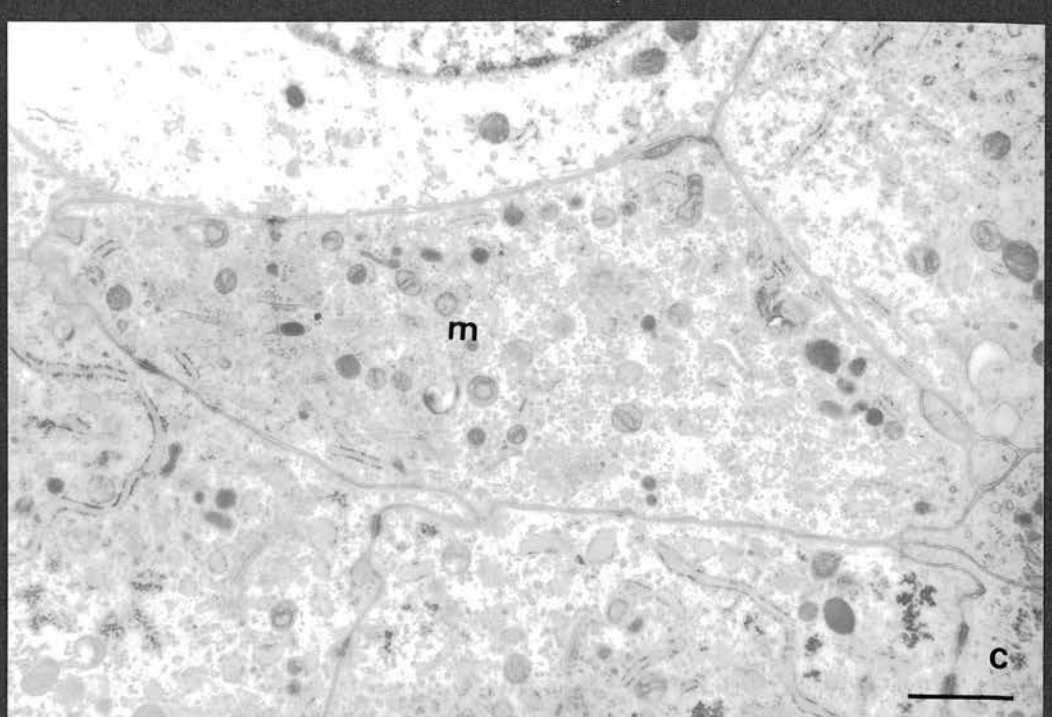

m
c

Figure 27.

Outer pillar cell Day 6.

The centriole (c) is positioned apically and few microtubules (arrow) associate with this. Array microtubules are arranged in fascicles (f). Granular electron dense material (d) is closely apposed to the lateral plasmalemma.

This section is grazing through the apical turret of an outer pillar cell. The cell, in this cross-sectional plane, is only as wide as the transcellular microtubule array. Compare this figure with Figure 29a-c, where, at lower cross-sectional planes, the width of the outer pillar cell is larger than the transcellular microtubule array.

Bar - 1 µm.

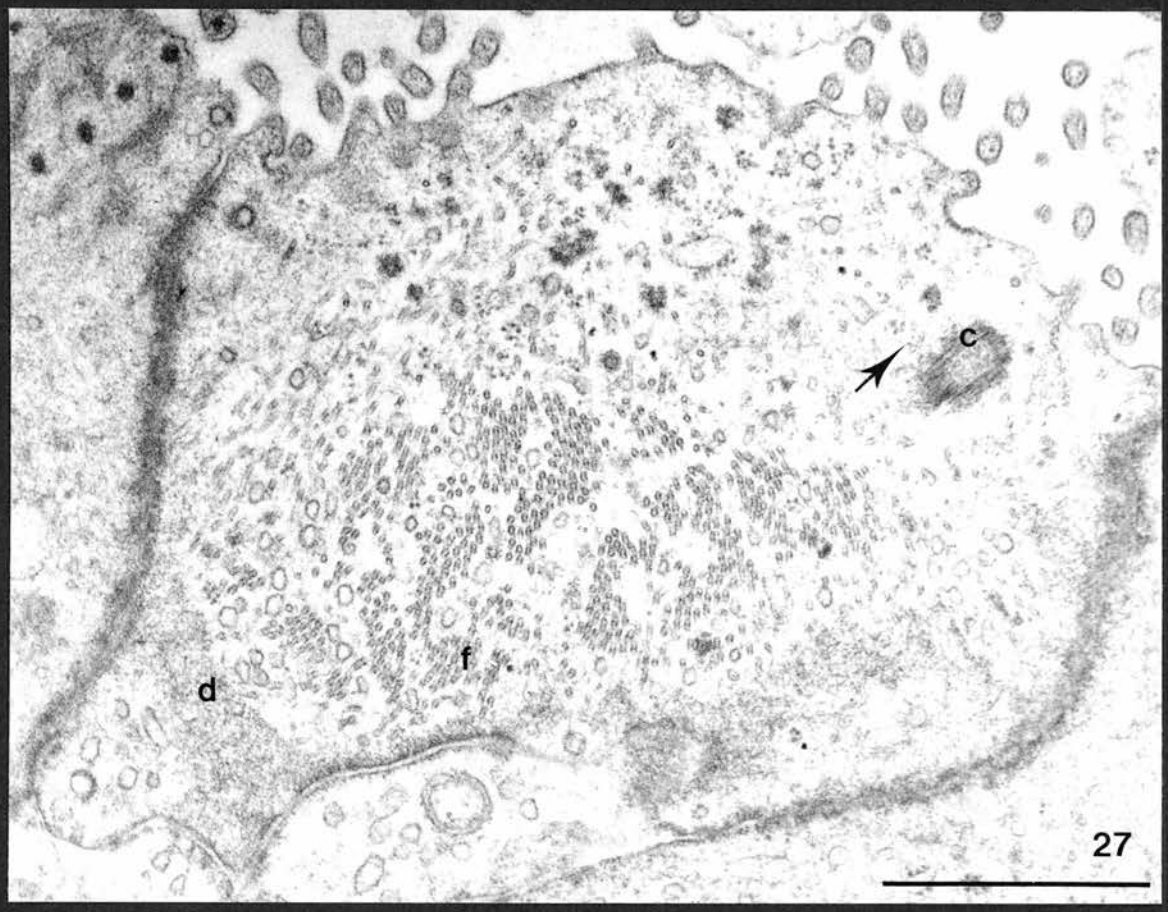

c
f
d
27

Figure 28.

Outer pillar cell Day 6.

Transcellular array microtubules (m) of the outer pillar cell have diameters of about 28 nm. Dense intertubular material lies between the microtubules and contains filaments of about 6 nm (arrow) in diameter.

The hair cell has a few microtubules of small diameter (about 21 nm) (d). Sub-membranous cisternae (which are characteristic of hair cells) are present (S).

Bar - 1 μm.

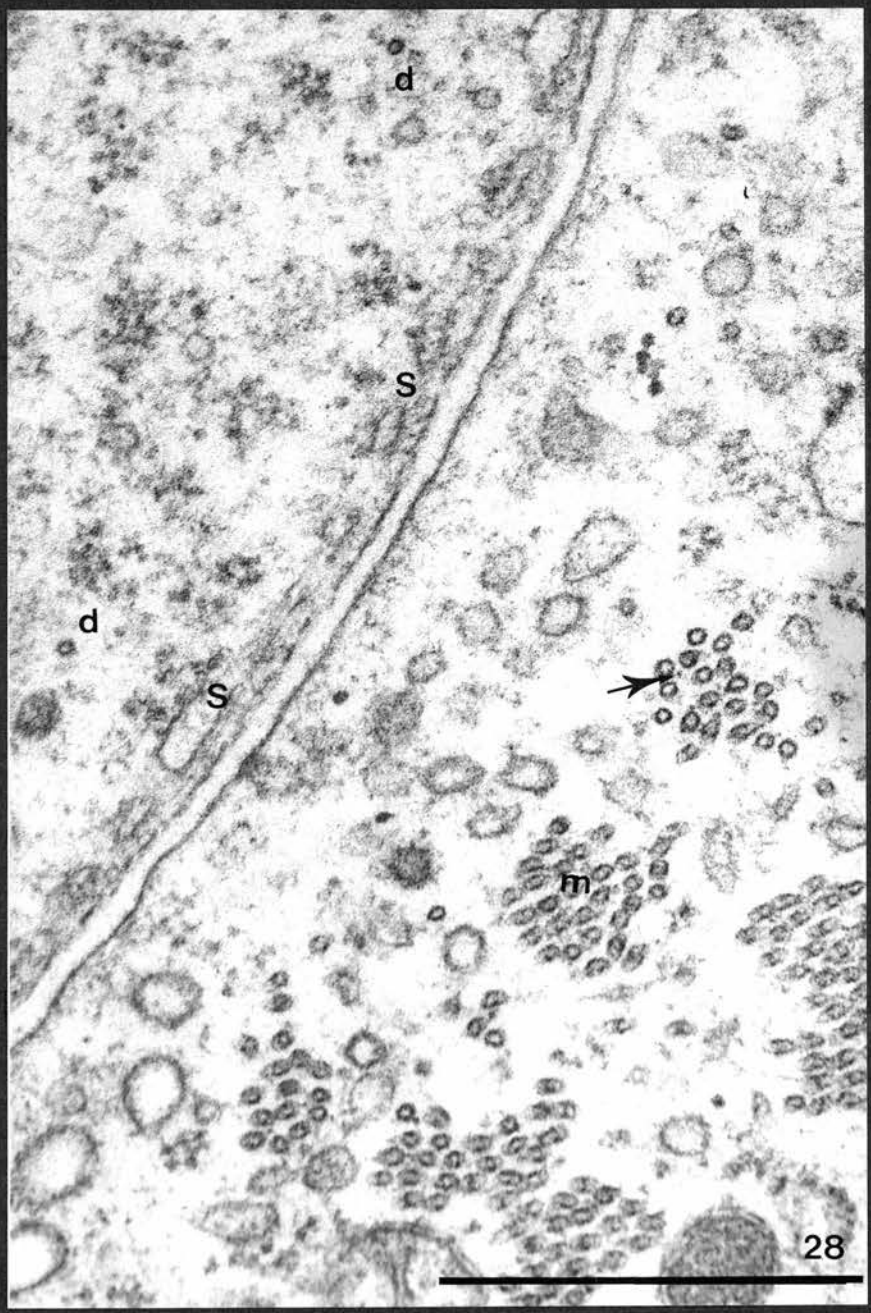

d
S
d
S
m
28

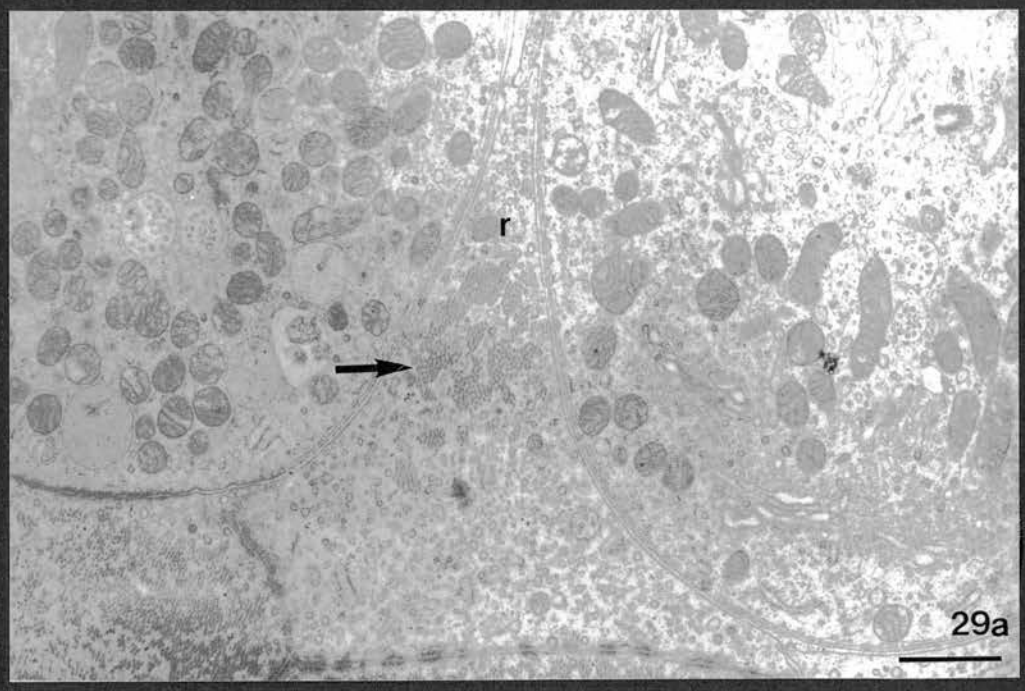
Figure 29a-c.

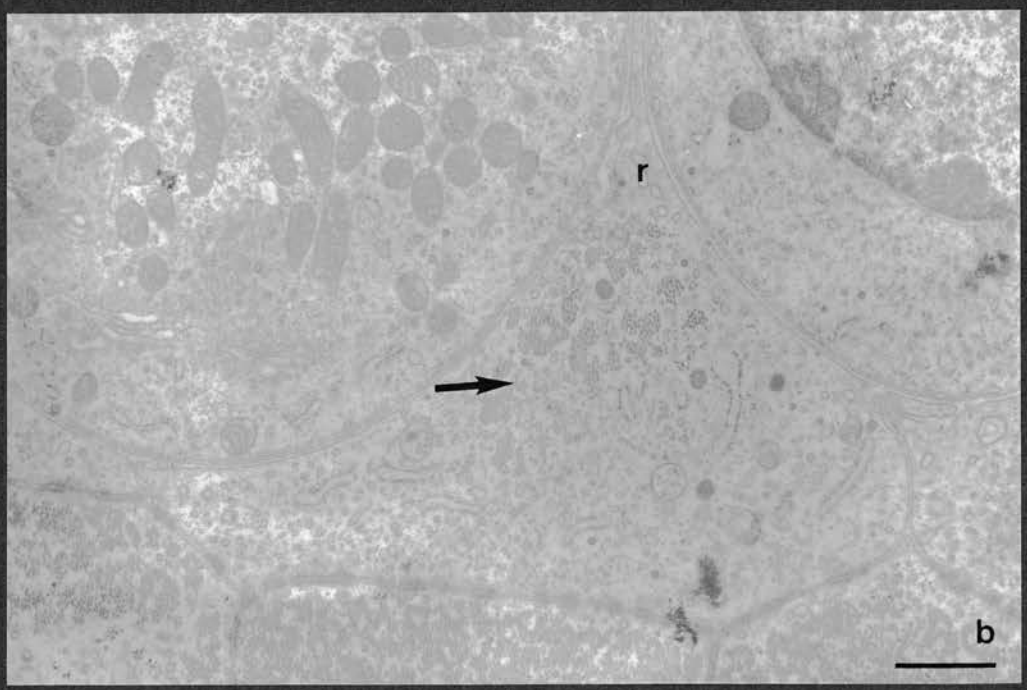
Outer pillar cell Day 6.

Microtubule numbers are high apically in the cell (a) but decrease at lower levels (b and c).

The transcellular array forms a solid rod of microtubules. Few microtubule profiles are observed between the array and the plasmalemma apposed to that of the inner pillar cell i.e. the microtubules are all clustered (arrow) in the 'tongue' (the lateral ridge)(r) between the first row of outer hair cells.

Bar - 1 μm.


r
29a


r
b

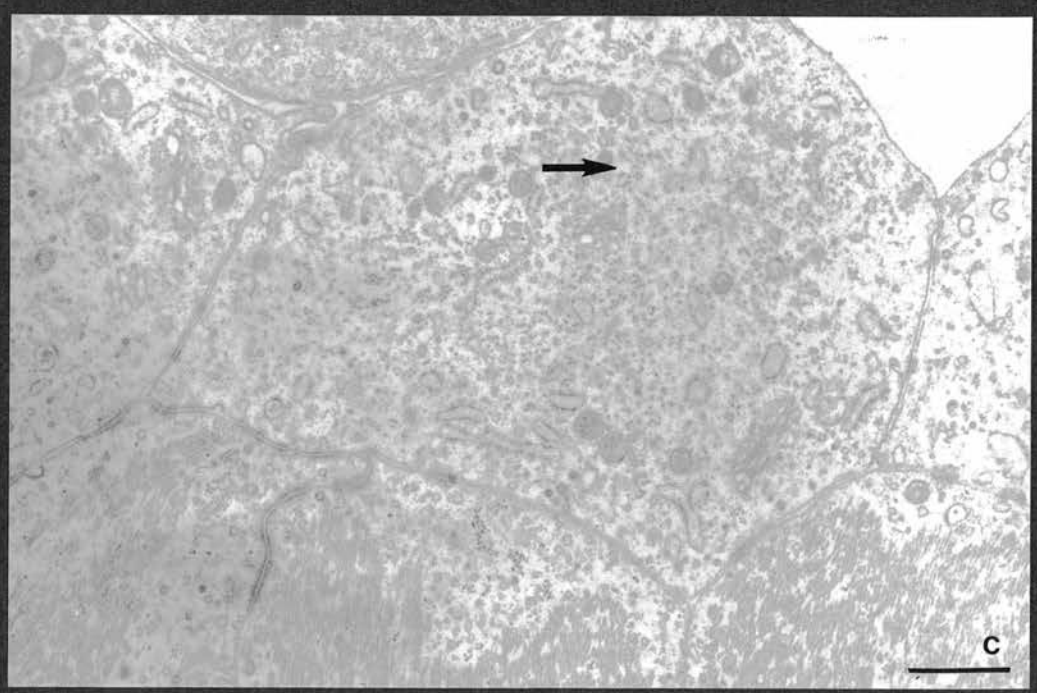

c

Fig. 30.

Schematic diagram of an outer pillar cell.

The turret (t) region is positioned apically. Outer pillar cells have a triangular shape apically with a lateral ridge (r) passing between outer hair cells of the first row.

Not to scale.

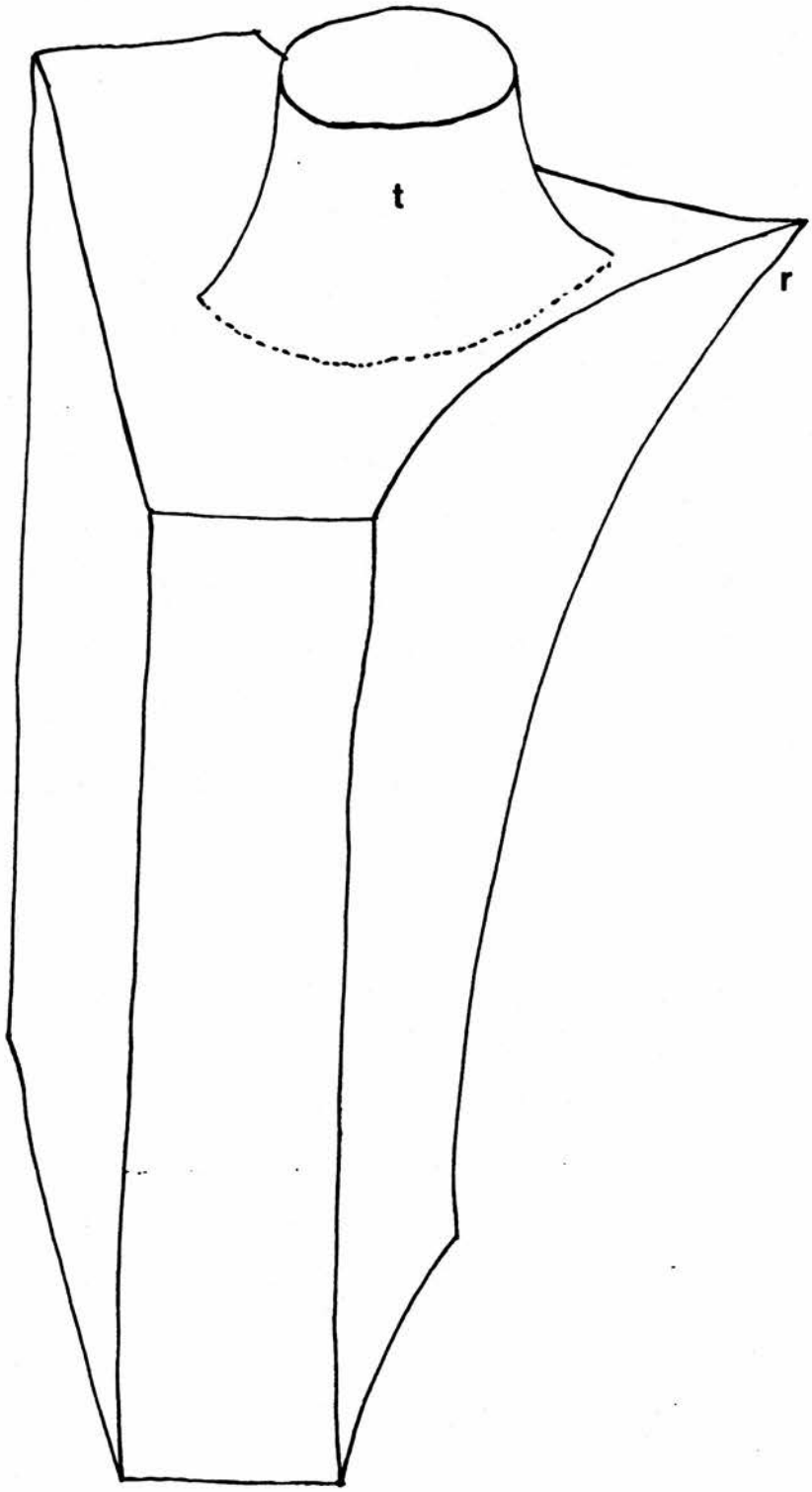


Fig.30

Fig. 31.

Schematic diagram of an outer pillar cell.

The turret region (t) contains the nucleating sites (s) for microtubules of the assembling transcellular array (m). The configuration of these sites produces a rod-like configuration of each array.

Line AB represents the cross-sectional plane illustrated in Fig. 27.

Line CD represents the cross-sectional plane illustrated in Fig. 29a.

Line EF represents the cross-sectional plane illustrated in Fig. 29b.

Line GH represents the cross-sectional plane illustrated in Fig. 29c.

Not to scale.

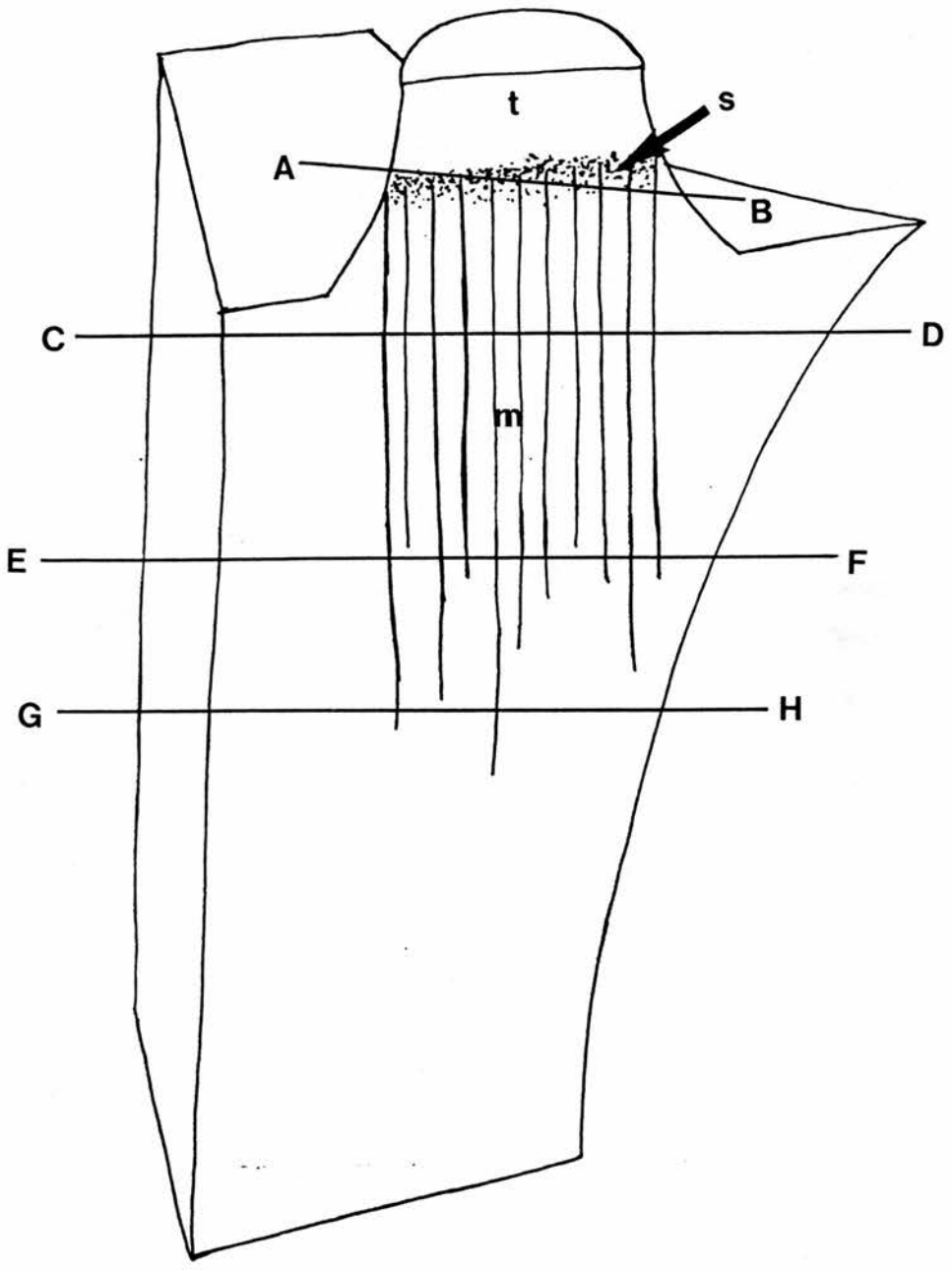


Fig. 31

DISCUSSION.

The development of the outer pillar cell of the organ of Corti of the mouse, like that of the inner pillar cell dealt with in Chapter 1, has not previously been described for the mouse or any other mammal.

Formation of the Tunnel of Corti.

The tunnel of Corti has not opened by Day 6. This contrasts to that found by Walsh and McGee (1986) who reported that the tunnel of Corti begins to open basally at Day 6. Subsequent findings suggest that the tunnel of Corti in the mice used in this investigation has not opened by Day 9 (personal communication - Neil Maidment). This may be due to the incomplete assembly of the transcellular array in each outer pillar cell. The tunnel may only open when microtubule assembly is complete. The tunnel of Corti reported by Walsh and McGee (1986) may have been mistaken for the spaces of Nuel which have opened by Day 6.

Apical Form of Outer Pillar Cells.

The apical portion of an outer pillar cell has different shape to that found for an inner pillar cell. A small turret-like process reaches the apical surface of the organ of Corti. It is this turret which contains the apical portion of the assembling microtubule array. At lower levels the cell expands into a triangular shape with the lateral ridge passing between pairs of first outer hair cells; the lateral ridge contains the elongating microtubules. The region between the microtubule array and the cell membrane adjacent to an inner pillar cell is devoid of microtubules.

Difficulty was experienced in definite identification of the outer pillar cell up to, and including, Day 1. This was because, in many instances, the apical turret region of the cell was not observed. Identification of the outer pillar cell

was not aided because of the interdigitations of the outer pillar cells and the interphalangeal processes of Deiters' cells.

Structure of the Microtubule Array and its MTOC.

In general, during days 3-6, the structure of the microtubule array of an outer pillar cell is similar to that of an inner pillar cell; the microtubules are grouped in small fascicles and there is dense intertubular material which contains filaments with diameters of about 6 nm. The major difference between the two cells is the positioning of the assembling microtubule array within the cytoplasm.

A lateral ridge of each outer pillar cell passes into spaces between outer hair cells of the first row. This contains the bundle of microtubules. The first indication that this is present is at 6 Days when small fascicles are observed. These fascicles are similar to those described and discussed in Chapter 1 in the inner pillar cell.

In the outer pillar cell, up to Day 6, the microtubule array has a rod-like configuration (Fig. 31). This contrasts to the tubular configuration of array microtubules found in inner pillar cells. The array microtubules of the inner pillar cell are found early in development from 1 Day onwards.

The microtubule array of an outer pillar cell starts to assemble slightly later than that of an inner pillar cell: it was not detectable until after 1 Day. Microtubule numbers increase in outer pillar cells from Day 3 to Day 6. The number of microtubules is highest in the apical region than at lower levels. This is a substantial indication, like that for inner pillar cells, that nucleation occurs apically within outer pillar cells and that microtubule elongation occurs in a basal direction. Microtubules which form the developing transcellular array are well aligned and the sites of nucleation appear to span, between the lateral plasmalemmas, the turret region sub-apically (Fig. 31). This assembly

pattern is similar to that observed in the developing transcellular array of an inner pillar cell (see Chapter 1). However, the major difference appears to be in the arrangement of microtubules which are nucleated at these sub-apical nucleation sites. The turret of outer pillar cells contains diffuse granular material which is similar in density and ultrastructural configuration to pericentriolar material which is present. This appears to fill this region and the apical portions of the microtubules of the assembling array are nucleated by it. This contrasts to the situation in inner pillar cells where tops of microtubules are evenly distributed between both lateral plasmalemmas in an annular arrangement around all sides of each cell.

This would suggest that the microtubules are nucleated by this dense granular material and elongate from it towards the basal region of the cell. This may also explain why the microtubules are closely packed in the array and the tubular configuration found in inner pillar cells is not generated. In addition, it would suggest a mechanism for the formation of the tunnel of Corti. The lateral plasmalemma of each outer pillar cell (adjacent to an inner pillar cell) may retract to form the tunnel of Corti. It would appear unlikely that the inner pillar membrane adjacent to the outer pillar cell would retract since it is situated closely against the microtubules of its own array.

In a number of mouse mutants with hearing abnormalities, development of the organ of Corti appears normal at birth (Kikuchi and Hilding, 1965; Osako and Hilding, 1971; Bock and Steel, 1983; Steel *et al.*, 1983; Steel and Bock, 1985). Hearing abnormalities are attributed in these cases to failure of formation of the tunnel of Corti and Nuel spaces. Gabrion *et al.* (1984) reports the failure of development of these structures to be due to the altered morphogenesis and reduced numbers of microtubules within pillar cells of hypothyroid rats. This suggests that in some mutant strains of mice there is failure of development of pillar cell transcellular microtubule arrays. If the

auditory epithelium fails to reach its normal height, perhaps mechanical coupling between the stereocilia of hair cells and the tectorial membrane (Slepecky and Chamberlain, 1983) does not occur. Hence, excitation of hair cells fails to occur in response to acoustic stimulation.

Microtubules of the outer pillar cell array, like those of the inner pillar cell (see Chapter 1), the transcellular microtubule arrays of *Drosophila* wing epidermal cells (Mogensen and Tucker, 1987) and *Drosophila* epidermal muscle attachment cell microtubule arrays (Paton, 1988) elongate from the apical region of the cell. The putative nucleating sites in outer pillar cells are also non-centrosomaly associated. They are plasmalemma-associated and, in this respect, similar to those sites described by Mogensen and Tucker (1987).

Microtubules in outer pillar cell arrays have similar diameters (about 28 nm) to those of inner pillar cell arrays. This is similar to that reported for pillar cells in the rat organ of Corti (Engstrom and Ades, 1973).

The centriole is associated with a microtubule population which has a different orientation to that of the assembling transcellular microtubule array which does not extend right up to the cell apex. The centriole-associated microtubule population lies more apically; its microtubules radiate from the centriole and the basal body. As in inner pillar cells, pericentriolar material probably acts as the nucleating site for the centrosomally-associated microtubules. However, there appears to be a dispersal of MTOC-like material within the turret of the outer pillar cell. This contrasts to that found in the inner pillar cell where the dispersal of MTOC-like material occurs to the lateral plasmalemmas only to form an annular configuration only.

In an outer pillar cell, microtubules which are centriole-associated have diameters of about 21 nm while the microtubules which are non-centrosomally nucleated have diameters of about 28 nm. These diameters are similar to

those found in centriole-associated and lateral plasmalemma-associated microtubules of inner pillar cells respectively.

Microtubule Stability within the Transcellular Array of Outer Pillar Cells.

Microtubule stability has previously been discussed in relation to inner pillar cells (see page 31). The transcellular array microtubules of outer pillar cells most likely have a similar stability: that of tempered stability as described by Sammack and Borisy (1988) This would permit a similar rearrangement of transcellular array microtubules during the final stages of development of outer pillar cells to that which may be occurring in inner pillar cells.

Microfilaments within the Transcellular Array.

Dense intertubular material and its filaments, like that described and discussed for inner pillar cells in Chapter 1, is present between microtubules of the outer pillar cell transcellular array. Filaments present within this material have diameters of about 6 nm. This dense intertubular material is similar to that observed in the rat organ of Corti (Engstrom and Ades, 1973) and in the transcellular arrays of *Drosophila* wing epidermal cells (Mogensen and Tucker, 1987) where it is predominantly composed of 6 nm diameter actin filaments (Mogensen and Tucker, 1988). These actin filaments in *Drosophila* all have the same polarity within each cell. Do actin filaments within the outer pillar cells have the same polarity?

In pillar and Deiters' cells in the organ of Corti of the chinchilla, 6 nm diameter filaments within the dense intertubular material are composed of actin (Slepecky and Chamberlain, 1983) and exhibited antiparallel polarities. These workers have suggested that this actin/microtubule complex, as well as maintaining cell shape,can alter the rigidity and thereby the mechanics of the basilar membrane after acoustic stimulation.

The mechanisms which may govern the alteration of the rigidity and mechanics of the basilar membrane have previously been considered for inner pillar cells in Chapter 1. They may involve tempered instability (Sammack and Borisy, 1988). This would permit modulation of hair cell excitation as described by Slepecky and Chamberlain (1983) in response to acoustic stimulation.

Opportunities for Further Analysis.

Thus, from the data presented above, it would appear that the development of the outer pillar cell is very similar to that of the inner pillar. The microtubule transcellular array does, however, develop slightly later than the transcellular array of the inner pillar cell.

The outer pillar cell microtubule array is still assembling at 6 Days post-partum. When does this assembly cease? Is the triangular cross-sectional profile of an outer pillar cell tightly packed with microtubules when this elongation has ended? Or, does the outer pillar cell membrane, in close proximity to the inner pillar cell, retract to form the tunnel of Corti? If this occurs, does the retraction begin basally, near the basement membrane or, in the apical region of the organ of Corti?

It has been reported by Lim (1980), that in the mature organ of Corti, a process passes from the outer pillar cell to emerge between the first and second rows of outer hair cells. Does the turret eventually emerge between the first and second rows of outer hair cells? Or, alternatively, does another array of microtubules assemble within the process, which is both spatially and temporally distinct from that described in this chapter?

In any future analysis of the mouse organ of Corti, the number of protofilaments present in the large diameter microtubules of the outer pillar cell transcellular array should be ascertained. It would appear from the results obtained in this study that, since the microtubules have diameters which are

greater than 24 nm, they are composed of more than 13 protofilaments. Are they similar to microtubules of the inner pillar cell array in probably being composed of 15 protofilaments (since microtubules of the transcellular arrays of both these cells have diameters of about 28 nm)?

Conclusions.

Microtubule Assembly in Pillar Cells - A Resume.

The data presented in Chapters 1 and 3 show that the transcellular microtubule arrays of inner and outer pillar cells have a similar pattern of assembly. Initially, in the apical region of both cell types, a centrosome is present which has microtubules associated with it. These microtubules have diameters of about 21 nm. Later, the transcellular arrays of the two pillar cells start to assemble.

The microtubule arrays of both pillar cells are similar in that they are nucleated at non-centrosomal sub-apical sites and the microtubules have diameters of 28 nm and are probably composed of 15 protofilaments. However, the configuration of each array is different; inner pillar cell arrays have a tubular configuration while the array of each outer pillar cell has a rod-like configuration. This marked difference is correlated with the configurations of the MTOCs of each cell. The MTOC of an inner pillar cell is associated with the lateral plasmalemma, has an annular configuration and so produces the tubular formation of the microtubule array. This contrasts with the MTOC of an outer pillar cell which is contained within a small apical turret-like projection of each outer pillar cell. The microtubules project from this turret and retain their rod-like configuration within the lateral ridge of each outer pillar cell.

Elongation of the inner pillar cell array begins during the first day post-partum. This contrasts with the assembly of each outer pillar cell array; little assembly appears to have occurred before the third day post-partum. Therefore, development of the transcellular array of each pillar cell is temporally distinct. This finding may be extremely important. The tunnel of Corti has not developed by the sixth day and may not do so until the microtubule array of each outer pillar cell has fully assembled.

The structure of each of these microtubule arrays is similar to those found in mature pillar cells of the chinchilla organ of Corti (Slepecky and

Chamberlain, 1983). These workers found a tubular configuration of microtubules in inner pillar cells and a rod-like configuration in outer pillar cells. Hence, the findings of this study of initial assembly stages in mouse are relevant to rodents and, perhaps, to mammals in general.

An important aim of this investigation has been to evaluate the effect of culturing explants of neonate organs of Corti on microtubule assembly. Russell and Richardson (1987) stated that this procedure has no effect on the overall morphology of the organ. This has been confirmed ultrastructurally in this investigation, provided the tissue is isolated within 24 hours of birth and cultured for not more than two days. Importantly, for future studies, this is the period when pillar cell microtubule arrays are being nucleated.

Microtubule assembly in the inner pillar cell is relatively unaffected by the culture procedure. Nucleation of microtubules for each array occurs at lateral plasmalemma-associated sites in these cells although the numbers of microtubules constituting an array are reduced.

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