# Electron microscopic studies of ion- and $H_2O$-transporting epithelial cells in the horizontal ampulla of the pigeon

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**Summary.** Earlier morphological studies of the epithelial structure in the semicircular canals of mammals have focused on the sensory cells of the crista ampullaris. This report draws attention to the fact that there exist at least seven further cell types in the horizontal ampulla walls of pigeon with various functions; the role of ion- and $H_2O$-transporting epithelial cells is dealt with here in detail. While the dark cells appear to play a decisive role in the regulation of ionic composition, the cells in the planum semilunatum may transport $H_2O$ and assist in the regulation of endolymph volume. In addition, protein-secreting structures are located in the apical region of the cells of the planum semilunatum. The question whether the proteins are dispersed in the endolymph or contribute to cupula formation remains unclear. The morphology and possible functions of these two cell types are discussed on the basis of electron microscopic results.

**Key words:** Ampulla, Planum semilunatum, Dark cells, Pigeon, Electron microscopy

## Introduction

Earlier electron microscopic studies on the ampulla in the inner ear of various species focused on the sensory cells, their functional relationship to the cupula and the associated physiological problems (Wersäll, 1956; Engstrom, 1960; Flock, 1965; Kimura, 1966; Wersäll and Bagger-Sjoback, 1974; Watanuki and Schuknecht, 1976; Trevisi et al., 1980; Hunter-Duvar and Hinojosa, 1984; Goldberg, 1991; Ross, 1993). Little attention was given to the morphology and function of the epithelial cells that lie outside the sensory epithelium. The findings from these previous studies demonstrate the existence of a pseudostratified epithelium consisting of several cell

types (Bairati and Iurato, 1960; Dohlman and Ormerod, 1960; Dohlman, 1964, 1965; Kimura, 1964, 1969; Iurato, 1967; Nakai and Hilding, 1968; Ishiyama and Keels, 1971; Hunter-Duvar and Hinojosa, 1984; Igarashi, 1989; Becerra and Anadon, 1992, 1993). Thus, in addition to the sensory cells, there exist for example supporting cells, transitional cells, so-called dark cells and the cells of the planum semilunatum. It has been postulated that these cell types, particularly the dark cells and the cells of the planum semilunatum, are instrumental in the generation of endolymph in the semicircular canals, in the regulation of the specific ionic composition and in the maintenance of homeostasis (Bairati and Iurato, 1960; Dohlman and Ormerod, 1960; Dohlman, 1964, 1965; Kimura, 1964, 1969; Iurato, 1967; Nakai and Hilding, 1968; Ishiyama and Keels, 1971; Cornford and Barajas, 1980; Igarashi, 1989; Becerra and Anadon, 1992, 1993).

Histochemical evidence of the existence in these cells of Na-K-ATPase, $H^+$-ATPase and further ATPases, carbonic anhydrase, Na-K-Cl-cotransporter (NKCCl), $Cl^-/HCO_3^-$-exchanger and special lectins, supports the hypothesised $H_2O$- and ion-transporting function (Ishii and Nomura, 1968; Mees, 1983; Yoshihara and Igarashi, 1987; Spicer et al., 1990; Sugiyama et al., 1991; Marcus et al., 1992, 1993; McGuirt and Schulte, 1994; Schulte and Steel, 1994; Crouch et al., 1997; Stankovic et al., 1997).

Despite the importance of these functions for endolymph homeostasis, the associated electrical properties of the sensory cells, and the mechanical behaviour of the cupula and cilia, there is a lack of systematic electron microscopic examination of the dark cells and the cells of the planum semilunatum. The present study was intended to complement these earlier findings using transmission electron microscopic techniques. This provides a new basis for a functional interpretation of these cells.

It was for the following reasons that we chose the pigeon as experimental model: a) Preparation of the horizontal ampulla in this species is achieved without

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too great technical difficulties. b) Further investigations should be performed in the inner ear of the pigeon; our results are the morphological basis for these experiments. c) There is enough material available from pigeon breeding. d) A number of publications on the pigeon are available allowing orientation and comparison (Igarashi and Yoshinobu, 1966; Ishiyama and Keels, 1971; Landolt et al., 1972, 1975).

## Materials and methods

Domestic pigeons (*Columba domestica*) of no specific strain from the Department of Applied Zoology of the Freie Universität Berlin were examined. The pigeons were anaesthetised with Ketanest® (PARKE-DAVIS, 10 mg/kg) and decapitated. The skin was immediately removed and the skulls were prepared. The bony labyrinth was isolated using a small, sharpened operating rasper to expose the ampulla of the horizontal semicircular canal. After opening the bony ampullary wall, in situ fixation was performed. After 30 minutes the hardened membranous ampulla was removed and placed in fixative solution: either Karnovsky solution (3% glutaraldehyde plus 3% paraformaldehyde in 0.1M phosphate buffer, pH 7.2) or in 1% glutaraldehyde in phosphate buffer with 0.5% tannic acid. The preparations were left in the fixation solutions for 2-4 hours.

After washing in buffer solution, post-fixation was carried out using 1% buffered $OsO_4$. The samples were rinsed and dehydrated in a series of graded alcohols and embedded in Epon. Semithin 0.5-1.0 $\mu$m sections were stained with 1% Toluidine blue and examined using light microscopy. Selected levels were thin-sectioned (50 nm) with a LEITZ Ultracut E, stained with uranyl acetate/lead citrate and evaluated using a ZEISS transmission electron microscope (EM10).

## Results

In addition to the sensory cells and nerve endings, at least 7 other cell types were found. Their location can be recognised readily in light microscopic images of the plastic-embedded samples (Fig. 1): 1) Cylindrical supporting cells were located between the sensory cells. 2) The connecting cells were located at the edges of the sensory epithelium, i.e. at the base of the crista ampullaris. These were not present in all preparations because they were not found at all parts of the crista. 3) Immediately adjacent was the area containing the dark cells (see below for details). 4) Light supporting cells were seen between the dark cells. 5) The transitional cells delimited the planum semilunatum. 6) The long, elevated cells of the planum semilunatum appeared in certain sections (see below). 7) These cells flattened out gradually and towards the roof of the ampulla there was a transition to a flat, i.e. mesothelium-like epithelium.

Only the dark cells and the cells of the planum semilunatum are described here. In accordance with previous literature, these were found to have morphological features of ion- and $H_2O$-transporting cells.

The electron microscopic findings indicate that the dark cells, in contrast to the others, were characterised by a high electron density (Fig. 2) and a very large surface area. This surface enlargement resulted from apical projections and deep basal and lateral infoldings in the infranuclear part of the cells (Figs. 2, 3, 4). Evidence of interlocking with neighbouring cells and short coarse projections was found supranuclearly. The finger-like apical projections were irregularly distributed, relatively short (800 nm) and varied in thickness (50-200 nm). In many cases they became progressively thicker towards the tips. The basolateral infoldings varied in depth, in some cases reaching the perinuclear area.

The cell membrane appeared to have a course similar to that of the epithelial cells in the proximal and distal tubules in the kidney or the stria marginalis cells. The similarity to these cells was also supported by the existence of large, crista-rich mitochondria in the cytoplasmic plates between the infoldings. In a few cells the basal infoldings were arranged concentrically (Fig. 3). In cross-section they showed a layered structure. In the cytoplasmic plates of some cells, rows of 120-160 nm large membrane-bordered vesicles or vacuoles were located between the mitochondria and the cell membrane. These vesicles occasionally converged in longer cavities of up to 600 nm in length (Figs. 4, 5). The width of the intercellular spaces (ICS) between the plates was in the range 50-500 nm.

Loosely packed myelin-like structures (Fig. 2), often up to 700 nm in size, were found in the area of infoldings. The images gave the impression that the cytoplasmic plates became increasingly thinner, then coiled up together to form these myelin-like structures. In addition, smaller (<150 nm), densely packed, typical myelin structures were present, whose localisation i.e. intra- or extracellular, could not always be defined.

The perinuclear and apical cytoplasm without infoldings occasionally contained many mitochondria, one to two large, often vesicular Golgi areas, some profiles of rough endoplasmic reticulum (RER), isolated vesicles and in many cases one to two membrane-bordered vacuoles of up to 600-800 nm in diameter with heterogeneous content. They contained round and lipid-like droplets, 50-100 nm in size, of variable electron density, membrane-like material, vesicles and small granules (<12 nm). Their appearance was similar to that of secondary lysosomes or residual bodies (Fig. 4).

The dark cells very rarely contacted one another. They were usually located between the light supporting cells. In the centre of the dark cell region those sections which were arranged perpendicularly to the epithelial surface (Fig. 2), contained dark cells and light supporting cells in a ratio of approximately 1:1. The proportion of supporting cells respective to dark cells increased progressively towards the periphery of this

region.

The light supporting cells were generally of columnar shape (Fig. 2). The lateral and apical parts of the cell surface were relatively smooth over long stretches. This changed systematically towards the basal region, where the light cells widened and occupied the spaces between the dark cells and the basal lamina with plate-like projections. These plates often possessed a pore-like opening of variable width, through which the dark cells were in contact with the basal lamina (Fig. 5). The basal infoldings of the dark cells converged towards these openings. Due to their wider ICS near the nucleus, the dark cells were of goblet shape. A further description of the contacts between the various cell types is provided later in the report. Light cells were characterised by the normal, loosely distributed cell organelles and the

absence of microvilli.

The cells of the planum semilunatum (p.s.) could not always be demonstrated. They did not form a continuous band on the inner surface of the ampulla, so that they only appeared in certain sections in dependence on the plane of the section. These cells were typically long and slender (Figs. 6, 7). The ICS were often up to 3 $\mu$m wide in the central and basal regions, but were always closed at the apex (see below). The wide ICS were bridged by broad-based, flat projections so that an irregular cell contour resulted. Long gap junctions were found at areas of contact between these projections. Isolated microvillus-like processes were seen in the immediate vicinity. Glycogen particles and cell organelles, e.g. large mitochondria, short ER cisterns and free ribosomes were distributed asymmetrically towards the apex of the
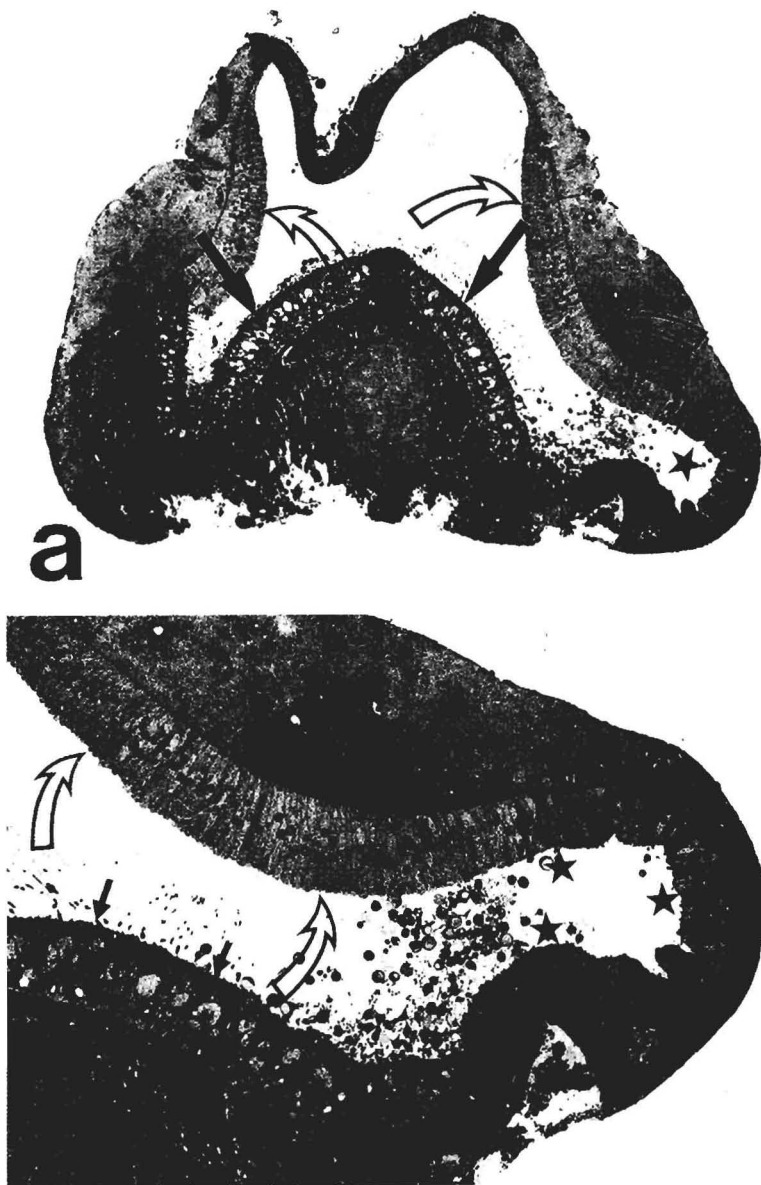


**Fig. 1.** Horizontal ampulla of the pigeon. **a.** Plastic embedding, Toluidine blue staining. Light microscopy of the ampulla with cross-section through the crista ampullaris (black arrows). A mixture of dark and light cells can be observed at the base of the crista (star). The planum semilunatum, consisting of a band of columnar cells, lies on the ampullary wall at some distance from the crista (arched arrows). x 50. **b.** Higher magnification. x 120
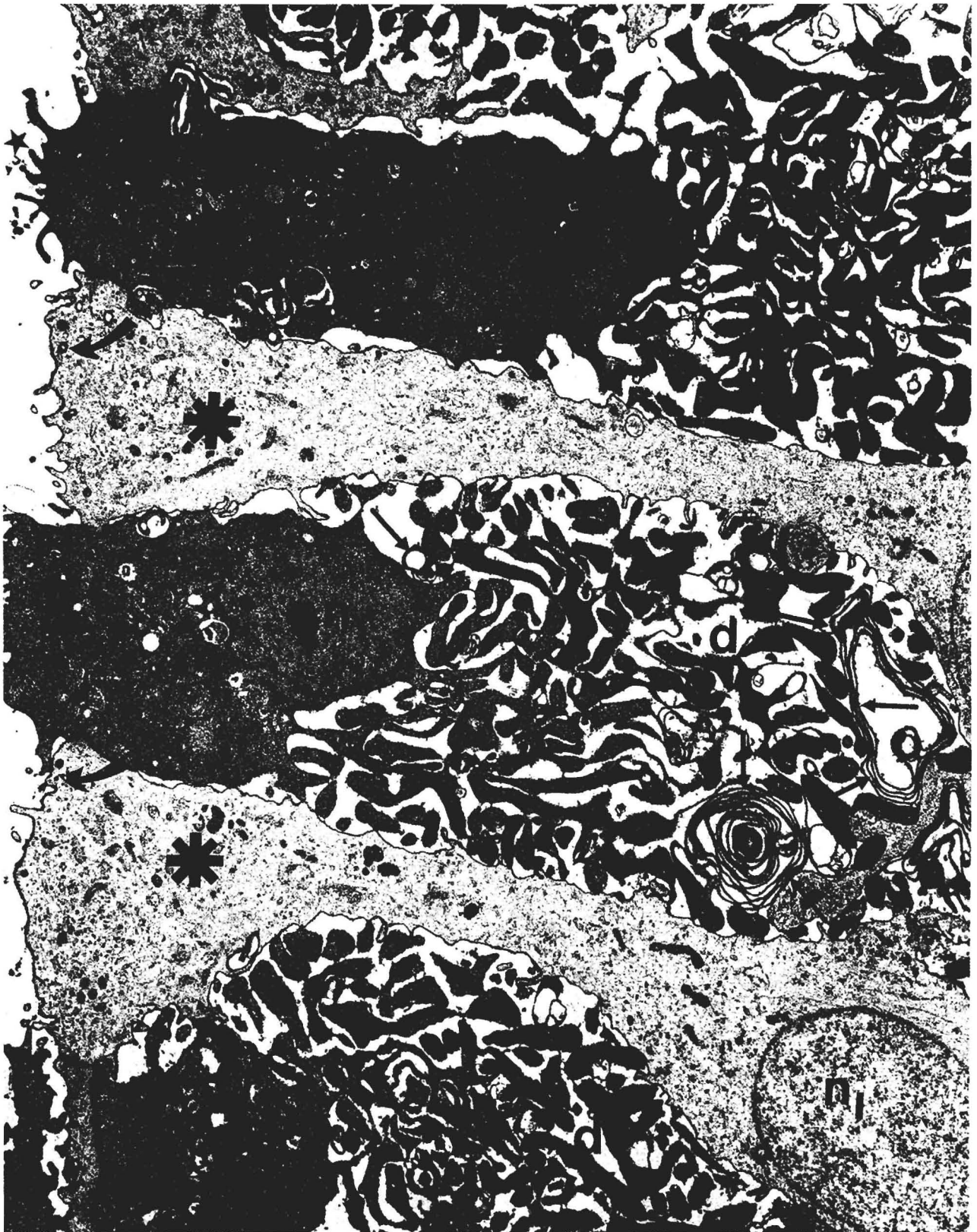
**Fig. 2.** Horizontal ampulla of the pigeon. Electron microscopic picture taken near the base of the crista ampullaris, illustrating the regular pattern of dark cells (d) and light supporting cells (asterisk). The basal part of the dark cells is characterised by deep infoldings and numerous mitochondria. Myelin figures (arrows) of varying size and density are situated between the infoldings. n: dark cell nucleus; $n_1$: supporting cell nucleus; arched arrow: tannic acid-positive surface coat and endocytotic structures on the apex of the cells. x 6,600

**Fig. 3.** Example of dark cells in the horizontal ampulla of the pigeon, with deep infoldings in the basal section of the cells (stars). Numerous mitochondria (m) are localised in the resulting, narrow cytoplasm plates. **a.** Longitudinal section through infoldings. x 30,000. **b.** Cross-section with concentric structure. x 17,000
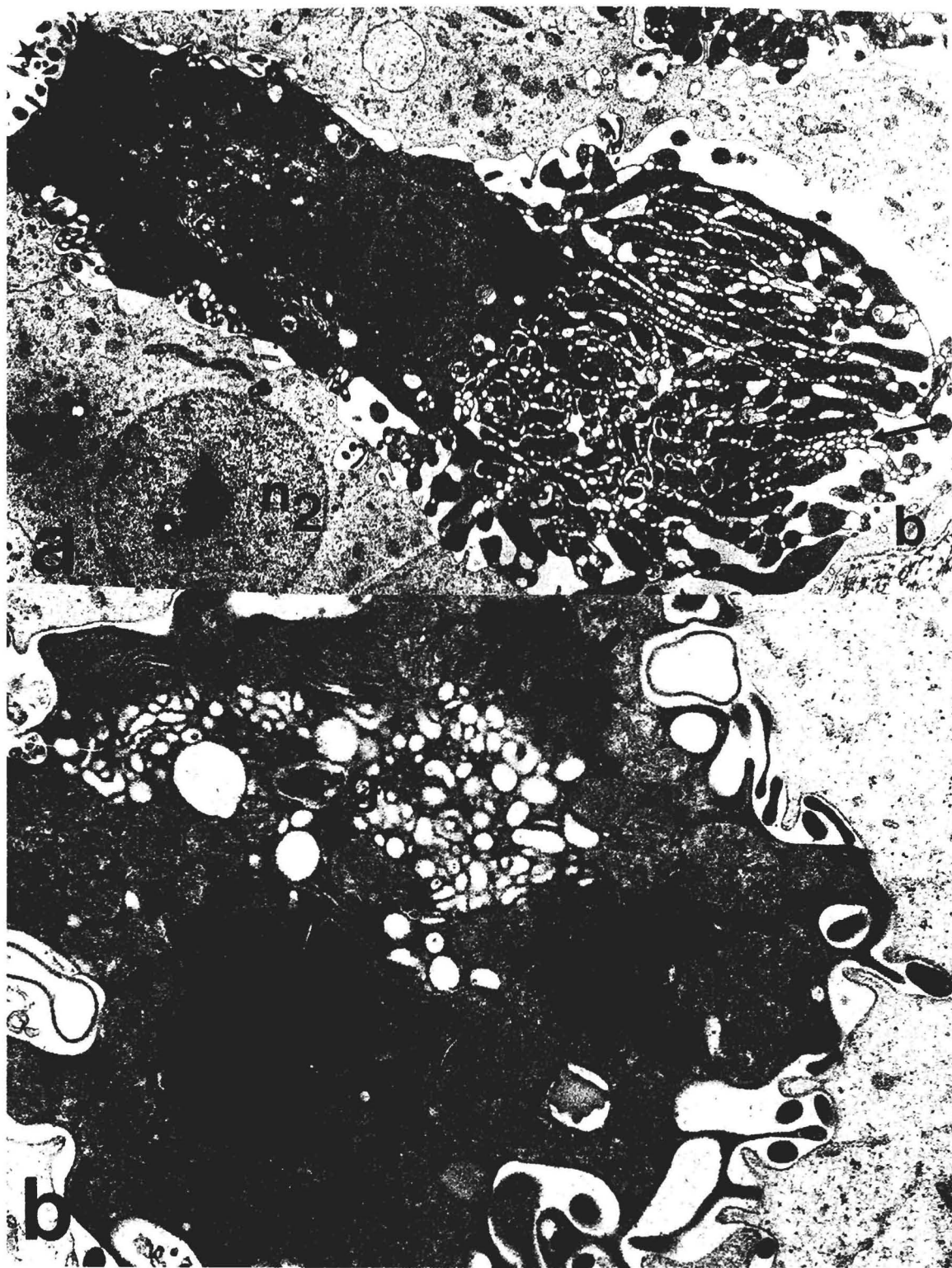
**Fig. 4.** Horizontal ampulla of the pigeon. **a.** Dark cells with apical microvilli (star). Compact, apical end of the cell with Golgi apparatus (g). The basal area is characterised by deep infoldings and long rows of vesicles can be recognised in the cytoplasm plates between the infoldings (arrows). A thin, basal cytoplasmic plate (b) formed by the light supporting cell separates the dark cell from the connective tissue. $n_1$: dark cell nucleus $n_2$: supporting cell nucleus. x 6,000. **b.** Section through a dark cell at the level of the nucleus (asterisk) with numerous mitochondria (m), a large vesicular Golgi apparatus (g) and lysosome-like inclusions with lipid droplets (arrows). x 25,000
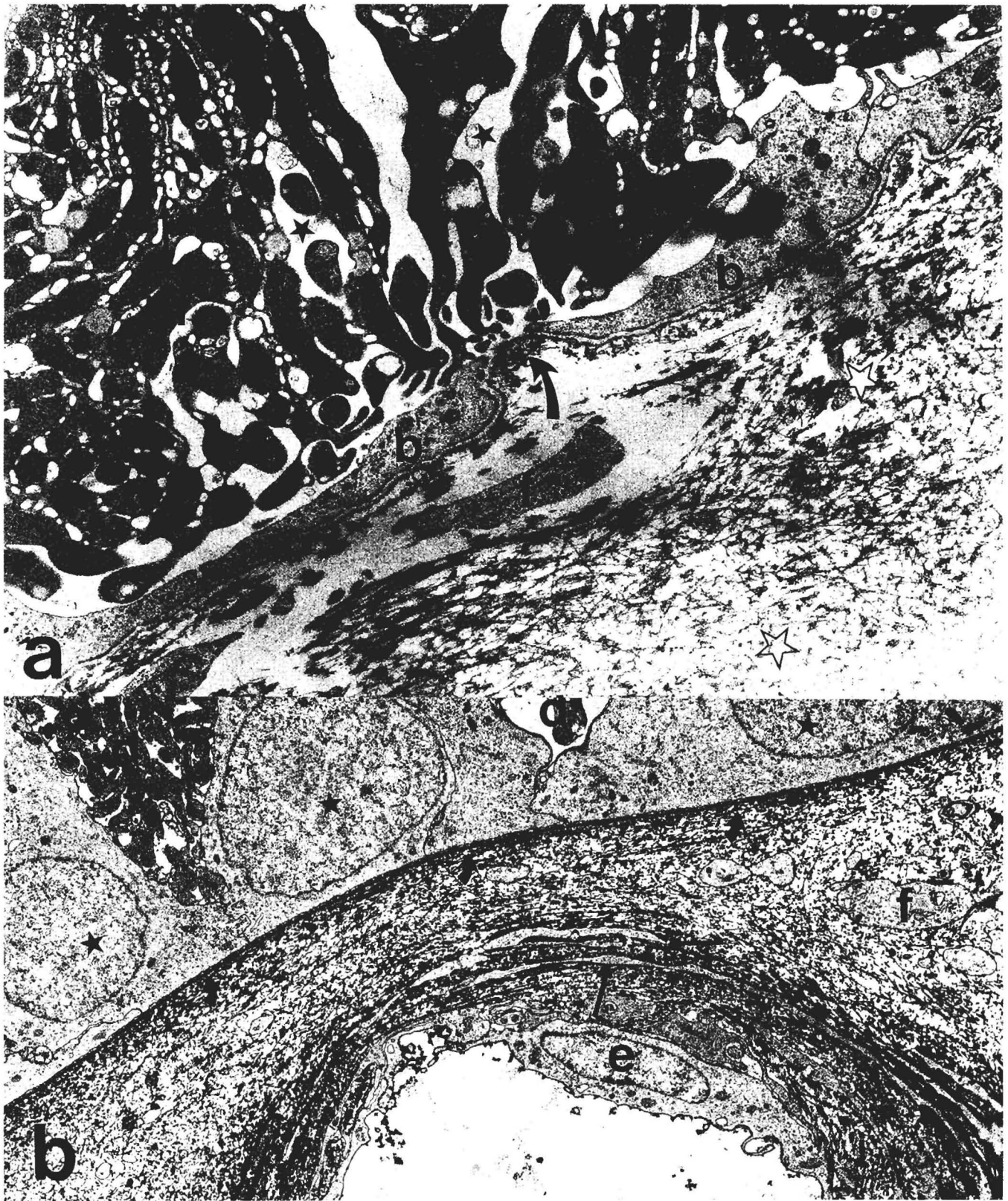
**Fig. 5.** Horizontal ampulla of the pigeon. **a.** Base of a dark cell with deep infoldings (stars), vesicle rows (arrows) and mitochondria (m). A thin, basal cytoplasmic plate (b) from the neighbouring supporting cell separates the dark cell from the connective tissue (open stars). The dark cell directly contacts the basal lamina at one point only (arched arrow). f: fibrocyte. x 12,000. **b.** The dark cells (d) are separated from the basal lamina (short arrows) by the light supporting cells. A capillary with endothelium (e), bounded by several layers of thin pericyte-like cells (arrows). f: fibrocyte processes. x 8,000
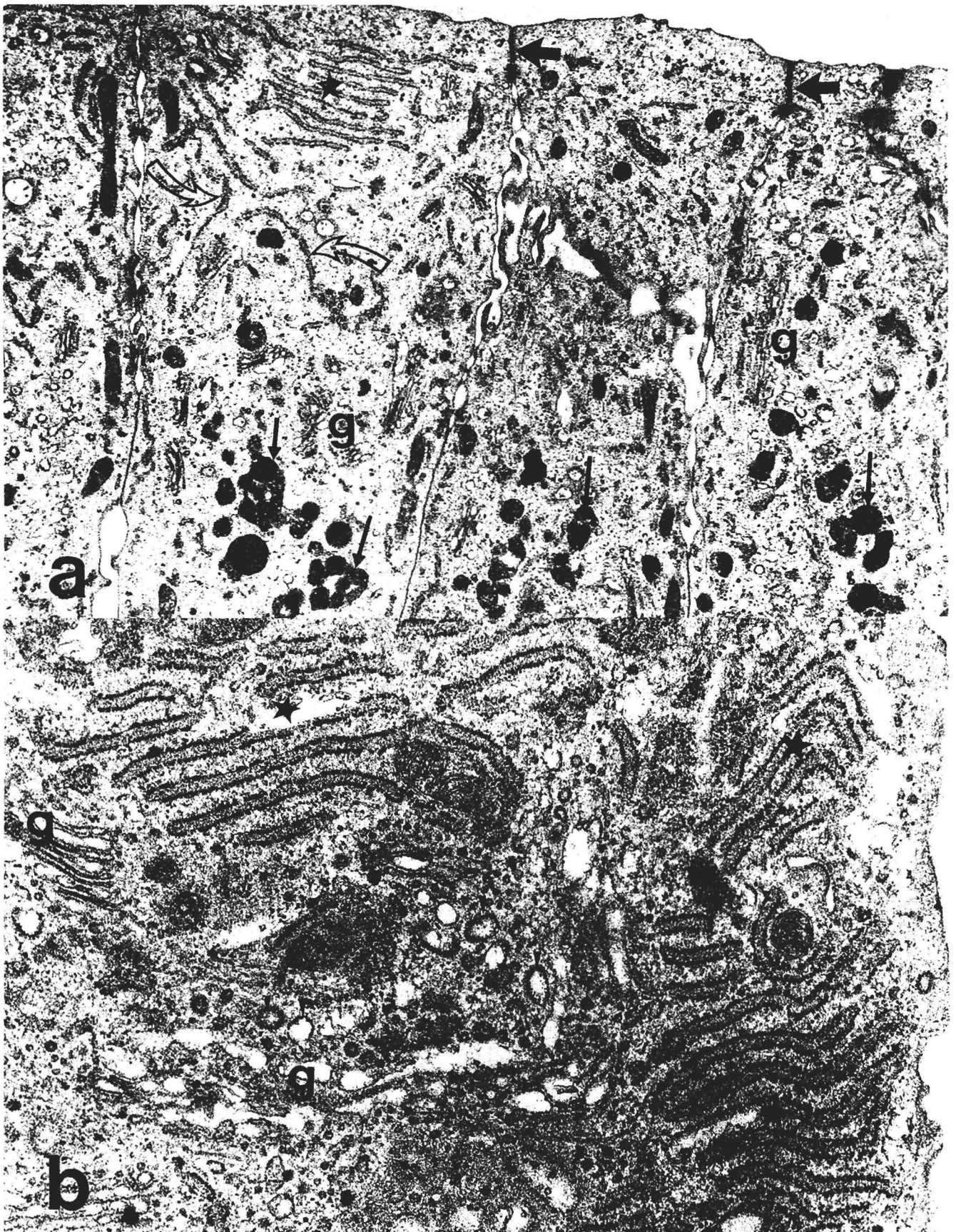
**Fig. 6.** Horizontal ampulla of the pigeon. Planum semilunatum. **a.** Apical area of cells with typical contact zones (short, thick arrows), Golgi apparatus (g), single rER profiles (arched arrows) and parallel-arranged cavities of rER (star). Thin arrows: region with numerous lipid droplets. x 12,000. **b.** as a) at higher magnification. Numerous coated and uncoated vesicles (arrowheads) in the vicinity of the Golgi apparatus (g) and numerous cisterns of the rER (stars). x 30,000
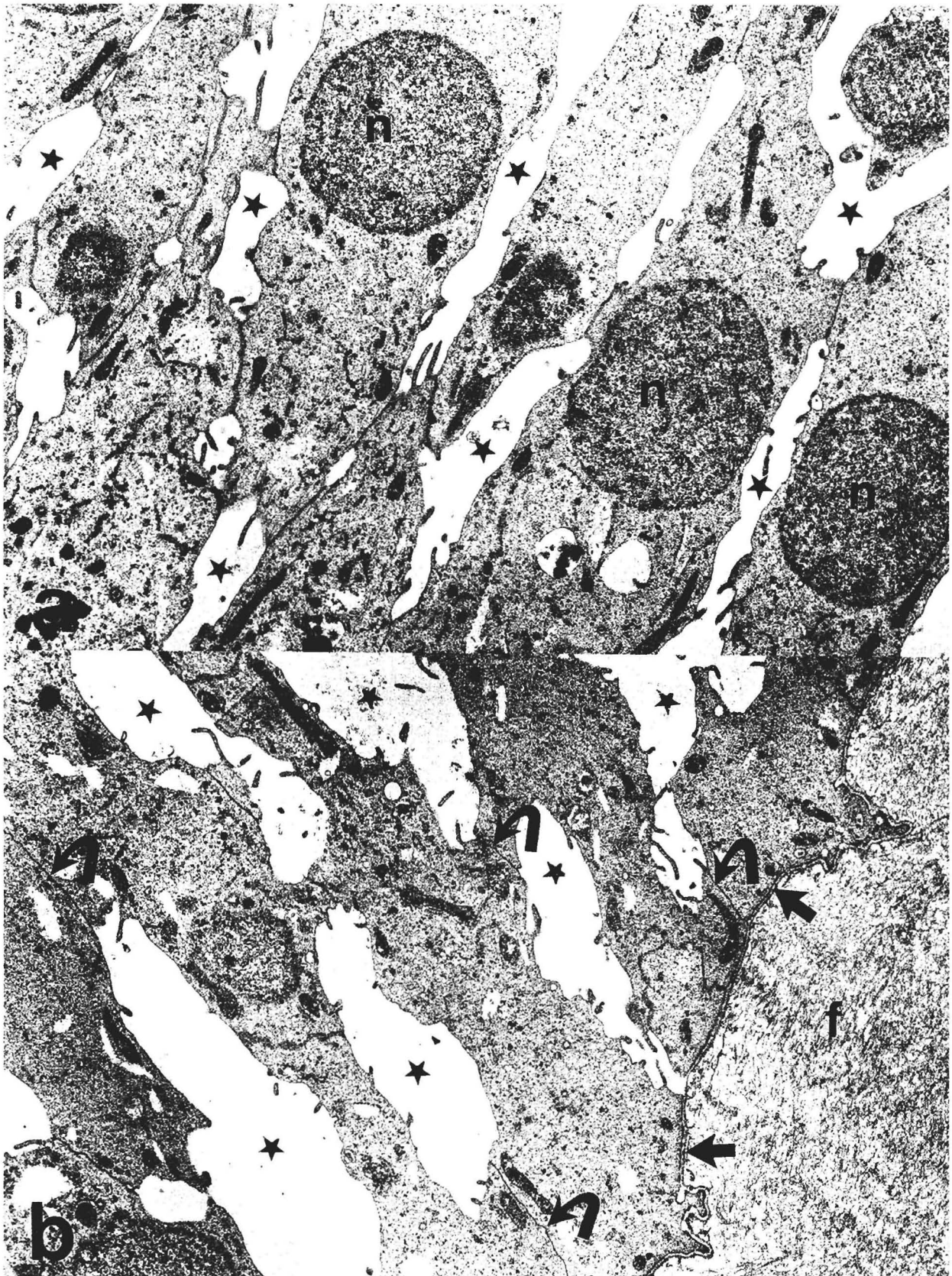
**Fig. 7.** Horizontal ampulla of the pigeon. Planum semilunatum. **a.** Section through cells with nuclei (n) and dilated intercellular spaces (stars). Few organelles. x 6,000. **b.** Basal region of the cell. Epithelial border (short arrows). Large intercellular spaces (stars). Gap junctions in the contact zones (arched arrows). f: fibrils. x 6,000

cells.

The apical part of the p.s. cells above the nucleus was characterised by three other structures: 1) Densely packed cisterns of RER (Fig. 6). In many cases up to 15 of these long and narrow cavities were arranged in parallel below the smooth apical cell membrane. 2) A large number of round lipid-like structures of variable electron density (Fig. 6). These were either isolated or arranged in groups enclosed by a membrane. Fine-granular material was often observed within such structures. 3) Many vesicles and vacuoles were present in the apical cytoplasm. Over long distances the basal cell membrane was smooth.

The course of the cell membranes and the contacts of the dark cells and of the planum semilunatum are all described together for better comparison (Fig. 8). The shape of the cells and the course of the membranes were dealt with in the description of the various cell types.

After tannic acid fixation, a distinct difference could be observed between the dark cells and the light supporting cells. The cell membrane of the dark cells had no tannic acid-positive surface coat and almost no vesicular activity in the apical part. In contrast, the surface of the supporting cells was distinctly tannic acid-positive. However, the thick surface coat was often discontinuous at the lumen, taking the shape of coated pits of various depth, or in some cases coated vesicles. In the lateral region of the cell membrane of the supporting cells continuous areas of the surface coat could be found, which were lightly tannic acid-positive. However, vesiculation processes were not observed. The cells of the planum semilunatum exhibited a typically thin and continuous surface coat after tannic acid fixation. With our material we were not able to observe any melanocytes.

The types of contact between the various cells were very similar (Fig. 8). Between the supporting cells, between the supporting and the dark cells, and between the cells of the planum semilunatum, a region of up to 200 $\mu$m in length existed near the lumen showing tight junctions. This type of contact was difficult to be demonstrated directly in sections fixed with tannic acid, because tannic acid does not penetrate between the cells and the surface coat of the cell membranes in these regions was missing (Fig. 8). Higher magnifications revealed a convergence of the outer lamellae of the cell membranes at the ends of this zone of contact. More basally, zonulae adhaerentes and desmosomes with distinct staining of the intercellular substance were seen. A few, irregularly distributed desmosomes could also be observed even more basally on the lateral membranes of all cell types. Striking were the large number and the length of the gap junctions between the supporting cells in the vicinity of the dark cells and between the cells of the planum semilunatum. Again, this type of contact became very clear after tannic acid fixation. It was particularly common around the basal, plate-formed regions of the supporting cells, which formed a basal border for the dark cells. In contrast, no gap junctions were found between the dark cells and their supporting cells.

## Discussion

A comparison of the ampullae of the different vertebrate species reveals, on the one hand, a great conformity but, on the other hand, also certain differences. Four aspects can be distinguished in this respect: 1) the actual anatomy of the inner ear or the ampulla, 2) the occurrence of certain cell types in the ampulla, 3) the distribution, or arrangement, of these cell types, and 4) the fine structure of the occurring cells.

In the following we want to focus on the 4th aspect, especially on the ion-transporting cells, i.e. the dark cells and the cells of the planum semilunatum. The dark cells show distinct species differences. In fish, these cells contain membrane-bordered vesicular and tubular cavities that correspond to smooth endoplasmic reticulum, but also exhibit orifices into the extracellular space and resemble in these cases a cell membrane (Becerra and Anadon, 1993). A similarity with chloride- and proton-transporting cells in HCl-secreting tissues appears to exist (Philpott and Copeland, 1963).

By contrast, the dark cells of mammals do not have this intracellular membrane system, but are characterised by deep basal and basolateral invaginations, as they are expressed in the basal labyrinth of the tubules of the kidney and other ion-transporting cells (Pease, 1956). The dark cells in the ampulla of the pigeon, however, have a position between these two species: in addition to the basal and basolateral invaginations they also have rows of vesicles known to exist in fish. The functional significance of this intermediate position is, according to our knowledge, not known with certainty.

Another problem is the composition of the region that contains the dark cells. The surprisingly regular distribution between light supporting cells and the dark cells that has so far not been described might be explained by the occurrence of this arrangement in the pigeon alone.

Of interest are the morphological differences between the dark cells and the cells of the planum semilunatum, given that similar functions are attributed to both cell types (Kimura, 1964; Dohlman, 1965, 1974; Iurato, 1967; Mees, 1983; Yoshihara and Igarashi, 1987). The dark cells exhibit the typical structure of ion- and $H_2O$-transporting cells as is the case in the proximal and distal tubule cells in the kidney, in some regions of the collecting ducts of salivary glands, and in marginal cells of the stria vascularis (Ishiyama et al., 1970; Mees, 1983; Forge et al., 1987; Holthofer et al., 1987; Winston et al., 1988; Bloom and Fawcett, 1994; Wangemann, 1995). They are characterised by deep basolateral membrane infoldings and apical processes. The flat cytoplasmic plates between the infoldings contain large cristae-rich mitochondria. In contrast, the cells of the planum semilunatum possess no indentations, infoldings or apical processes. Instead, they are characterised by the
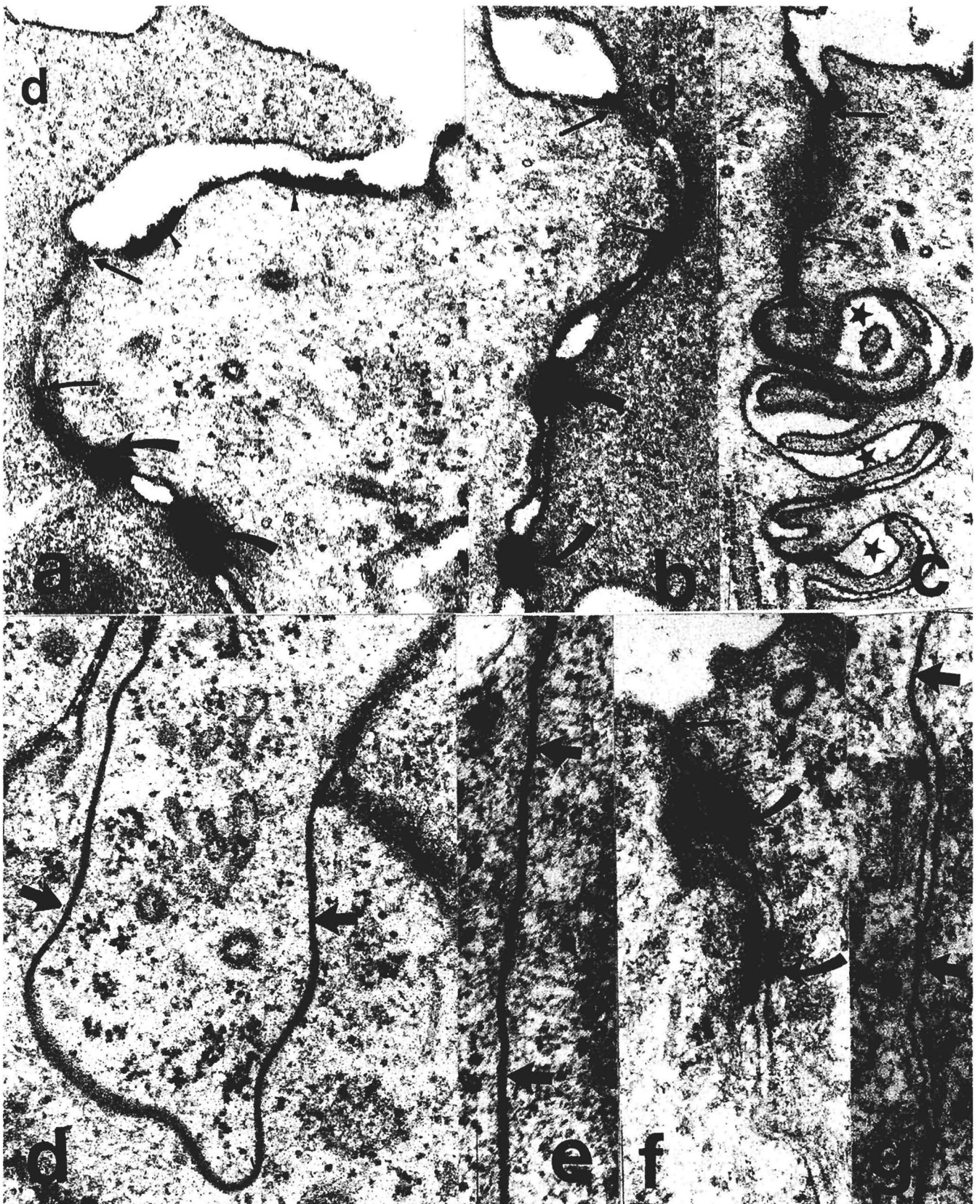
**Fig. 8.** Horizontal ampulla of the pigeon. Cell contact zones, **a-e:** tannic acid fixation. **f-g:** without tannic acid. **a.** Tannic acid-negative tight junctions (arrows) and desmosomes (arched arrows). On the left a dark cell (d) without surface coat, right a light supporting cell with thick surface coat (arrowhead). x 60,000. **b.** As a) but without luminal boundary. x 60,000. **c.** Contact between two light supporting cells. The zone with tight junctions lies between the two arrows. Directly beneath, a zone with indentations (stars). x 60,000. **d.** Gap junctions (arrows) in the basal region of cells in the planum semilunatum. x 6,000. **e.** As d) with magnification of gap junctions (arrows). x 75,000. **f.** Apical contact zone between cells of the planum semilunatum with tight junctions (arrows) and desmosomes or adhesion plaques (arched arrows). x 60,000 **g.** Lateral membrane of a planum semilunatum cell. With alternating tight junctions (arrows) and non-specific contacts. x 55,000

broad ICS. A similar picture is to be found in $H_2O$-transporting cells, e.g. in the colon and in the epithelium of the gall bladder (Ellis and Abel, 1964; Diamond and Tormey, 1966; Kaye et al., 1966; Diamond and Bossert, 1968; Herzer et al., 1969, 1970; Holthofer et al., 1987). Further investigations, including the immunohistochemical detection of aquaporins in the cells of the planum semilunatum, are needed in order to elucidate the water transport activities in the planum semilunatum. It must therefore be questioned whether the two cell types (dark cells and cells of the planum semilunatum) do perform identical functions. It is more likely that the dark cells secrete $K^+$, $Na^+$, $H^+$ and $Cl^-$ by means of Na-K-ATPase and other transport-ATPases, co-transporters, $Cl^-/HCO_3^-$ exchanger and, with the exception of $K^+$, most likely resorb these ions (Marcus et al., 1992, 1993; McGuirt and Schulte, 1994; Schulte and Steel, 1994; Crouch et al., 1997; Stankovic et al., 1997). In contrast, the cells of the planum semilunatum may function primarily in the transport of $H_2O$ (Diamond and Bossert, 1968; Herzer et al., 1969, 1970). The necessary osmotic gradient is most likely provided by a $Na^+$ gradient (Diamond and Tormey, 1966; Dibona and Mills, 1979; Greger, 1996). The larger basolateral surface area of the dark cells can be regarded as necessary for the integration of a highly developed ion transport system in the cell membrane.

The function of the compartments, or partial compartments, formed by the infoldings and the basal plates of the supporting cells, remains unclear. It could be regarded as an ion reservoir or a regulatory mechanism for ion flow. The existence of such an additional compartment underlines the similarity to the stria vascularis, where additional compartments are formed by the basally located basal cells (Sterkers et al., 1984; Wangemann, 1995).

Additional functions for the two cell types can be surmised from their other component parts: the dark cells possess lipid-enriched, lysosome-like inclusions, while the planum semilunatum cells exhibit not only similar inclusions but also membrane staples of RER and secretion vesicles.

The well-developed RER, the presence of a large Golgi apparatus in the apical cell region in the planum semilunatum cells and also apical secretory vesicles are strong indication for protein and proteoglycan secretion into the endolymph, either for the maintenance of protein components in the endolymph, or for the formation of cupula material (Dohlman and Ormerod, 1960; Cornford and Barajas, 1980; Munyer and Schulte, 1991).

The epithelium of the ampulla appears to be highly specialised. The distinct morphologies of each of the identified cell types indicate that they serve one or more specific functions. In this context the differences in surface coat properties in the region of the dark cells are of interest. The tannic acid-positive layer is absent in the dark cells, while in the light supporting cells it is very distinct over large stretches with apical vesiculation processes. It can therefore be speculated that one

function of the light cells is the endocytotic absorption of substances from the endolymph. The presence of coated pits and vesicles would indicate an absorption process mediated by receptors. The absence of the otherwise universal surface coats on the dark cells is particularly surprising. A search for parallel findings yielded the reports by Fawcett (1966) and Ghadially (1988) of a similar absence of surface coats in the parietal cells in the stomach. These cells and the dark cells of the ampulla have a $H^+$ and $Cl^-$ transport activity in common (Lim et al., 1983; Hsu and Nomura, 1985; Schmid et al., 1989; Marcus et al., 1993; Wangemann, 1995; Crouch et al., 1997; Stankovic et al., 1997).

The contact structures in the apical regions of the three cell types described here (dark cells, supporting cells and planum semilunatum cells) are identical: tight junctions, adhesion plaques and desmosomes, i.e. a junctional complex. Therefore, the endolymph space is more or less sealed. An ionic flow is primarily facilitated by active transport. In contrast, $H_2O$ is transported passively when a sufficient osmotic gradient is present (Greger, 1996). From the present findings it may be suggested that this $H_2O$ transport takes place predominantly in the area of the planum semilunatum. As is the case with other water-transporting epithelial units this may be driven by a $Na^+$ gradient (Diamond and Bossert, 1968; Herzer et al., 1969, 1970).

The occurrence of vesicles in the cytoplasmic plates of the dark cells between the infoldings is more difficult to interpret. They can be compared with the membrane-bordered spaces in other HCl-producing cells and with the endosomes involved in the endocytotic cycle (Eveloff et al., 1978; Schmid et al., 1989). As is the case with the dark cells of the ampulla, both of these cell types transport $H^+$. Since a carbonic anhydrase was observed in the dark cells, this function could be localised in the vesicles (Lim et al., 1983; Drescher and Kerr, 1985; Stankovic et al., 1997). However, an alternative interpretation is also possible. It is known that regulation of the surface area involves vesicle incorporation or new formation of vesicles at the cell membrane. If the basal membranes are considered as carrier membranes for enzymes and channel proteins in the previously described ionic transport systems, then the ion-transport activity could be regulated by the varying number and depth of the infoldings. The vesicle rows would represent a morphological equivalent of these processes. Furthermore, such processes would facilitate a faster regulation than a mechanism involving the new synthesis and decomposition of transport systems.

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