Electron Microscope Observations on *in Vitro* Cultures of the Isolated Fowl Embryo Otocyst*

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PLATES 113 TO 118

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ABSTRACT

In vitro cultures of isolated fowl embryo otocysts were studied with the electron microscope. Hair cells of the developing organ of Corti and crista ampullaris have been examined with particular reference to the structure of the cilia and of the cell membrane. Two types of hair cells could be distinguished on the basis whether or not they possessed a "kinocilium" and "stereocilia," or "stereocilia" only. The cytoplasmic membranes were simple and there were no multiple vesicular layers in any of the hair cells. The supporting elements consisted of supporting cells flanking the hair cells, fibroblasts, and the cartilaginous otic capsule.

Both the cochlear and vestibular sensory area showed rich innervation by mainly non-myelinated fibers with partial myelinization in others. There were well developed ganglion cells present. Bare axons penetrated the basement membrane and spread, amongst the supporting cells sheltering them, to the base of the hair cells where they formed bud-shaped nerve endings but, at the stage of development examined, no calyces. These *in vitro* cultures of the isolated fowl embryo otocyst provided convenient and suitable material for the electron microscope study of the sensory epithelium of the ear and revealed further that the isolated fowl embryo otocyst possesses great powers of self-differentiation also at the ultrastructural level.

The ultrastructure of the sensory epithelium of the inner ear has only been studied by a comparatively small number of workers. For example, the organ of Corti of the guinea-pig has been examined with the electron microscope by Engström and Wersäll (1953 *a*, *b*, and *c*), Engström and Sjöstrand (1954), Smith and Dempsey (1957), and Spoendlin (1957). The ultrastructure of the macula has been studied by Wersäll, Engström, and Hjorth (1954) and Smith (1956), and that of the cristae ampullares by Wersäll (1954 and 1956).

All these studies have been carried out on guinea pigs, the tissues removed after killing the animal (with or without an anaesthetic) as rapidly as possible so as to avoid any untoward post-mortem change. In order to try to overcome this difficulty tissue culture material was used in the present electron microscopical investigations, based on the results of light microscopical studies of *in vitro* cultures of the isolated fowl embryo otocyst (Friedmann, 1956). Using the watch-glass technique first developed by Fell and Robison (1929) for the study of the self-differentiating capacity of avian organ rudiments, Friedmann (1956) has shown that, as first suggested by Fell (1929), the isolated otocyst possesses great powers of self-differentiation leading to the *in vitro* development of the organ of Corti, the crista, and the macula. It was thought that further information on the extent of differentiation could best be obtained by means of electron microscopical methods.

* Based on a communication presented at the Summer Meeting of the Pathological Society of Great Britain and Ireland in Newcastle-upon-Tyne on July 4, 1958.

Methods

The technique of tissue cultures employed in the present experiments has been modified from what is customary by the use of sterile *acetone-treated* terylene-voile strips for the support and maintenance of the developing cultures. Moreover the technique of excision of the explants has been improved.

Tissue Culture:

The otocysts of 3½ to 4 day old fowl embryos were carefully dissected leaving a thin rim of mesenchymatous tissue around them, containing the primordial eighth nerve and auditory ganglion. The dissected otocysts were placed on sterile strips of terylene-voile, three otocysts on each strip.

Preparation of Terylene-Voile Strips:

Pieces of sheer terylene-voile, white (John Lewis & Co., Ltd., London, M6/101) about 2 × 4 inches are treated with acetone for a few seconds only and then placed on a sheet of glass and allowed to dry. When removed from the glass, the smooth flat voile will have changed from the original square meshwork to a *convex* sheet perforated with small round holes. This is cut to smaller shapes 1 × 5/16 inch. The strips are carefully washed in two or three changes of distilled water, dried on clean filter paper, and sterilized by dry heat at not more than 140°C. for 2 hours. The convex shape of the strip prevents the tissue from becoming submerged and allows it to obtain a good supply of oxygen; it also forms a channel between the surface of the clot and the strip which may be filled with extract; this may be exchanged if necessary between transplantations in order to control the pH of the culture. This modification of Shaffer's (1956) voile-technique, has proved of considerable advantage in the *in vitro* cultivation of the isolated fowl embryo otocyst.

Two otocyst-carrying strips were placed on the surface of a clot (in a watch-glass) consisting of two parts of fresh fowl plasma and one part fresh embryo extract made from 12 to 13 day old fowl embryos in Gey's balanced salt solution (pH 7.3–7.5). The cultures were incubated at 37°C. and transplanted every other day onto fresh plasma extract clots, remaining attached to the voile strip. Between transplantations any surplus fluid was pipetted off.

Fixation:

The otocysts were carefully detached from their strips at varying intervals between 8 and 12 days of incubation and immediately fixed. Veronal-acetate buffered (Palade, 1952) or non-buffered 1 per cent osmic acid containing 3 per cent dextran and 0.25 per cent sucrose (Engström and Wersäll, personal communication and 1958) proved satisfactory as a fixative. Fixation period was 2 to 3 hours in the refrigerator at 4°C.

After dehydration in graded alcohols, the cultures were embedded in methacrylate (9 parts butyl-methacrylate and 1 part methyl-methacrylate) and polymerized at 40° or 60°C. Some otocysts were embedded in araldite (Glauert, Rogers, and Glauert, 1956, and Glauert and Glauert, 1958). This latter proved particularly successful in experimental work on tissue cultures of otocysts (to be reported in another paper), although complete setting of araldite in otocysts was occasionally impeded owing to the cystic nature of the material, or due to the fact that older otocyst cultures are surrounded by fairly thick cartilaginous capsules which delay penetration by araldite (*ceteris paribus* of the fixative and of other fluids). On the whole, however, the advantages of using araldite have outweighed the difficulties encountered (Fig. 9).

Sections were cut on a Porter-Blum type microtome (silver and gold coloured) and mounted on Athene new 200 grids and examined in a Siemens Elmiskop I. Electron micrographs taken at magnifications of from 2500 to 20,000 were further enlarged photographically.

Tissue Cultures of the Otocysts

A brief summary in this place of the main features of the cultured otocyst may be of some interest.

After about 10 to 12 days *in vitro* (over-all age about 14 to 16 days), the otocyst has grown considerably and lies inside a semilunar or circular cartilaginous capsule, separated from this by a thin layer of fibroblasts. The epithelium rests on this layer, lying on a clearly defined basement membrane. To a great extent the cells of the epithelium are cubical or columnar but there are well defined areas of differentiation which resemble either the organ of Corti, the crista, or the macula of the fowl embryo of similar age.

It may, perhaps, be pointed out that *in vitro*, the organ of Corti has been the most frequently observed structure because it is easily recognisable by its tall hair cells and the tectorial membrane, given a positive periodic acid-Schiff reaction. The crista area has been frequently observed, with the macula proving comparatively more difficult to identify.

There are large numbers of mitochondria beneath the apical surface of the cell and in the supranuclear zone; there are usually fewer in the infranuclear portion of the cytoplasm. Membranes of the endoplasmic reticulum are present both in the supra- and infranuclear areas. Both types, the rough granular type and smooth surfaced membranes, have been observed. Endoplasmic membranes may occur in larger conglomerations

underneath the nucleus of both the hair and supporting cells. Golgi membranes have been observed in the paranuclear zone of the cytoplasm. There were no cells with multiple vesicular cytoplasmic membranes such as those observed in external hair cells of the guinea pig, possibly due to the fact that the organ of Corti of the fowl embryo has only one type of hair cell and does not differentiate into internal or external hair cells.

Supporting Cells

The hair cells are supported by several rows supporting cells (Figs. 3 to 5). Their shape in sections is elongate, columnar, or flask-shaped, with a basal globular portion containing a large nucleus, the whole resting on a conspicuous basement membrane. The top portion embraces the hair cells and often reaches the surface. Supporting cells possess no cuticle and no cilia on their free surfaces, but microvilli are numerous. (Figs. 4 and 5). Thin tonofibrils are present in the cytoplasm, which also contains a fair number of mitochondria, round or fusiform in shape, forming typical cristae. Vesicles of varied shape and size resembling secretory granules may also be present.

Electron Microscope Observations

Organ of Corti Area

Sensory Epithelium.—Figs. 1 and 3 show the light microscope appearances of this area. Fig. 4 shows an electron microscope survey picture and the general architecture of the region (12 days *in vitro*). The principal cells of the organ of Corti are hair cells and supporting cells.

Hair Cells

These are usually arranged in a single row on the surface and are readily identified by the nature of their ciliated surface. Clusters of cilia emerge from the greyish, apparently homogeneous cuticular plate (Figs. 4 and 5) which lies directly underneath the plasma membrane covering the surface of the cell. Small vesicles may be present in the cuticular plate, but mitochondria have not been observed.

The cilia, caught as they are here in a single thin section, appear more or less equal in length. In actual fact they reach considerable lengths in tissue cultures (1 μ to 10 μ). Long hairs may be seen projecting into the cyst (Figs. 4 and 5) which contains some homogeneous matter corresponding to the PAS-positive material forming the tectorial membrane. There is usually a clear, less dense area around each group of cilia (Fig. 4).

The shape of the hair cells is long, columnar, or flask-shaped, with a central or basal nucleus provided with long nucleoli (Figs. 4 and 5). The cytoplasm contains a number of mitochondria of variable size and shape with characteristic cristae. They may be round or fusiform when sectioned lengthwise. Some cross-cut mitochondria contain small round cross-sections of the cristae.

The Region of the Crista

The general pattern of the crista area is somewhat different from that of the organ of Corti. It forms a ridge-shaped structure covered by the cupola. Fig. 2 shows the crista in an 11 day old *in vitro* culture of the isolated fowl embryo otocyst.

Hair Cells

The slopes of the crista may be more or less covered with hair cells bearing clusters of cilia. The shape and size of the cells varies with conical or flask-shaped hair cells predominating (Fig. 6). No attempts have been made to classify the hair cells of the crista.

There appears to be a significant difference in the arrangement and composition of the cilia compared with those of the hair cells of the organ of Corti. Forty to 70 cilia are arranged in a more or less regular group lying in a clearance of the cupola material. With one exception they are long slender stereocilia with thin fairly long rootlets. The exception, a typical kinocilium, can easily be distinguished (*K*, in Fig. 6). With its deep thick walled basal corpuscle embedded in the surface of the cell and its characteristic internal organization of nine peripheral and double axial fibres, the kinocilium forms also *in vitro* a conspicuous feature of the hair cells of the cristae ampullares (Fig. 6).

Supporting Cells

The hair cells are surrounded by non-differentiated supporting cells arranged in a less regular fashion than in the organ of Corti area. Columnar or cuboidal in shape, the supporting cells have shown no unusual components.

Nerves and Ganglion Cells

The sensory areas of the developing otocyst can be readily identified in thick, unstained sections

under phase contrast by virtue of the subepithelial aggregation of nerve fibres and ganglion cells (Fig. 2). These latter are easily recognized in thin sections since there are normally large numbers of non-myelinated or partly myelinated nerve fibres present. The nerve fibres and their Schwann cells form large groups closely associated with small capillaries (Fig. 12). Myelinization, as a rule limited to a relatively small number of myelin layers (Fig. 7) may increase with the age of the culture.

All nerve fibres penetrate the basement membrane as non-myelinated axons containing large numbers of mitochondria embedded in a neuroprotofibrillar matrix (Fig. 8). There are also numbers of axons underneath and just above the double lined basement membrane (Figs. 8 and 9). They extend up from between the supporting cells into the sensory epithelium, where, on reaching the hair cells, nerve endings are formed (Fig. 11). No definite nerve calyces have been observed although there has been some indication that they might develop later.

Ganglion cells are easily recognisable by their size (2 μ to 10 μ), their large nucleus, and by widely scattered Nissl granules in the cytoplasm (Fig. 10). Nerve fibres have been observed emerging from the ganglion cells and spreading in the stroma.

Otic Capsule

Long slender fibroblasts, showing an elaborate development of the endoplasmic reticulum, form the subepithelial stroma separating the epithelium from the hyaline cartilage of the otic capsule (Fig. 9). Polygonal darkly stained cartilage cells, lying in a homogeneous matrix, form the otic capsule. In sections stained with 1 per cent phosphotungstic acid collagen fibres can easily be recognised in it.

Accessory Structures

Keratinizing squamous epithelium is often present and forms cystic structures (epidermoid cysts) which may present some difficulty and have to be trimmed off. Other cystic structures encountered are lined by ciliated secretory epithelium consisting of ciliated cells equipped with large numbers of kinocilia (with the typical internal structure) and secretory cells containing large secretory granules. They are almost certainly derived from the epithelium of the middle ear and pharynx (Figs. 13 and 14). Small sinusoids or capillaries lined by endothelial cells are numerous in the subepithelial tissue and are intimately interlinked with nerve fibres (Fig. 12).

DISCUSSION

In vitro cultures of the isolated fowl embryo otocyst are not difficult to maintain and provide excellent material which can be promptly fixed and processed for thin sectioning. The present investigation, the first electron microscopical study of *in vitro* cultures of the otocyst, has shown far reaching differentiation also on the ultrastructural level.

Although the structure of the avian inner ear may differ from that of mammalian ears, the sensory epithelium with its hair cells and supporting cells compares well in its basic features with that of mammalian cells. Since there have been no similar studies in adult avian material, the present observations have been compared with the pioneering studies of Engström and Wersäll (1953), Engström and Sjöstrand (1954), further developed by Smith and Dempsey (1957) and Spoendlin (1957) on the fine structure of the organ of Corti, and those of Wersäll (1956) regarding the ultrastructure of the cristae ampullares of the adult guinea-pig. Here some of the more important *in vitro* findings and the apparent discrepancies will be considered.

Hair cells, supporting cells—*e.g.* Deiters' cells, myelinated and non-myelinated nerve fibres, and ganglion cells, as well as simpler cells such as undifferentiated cuboidal or columnar cells lining the non-sensory areas, fibroblasts, and cartilage cells of the otic capsule have shown full development in tissue cultures of the otocyst.

The size and shape of hair cells show considerable variation. On the whole the conical or flask-shaped cells predominate over the tall columnar type in the surface layers of the respective sensory areas. Attempts at a classification based on shape or size have proved of only limited value. Two types of hair cells could be distinguished on the basis whether or not they possess, besides a large number of stereocilia, a kinocilium.

Wersäll (1956) has drawn attention to the absence of the kinocilium in hair cells of the organ of Corti of the guinea pig and to its presence in hair cells of the cristae and maculae. This seems to be an important distinguishing feature. On the whole the present *in vitro* investigations

favor the suggestion that all the hair cells of the vestibular apparatus possess a kinocilium whereas cells of the auditory apparatus are usually equipped only with stereocilia. Some cells of the organ of Corti of the fowl embryo otocyst, however, may possess hairs forming a basal corpuscle and internal filaments (Fawcett and Porter, 1954). Moreover, occasional isolated kinocilia may be seen on cells of the crista and also on some of the cells of the apparently non-sensory epithelium of the otocyst. Further studies possibly on older otocysts may provide additional information on this interesting point.

It is difficult to express any definite views on the function of the cilia; attempts at visualizing their movement in tissue cultures of the otocyst have not been successful. There is, however, no doubt that the cilia are specially differentiated structures of the hair cells and play some role in the function of the sensory epithelium.

As regards the terminology, it could be argued that the terms "kinocilium" and "stereocilium" ought to be abandoned in favour of the terms "cilia" for kinocilia, and "villi." The latter could further be subdivided into "macrovilli" for stereocilia, and "microvilli" for the less conspicuous cytoplasmic processes. In the present paper, so as to avoid confusion, the terms used by most workers in this field (Wersäll and others) have been retained.

The multiple vesicular layers described in the cytoplasmic membranes of external hair cells of the organ of Corti of the guinea pig by Engström and Sjöstrand (1954), Engström (1955), and confirmed by Smith and Dempsey (1957) and Spoendlin (1957) have not been observed. The avian organ of Corti, however, is a comparatively simple structure and does not differentiate into external and internal hair cells. The mitochondria also appear to be more numerous in the supranuclear area of the cytoplasm and are not marginally arranged, their distribution resembling that described in inner hair cells by Smith and Dempsey (1957).

Concerning the innervation, the sensory area of both the organ of Corti and of the vestibular apparatus is richly endowed with nerve tissue consisting, at the age examined, mainly of non-myelinated and partly myelinated fibres and ganglion cells. The nerve fibres are enclosed in Schwann cells and several fibres may be associated with the same Schwann cell. As in the sensory area of the inner ear of the adult guinea pig (Wersäll, 1956, Smith and Dempsey, 1957, and Engström and Wersall, 1958) only bare non-myelinated axons penetrate the basement membrane and branch out among the supporting cells, forming bud-shaped nerve endings at the base of the hair cells.

In the epithelium the axons are surrounded by the supporting cells which have assumed, as suggested by Wersäll (1956), the role of Schwann cells in isolating, and probably feeding the nerve fibres. The nerve endings usually contain mitochondria. No nerve calyces as described by Wersäll (1956) could be identified around any of the hair cells, but possibly these features are not fully developed at this stage. The over-all age of the cultures, *i.e.*, the stage of development, averaged 14 to 16 days, and it is possible that the calyces would develop at a later stage. Myelinization might also be delayed until later.

These apparent discrepancies may, of course, be due to the species studied, although over-all development of the isolated otocyst seemed to be complete when compared with the inner ear of fowl embryos of similar age.

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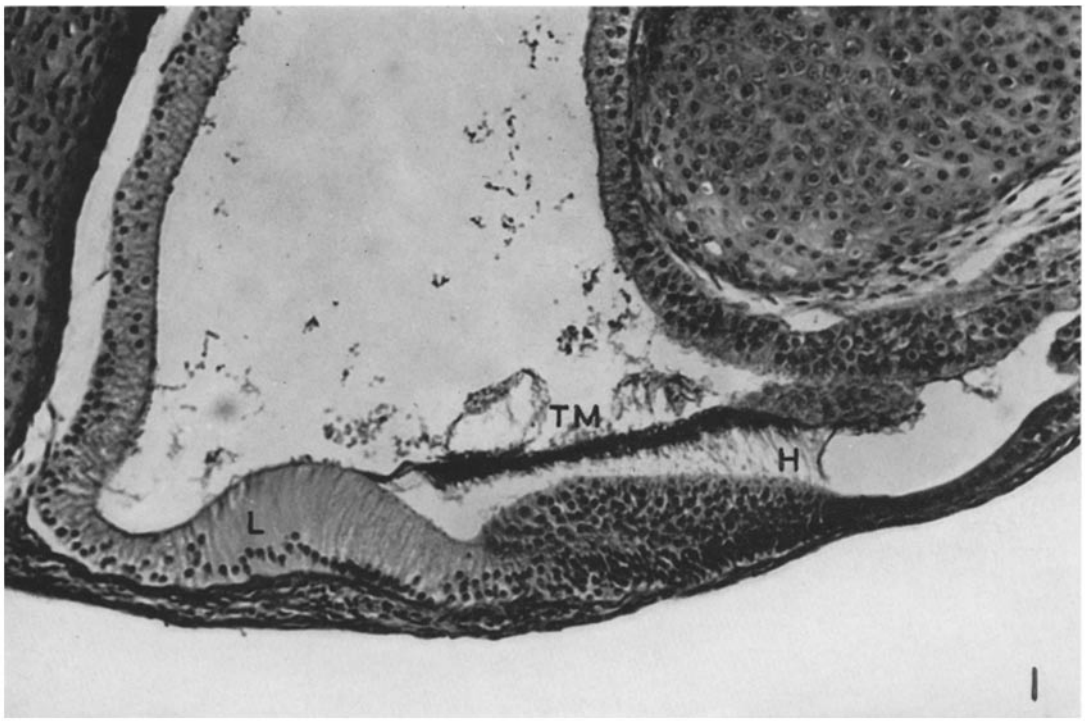
Explanation of Plates

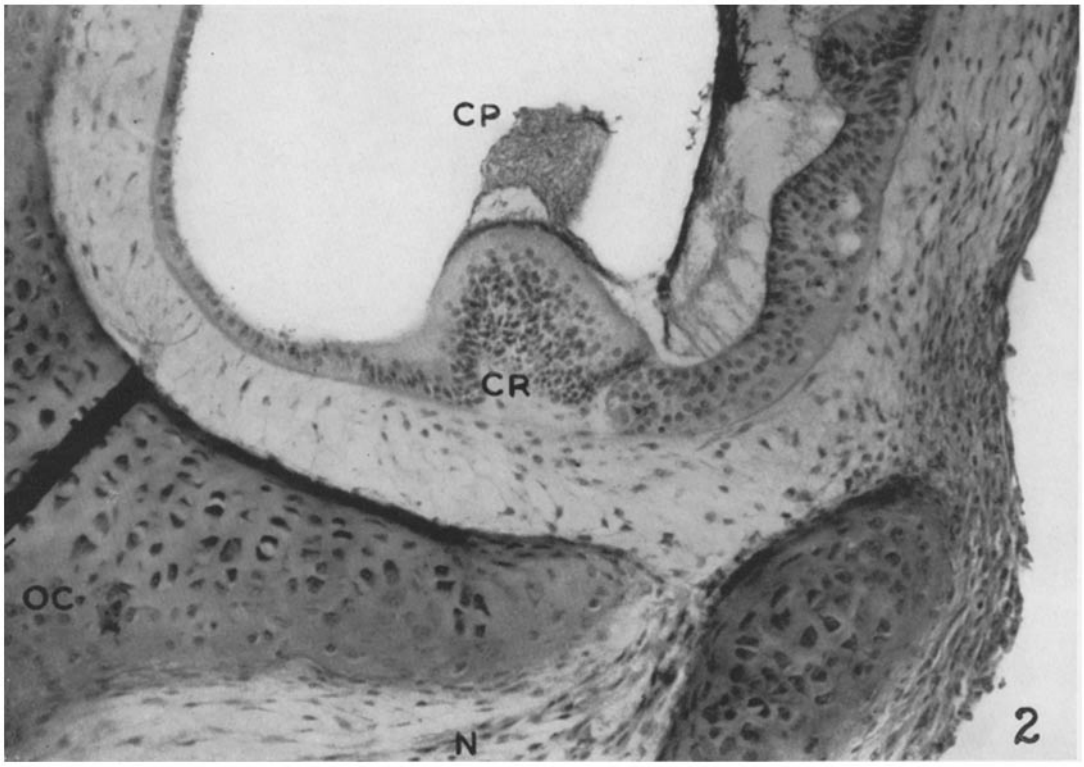
Plate 113

Fig. 1. 11 day old culture of fowl embryo otocyst, showing area of organ Corti. Note tall clear limbus cells (*L*) and hair cells (*H*) covered by the PAS-positive tectorial membrane (*TM*). Periodic acid–Schiff reaction. (OT 83.[1]) × 270.

Fig. 2. Tissue culture of the otocyst (11 days *in vitro*), showing well differentiated crista (*CR*) with cupola (*CP*). Note nerve (*N*) reaching the crista through an opening in the otic capsule (*OC*) (OT 83). Periodic acid–Schiff reaction. × 285.

[1] Serial number of our otocyst (OT) experiment.



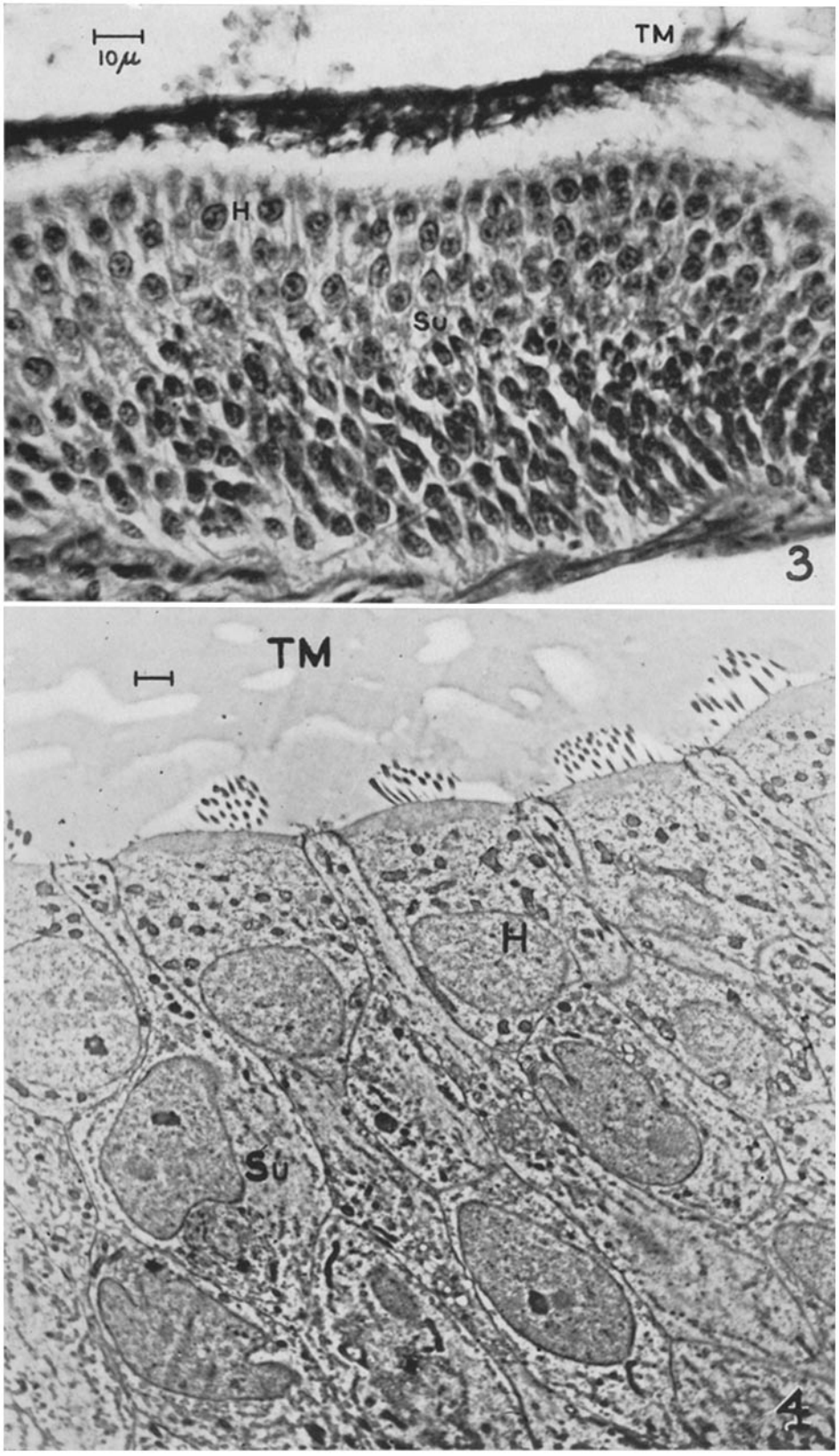


(Friedmann: *In vitro* cultures of fowl embryo otocyst)

PLATE 114

FIG. 3. 11 day old organ of Corti of isolated fowl embryo otocyst, showing hair cells (*H*) and supporting cells. (*Su*). Note PAS-positive tectorial membrane (*TM*). Periodic acid–Schiff reaction. (OT 83 *T*). × 850.

FIG. 4. Survey picture of organ of Corti area, showing characteristic arrangement of sensory hair cells (*H*) flanked by their supporting cells (*Su*). Note groups of cilia rising from greyish cuticular plates and lying in clearances of the greyish tectoral membrane (*TM*). (EM 335). × 6,250.

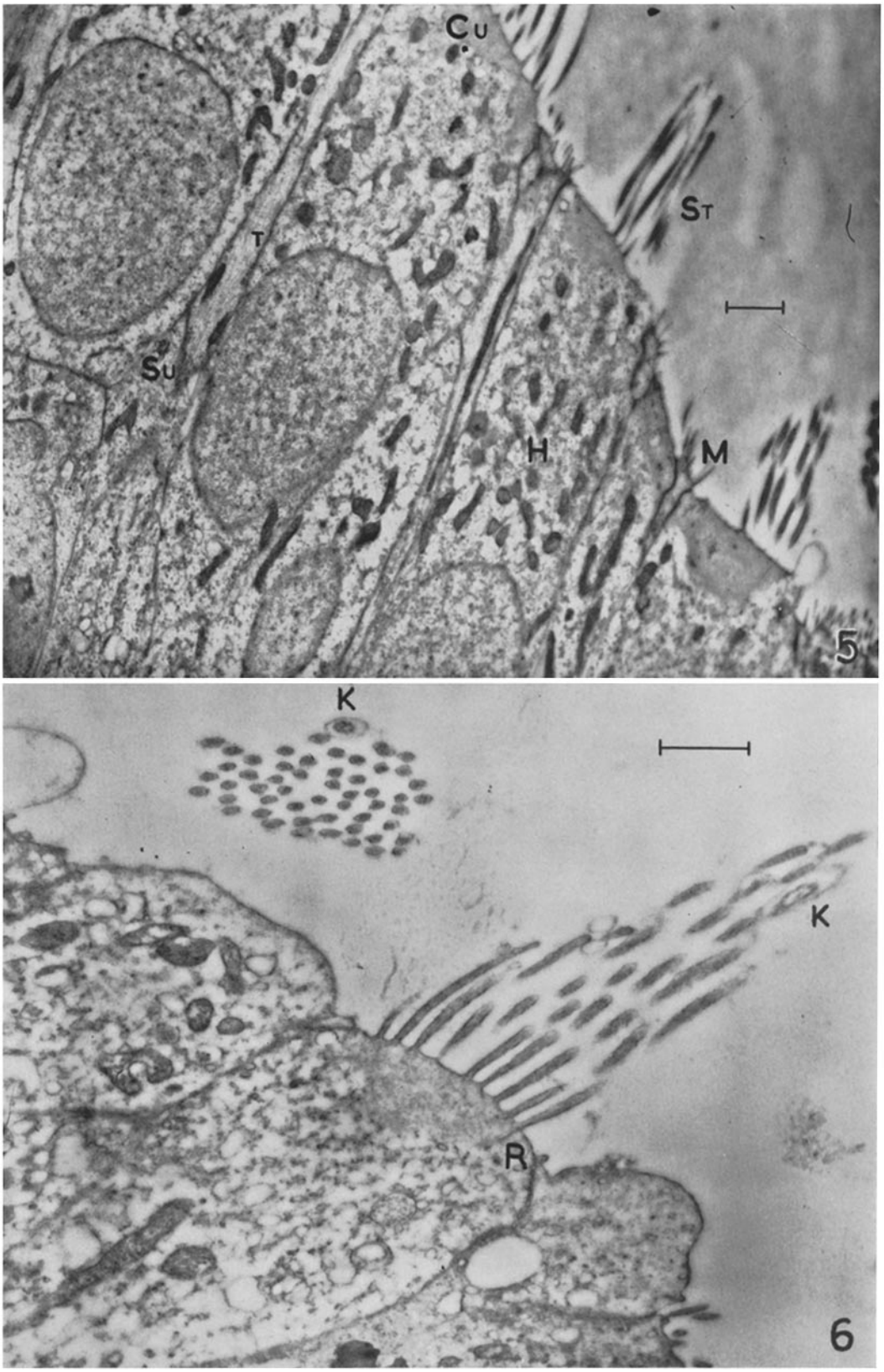


(Friedmann: *In vitro* cultures of fowl embryo otocyst)

PLATE 115

FIG. 5. Part of organ of Corti, showing hair cells (*H*) flanked by supporting cells (*Su*). Note large numbers of mitochondria in supranuclear parts of hair cells and tonofibrils (*T*) in supporting cells. There are only stereocilia (*ST*), ("Macrovilli"), on hair cells arising from the cuticle (*CU*) and microvilli (*M*) on supporting cells. (EM 341.) × 12,500.

FIG. 6. Slope of crista covered by hair cells endowed with large numbers of cilla. These form clusters or tufts consisting of approximately 30 to 50 stereocilia with a single kinocilium (*K*). The stereocilia arise from the greyish cuticle in which slender rootlets (*R*) can be seen. The cytoplasm contains a small number of mitochondria. (EM 313.) × 20,000.

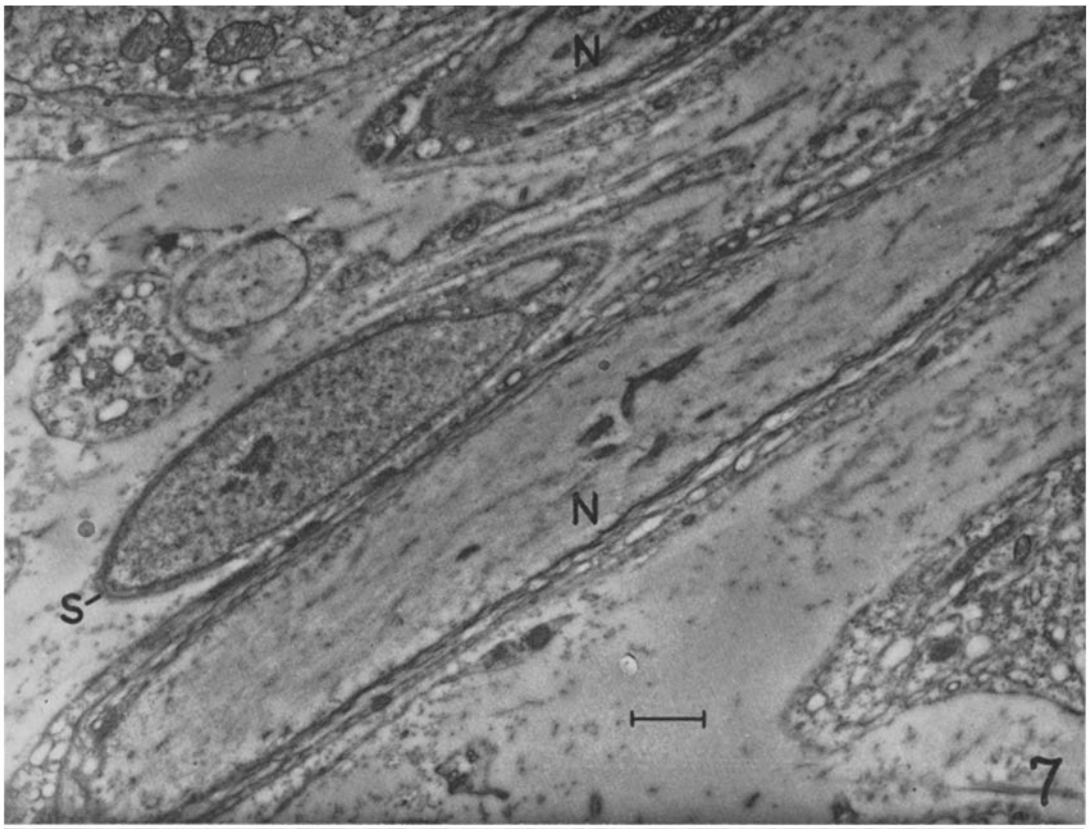


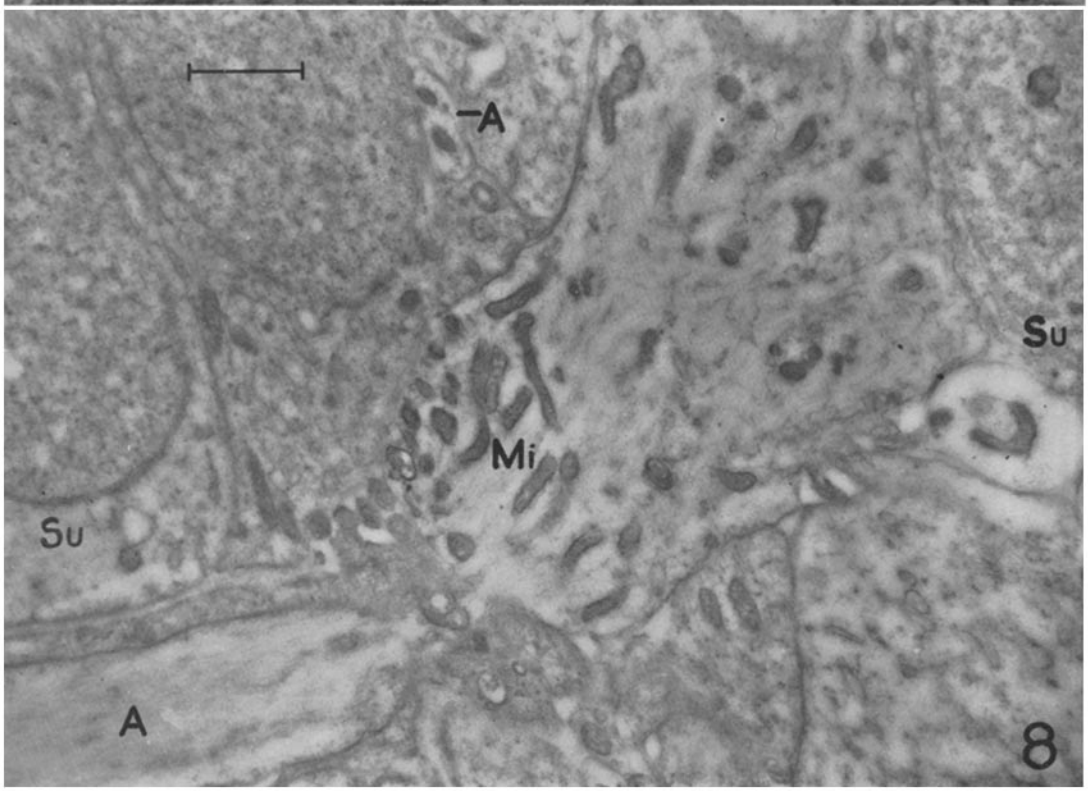
(Friedmann: *In vitro* cultures of fowl embryo otocyst)

PLATE 116

FIG. 7. Myelinated and non-myelinated nerve fibres (*N*) with Schwann cells (*S*) in sensory area. (EM 349.) × 12,500.

FIG. 8. Axon (*A*) penetrating basement membrane and expanding among supporting cells (*Su*). Note large numbers of mitochondria (*Mi*). (EM 257.) × 20,000.





(Friedmann: *In vitro* cultures of fowl embryo otocyst)

PLATE 117

FIG. 9. Base of organ of Corti, showing basement membrane (*BM*), with nerve axon (*A*) between supporting cells and fibroblasts (*F*), containing endoplasmic reticulum membranes underneath the basement membrane. Araldite. (EM 853.) × 12,500.

FIG. 10. Ganglion cell in tissue culture of the isolated fowl embryo otocyst (12 days *in vitro*). The slightly crenated and eccentric nucleus lies within the fibrillar cytoplasm containing Nissl granules (*Ni*) and mitochondria. The cell is enveloped by a Schwann cell (*S*) and lies in a collagenous stroma. The banded collagen fibers (*CF*) are clearly visible. (EM 252.) × 12,500.

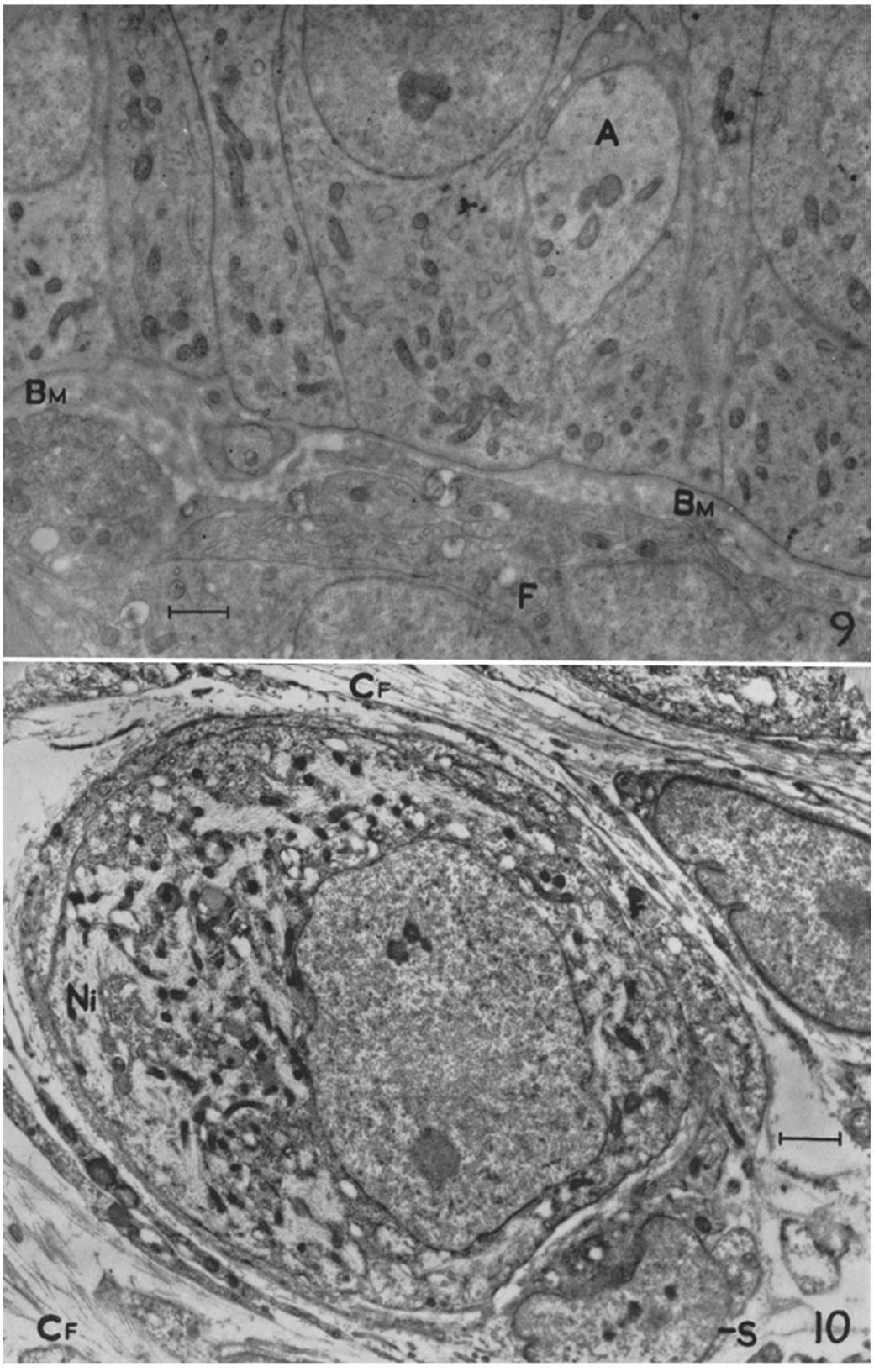


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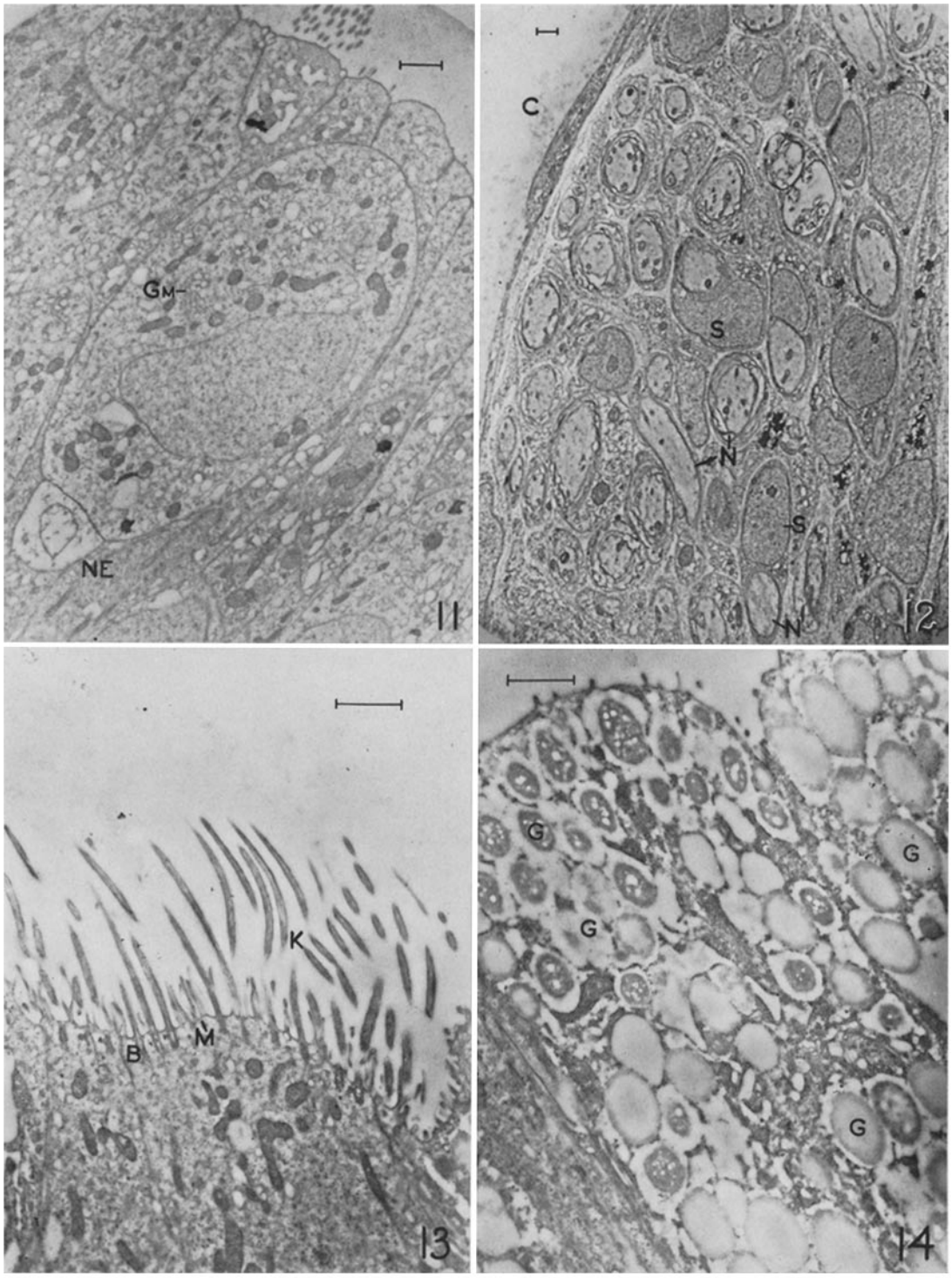
PLATE 118

FIG. 11. Hair cell with bud-shaped nerve ending (*NE*). Note Golgi membranes (*GM*) in paranuclear zone and scattered mitochondria. Supporting cells and other hair cells cut in rather irregular fashion. (EM 451.) × 12,500.

FIG. 12. Survey picture of richly innervated sensory area. There are large numbers of partly myelinated and non-myelinated nerve fibres (*N*) with Schwann cells (*S*) lying in the fibrous tissue closely linked with a capillary (*C*) whose wall lined by an endothelial cell is visible in the left upper corner. (EM 361.) × 6,250.

FIG. 13. Accessory cystic structure in tissue culture of the otocyst, showing ciliated epithelial cells covered by kinocilia (*K*) forming basal corpuscles (*B*) on the surface of the cell, also equipped with large numbers of microvilli (*M*). (EM 259.) × 20,000.

FIG. 14. Accessory cystic structure in tissue culture of the otocyst, showing secretory cells filled with mucoid secretory granules (*G*). (EM 160.) × 20,000.



(Friedmann: *In vitro* cultures of fowl embryo otocyst)