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## Degeneration and death, by apoptosis and necrosis, of the pavement and chloride cells in the gills of the teleost *Oreochromis mossambicus*

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**Summary.** Degeneration and death of branchial epithelial cells were studied in an African cichlid fish. In both freshwater and seawater fish the superficially located pavement cells are sloughed off at the end of their lifecycle. This process is preceded by degeneration via a process of cytoplasmic shrinkage and condensation related to apoptotic (physiologically controlled) cell death. The chloride cells are pleomorphic, i.e., accessory, mature, and degenerating cells. Degeneration of chloride cells mainly occurs by apoptosis. Degenerating cells show shrinkage and densification of cytoplasm and nuclei, and swelling of the tubular system; these cells are then separated from the ambient water by pavement cells. They are finally phagocytosed and digested by macrophages. Apoptosis of chloride cells, but not of pavement cells, is greatly stimulated when the fish are in seawater; this reflects an increase in cellular turnover of the chloride cells. Accidental cell death (necrosis) of pavement cells or chloride cells is rarely observed in fully adapted freshwater and seawater fish. Its incidence increases in the first few days following transfer of fish from fresh water to seawater.

**Key words:** Apoptosis – Necrosis – Gills – Chloride cells – *Oreochromis mossambicus* (Teleostei)

Fish gills are organs for respiratory gas exchange, osmoregulation, excretion of nitrogenous waste products, and acid-base regulation. This multifunctional character is reflected in the complexity of the branchial epithelium, which consists of at least four cell types: pavement cells, mucus cells, chloride cells and neuroepithelial cells (Laurent 1984). The epithelium covering the branchial filaments and their platelet outgrowths, the branchial lamellae, is exposed to a continuous flow of water that may contain potentially harmful substances. Many water pollutants are known to affect branchial structure (Mallatt 1985). Consequently, the gill epithelium has a high rate of cell renewal, with a half-life that in juvenile salmonids varies from 16 days in fresh water to 6 days in seawater (Conte and Lin 1967; MacKinnon and Enesco 1980; Chrétien and Pisam 1986; Zenker et al. 1987).

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Cell renewal implies that cell death and deletion occur in this tissue. In the last fifteen years it has become well established that there are two types of cell death: necrosis and apoptosis. They correspond to cell death by extrinsic or intrinsic factors, respectively (Kerr et al. 1972; Wyllie et al. 1980). Extrinsic factors include mechanical injury, the actions of infectious organisms, or toxic substances. Necrosis is structurally characterised by swelling of cells and cellular compartments, followed by disruption. Intrinsic factors are less well defined than extrinsic factors. In contrast to the latter, they are physiological in nature and include hormones, other humoral substances and tissue factors. Intrinsic cell death or apoptosis has become recognised as a physiological mechanism that is part of the genetic program controlling the balance between cell division, differentiation and deletion in many epithelia and other tissues (Wyllie 1981; Bowen 1981). Extrinsic factors may induce apoptosis indirectly, e.g. via the action of hormones. Apoptotic cells show a characteristic sequence of cellular shrinkage and increasing osmiophilia of the cellular components, ending in the transformation of each cell into one or more compact apoptotic bodies that are phagocytosed by adjacent epithelial cells or by macrophages (Kerr et al. 1972; Wyllie et al. 1980).

The branchial epithelium has been extensively studied in many fish species, and in fish from water of different osmolarity and ionic composition or water containing various kinds of pollutants (Laurent 1984; McDonald 1983). With respect to cell death, only the effects of extrinsic factors have been examined to date. There is in particular an extensive literature on the histopathological effects of pollutants on fish gills (Mallatt 1985). Intrinsic cell death in fish gills has received little attention, and no specific reference to apoptosis is known to us. However, knowledge not only of cell division and differentiation, but also of cellular ageing and deletion is a prerequisite for the understanding of the cellular dynamics and the histopathology of the branchial epithelium. The branchial epithelial cells, in particular the chloride cells, are pleomorphic in ultrastructure. The status of the accessory chloride cells, which have been considered to be developing or degenerating chloride cells (Coleman et al. 1977; Sardet et al. 1979; Hootman and Philpott 1980) or to be a functionally specific type of cell (Dunel and Laurent 1980; Chrétien and Pisam 1986), is still debatable.

In the present study, the branchial epithelium of the African cichlid teleost *Oreochromis mossambicus* (formerly



called *Sarotherodon mossambicus* and *Tilapia mossambica*) is described. This is a euhaline species found in fresh water and in seawater, in coastal areas. Chloride cell function has been studied extensively in this species (Foskett et al. 1983). Degeneration, death, and deletion of pavement cells and chloride cells were studied in freshwater fish and in fish transferred from freshwater to seawater. Using the latter group, it was possible not only to examine the structural changes associated with seawater transfer, but also to analyse cell degeneration and death under conditions of accelerated cell renewal.

### Materials and methods

Sexually mature male and female tilapia *Oreochromis mossambicus* of 8–20 g body weight were obtained from laboratory stock. They were kept in 100 l aquaria containing tap water, at 25° C with a daily 12 h photoperiod. The water was continuously circulated through charcoal filters and partially refreshed when necessary to keep nitrogenous waste at low levels. The concentrations of the main ions (in mM) were: Na<sup>+</sup>, 5.0; K<sup>+</sup>, 0.06; Ca<sup>2+</sup>, 0.8; Mg<sup>2+</sup>, 0.2; Cl<sup>-</sup>, 4.2; SO<sub>4</sub><sup>2-</sup>, 0.5; pH 7.6.

Freshwater fish were transferred to half-strength (one day) and subsequently to full-strength seawater prepared from Wimex artificial seasalt. Concentrations of the main ions (in mM) of full strength seawater were: Na<sup>+</sup>, 468; K<sup>+</sup>, 9.7; Ca<sup>2+</sup>, 10.2; Mg<sup>2+</sup>, 56; Cl<sup>-</sup>, 555; SO<sub>4</sub><sup>2-</sup>, 56; HCO<sub>3</sub><sup>-</sup>, 2.3. The fish were killed after a stay of 3, 5 or 28 days in full-strength seawater.

For electron microscopy the gill filaments were prefixed in cacodylate-buffered 3% glutaraldehyde (0.1 M, pH 7.4) for 15 min at room temperature. They were fixed in a similarly buffered mixture (1:1:1) of 2% osmium tetroxide, 3% glutaraldehyde and 5% potassium dichromate, for 1 h at 0° C, block-stained for 1 h in 1% uranyl acetate, and embedded in Spurr's resin. Sections were stained with lead citrate and examined in a Philips 301 or Jeol electron microscope.

### Results

The architecture of the branchial filaments and lamellae of *O. mossambicus* is similar to that described for other teleost species (see reviews by Laurent and Dunel 1980; Laurent 1984). Briefly, the filaments contain afferent and efferent arteries that are in connection with a venous blood space and that open into the vascular spaces of the lamellae via afferent and efferent arterioles. The filaments are covered by a multilayered epithelium of specialized and undifferentiated cells. The latter are located in the basal layers of the epithelium, and give rise to pavement cells, chloride cells and mucocytes. The pavement cells contain a network of microfilaments and are interconnected by desmosomes and tight junctions. This system is prominent in the cells covering the leading and trailing edges of the filaments. The apical membrane is folded into microridges (Figs. 1, 2). Intercellular spaces are often present between the more basally located cells; these spaces may contain an occasional leucocyte or macrophage (Fig. 9).

The lamellar epithelium mainly consists of two layers of pavement cells and, in the basal layer, undifferentiated cells. The two layers are partially separated by intercellular spaces. Chloride cells are common in the interlamellar parts

of the filament epithelium. They also occur on the leading and trailing edges of the filaments, and occasionally on the lamellae.

### Freshwater fish

The pavement cells of the filament and lamellar epithelium are well developed and pleomorphic. They all contain a network of microfilaments with a diameter of about 20 nm, a few mitochondria, and a single Golgi area or lysosome (Fig. 3). Several cells show signs of degeneration. Rarely, a cell displays swelling of the cytoplasm, mitochondria and nucleus; this may be accompanied by membrane fragmentation, and is interpreted as necrosis. More frequently, cells with signs of apoptosis are observed, i.e. cellular shrinkage, condensation of most cellular compartments including cytoplasm and nucleus, and condensation of nuclear chromatin (Figs. 3, 4). Such cells eventually lose desmosomal contacts with adjacent cells, and may disappear into the water.

The chloride cells on the filaments occur as isolated cells or, less frequently, in groups of two or three between the pavement cells (Figs. 5, 6). In the lamellar epithelium, an occasional isolated chloride cell may be found, mainly at the base of the lamellae. The most common form contains an extensive network of smooth anastomosing tubules, associated with numerous mitochondria, some strands of granular endoplasmic reticulum, a Golgi area, and a few small clear vesicles in the apical region of the cell that is in contact with the ambient water. The apical cell membrane is folded into small projections and may be slightly concave or form an apical cavity (Fig. 5). This type is considered a mature chloride cell. Several mature chloride cells are associated with a crescent-shaped cell having the characteristics of an accessory cell (Hootman and Philpott 1980; Laurent 1984), being small and electron dense, with many small mitochondria. In comparison with mature chloride cells, these crescent-shaped cells have a less

**Figs. 1–6.** Epithelium of the branchial filaments of freshwater fish; Figs. 1–3: trailing edge; Figs. 4–6: interlamellar region

**Fig. 1.** Pavement cells; the surface of the upper cell layer is folded into microridges. × 7200

**Fig. 2.** Tight junction (*tj*) and desmosomes (*d*) between two pavement cells located at the epithelial surface; *arrow* microfilaments. × 32000

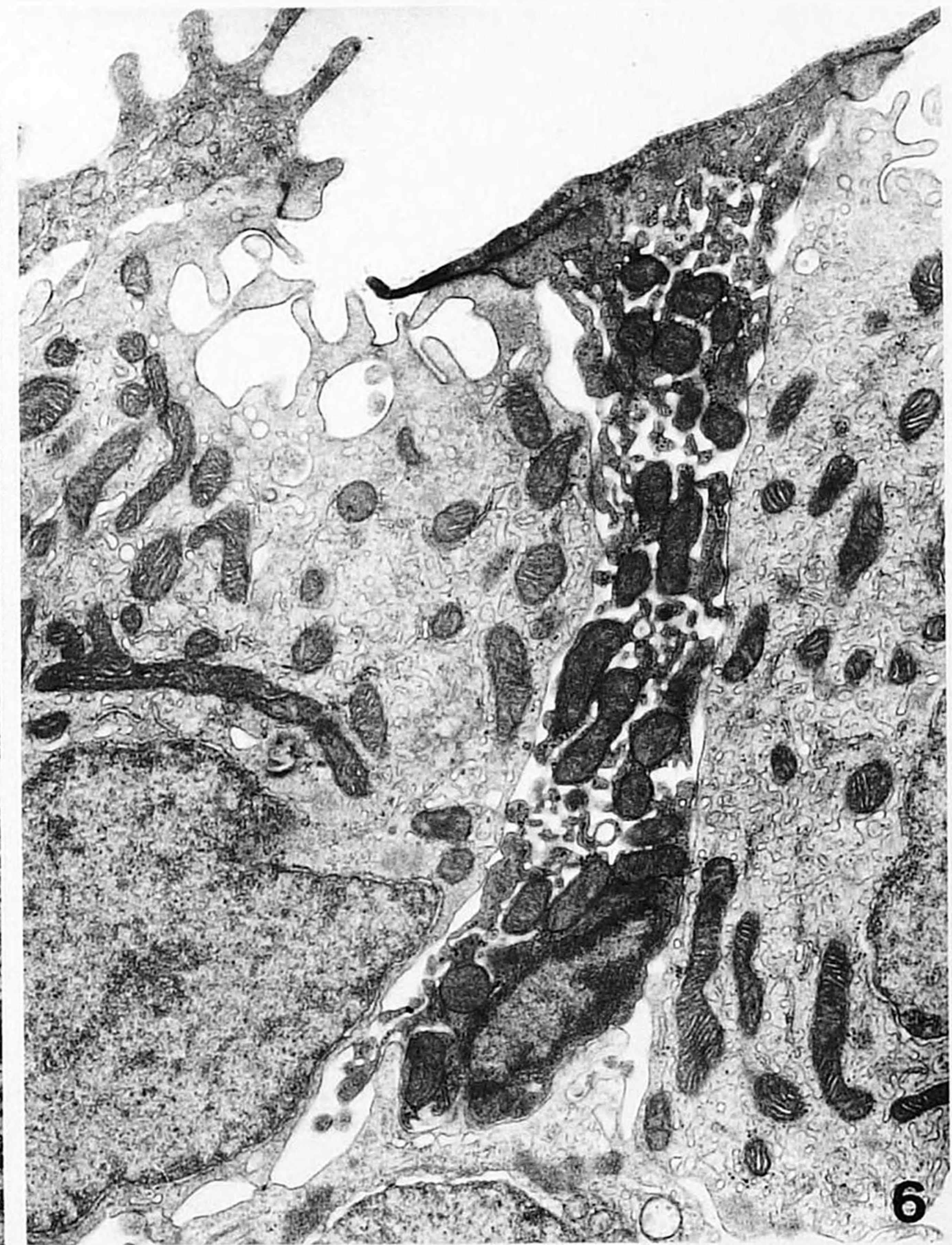
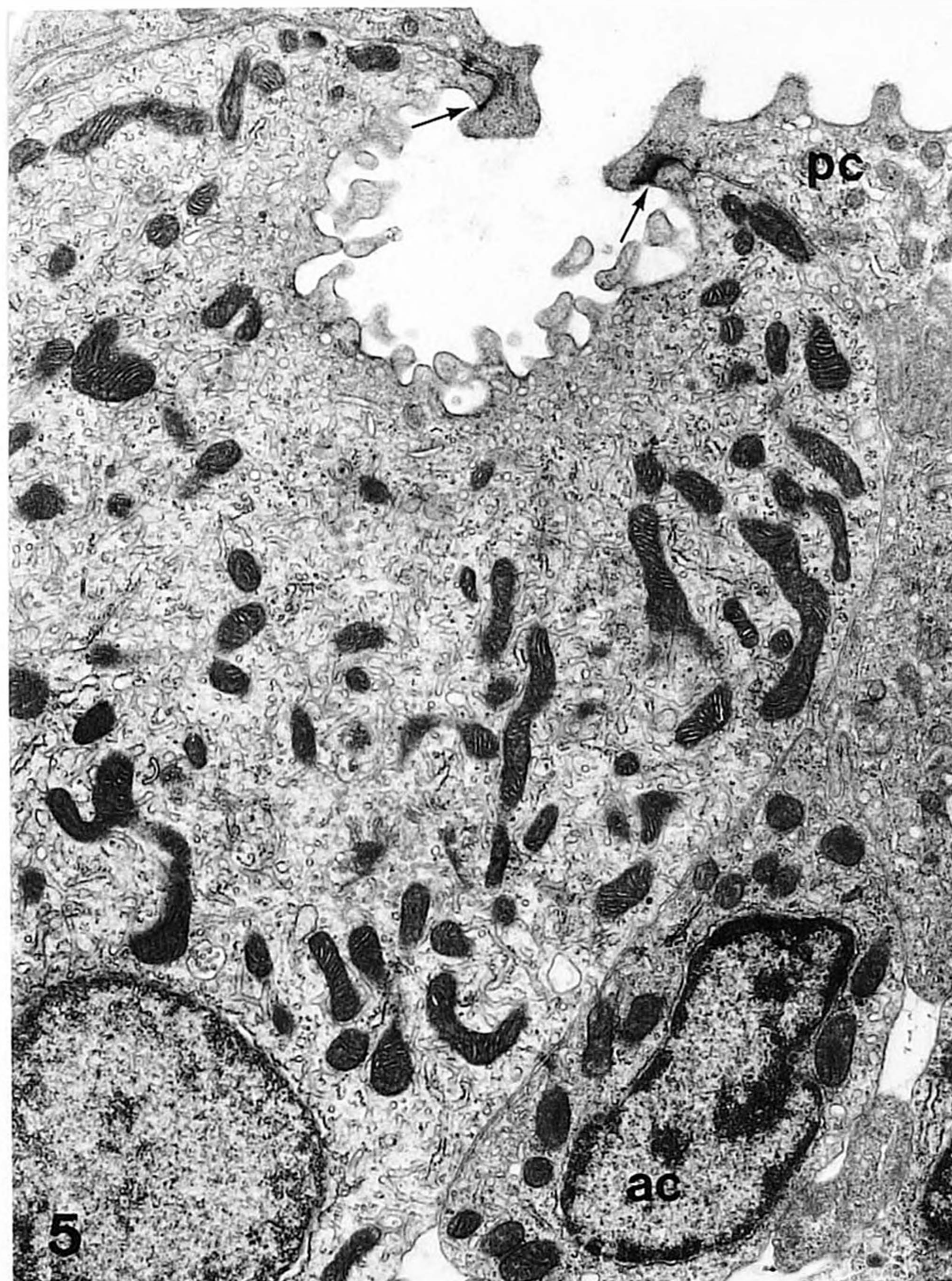
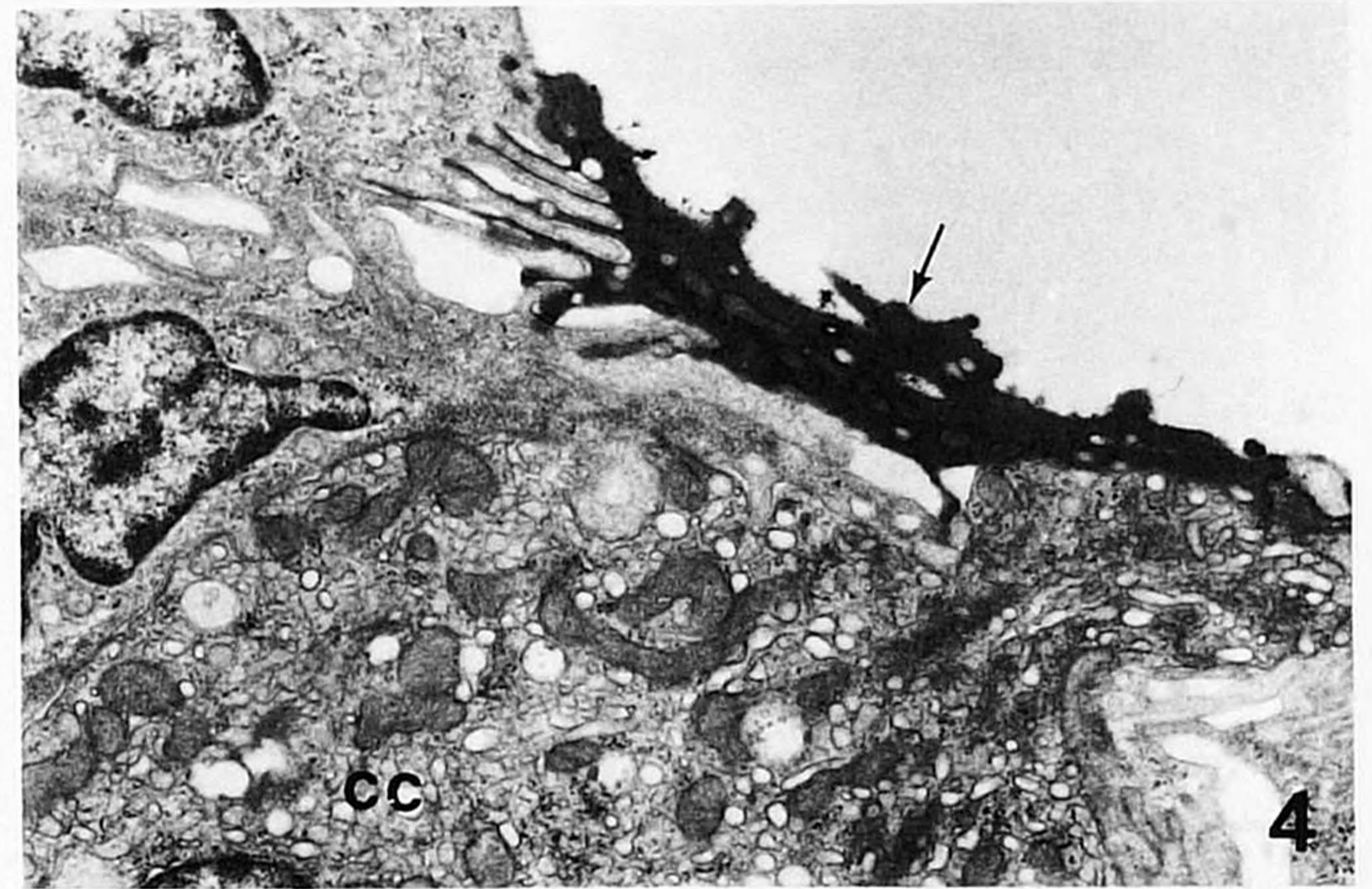
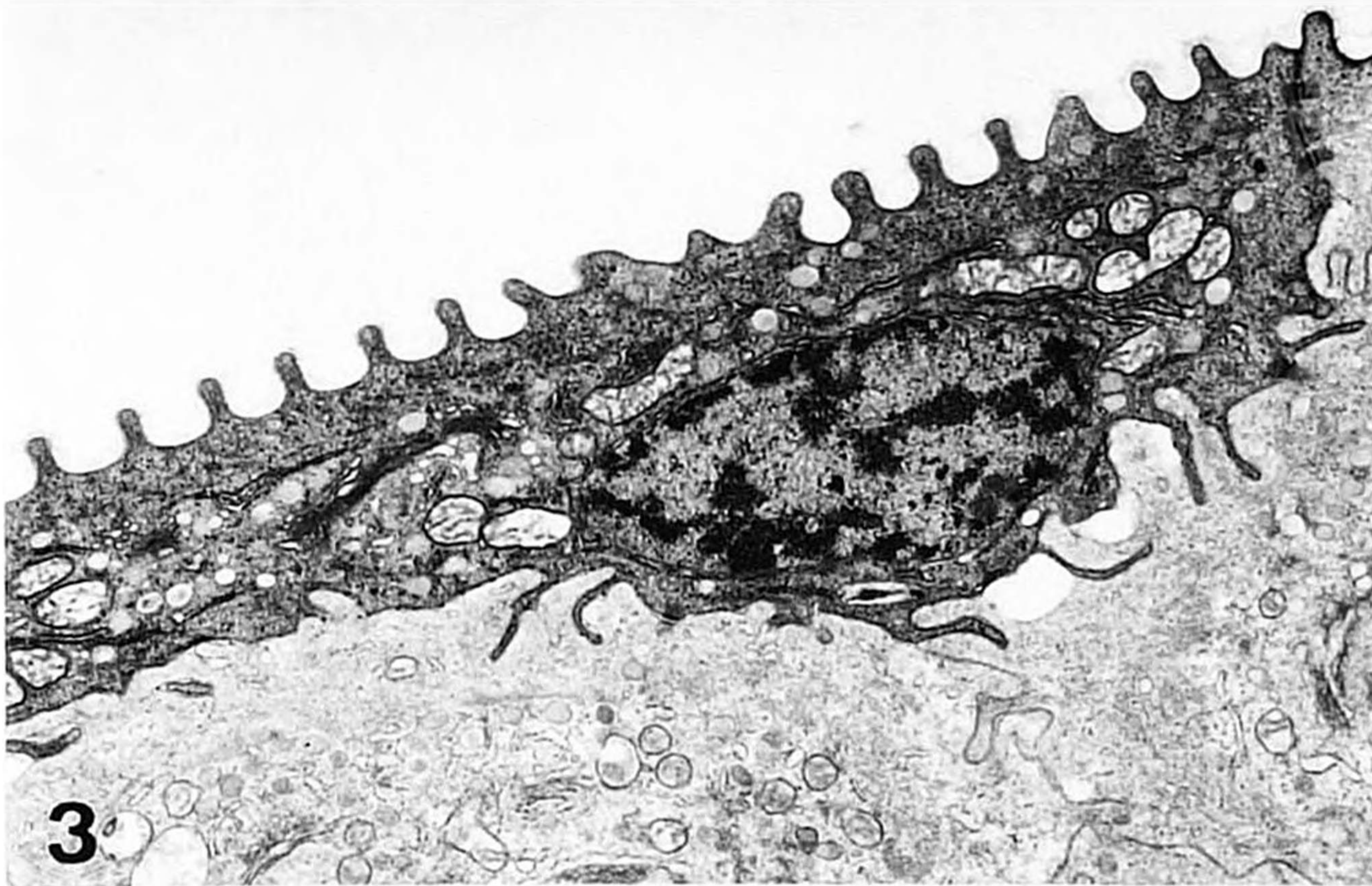
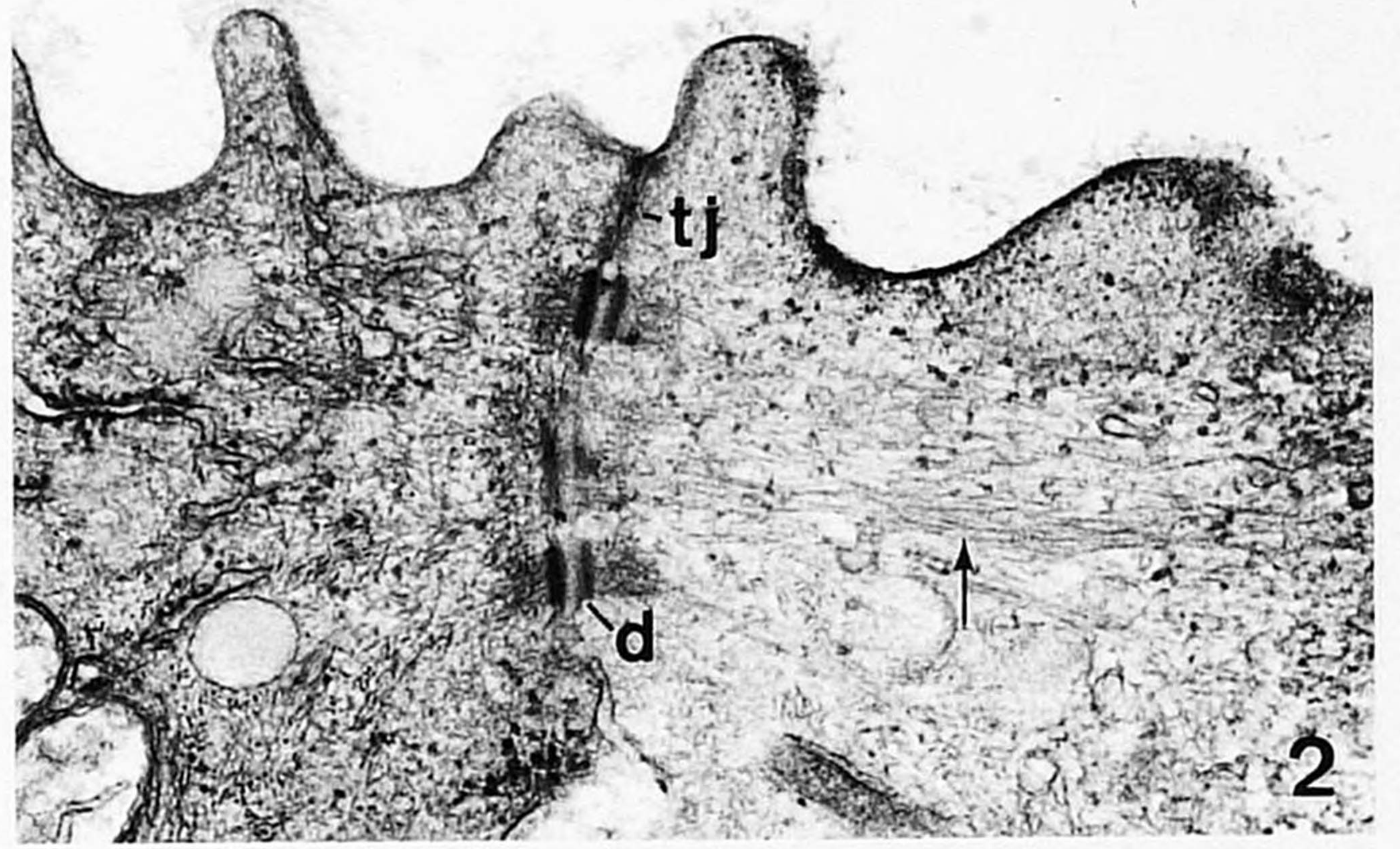
**Fig. 3.** Pavement cell at the epithelial surface showing the cytoplasmic and nuclear condensation characteristic of apoptosis. × 8000

**Fig. 4.** Electron-dense remnants of pavement cells at the epithelial surface (*arrow*), probably representing final stage of apoptosis; *cc* chloride cell showing distension of the tubular system typical for the start of apoptosis. × 10000

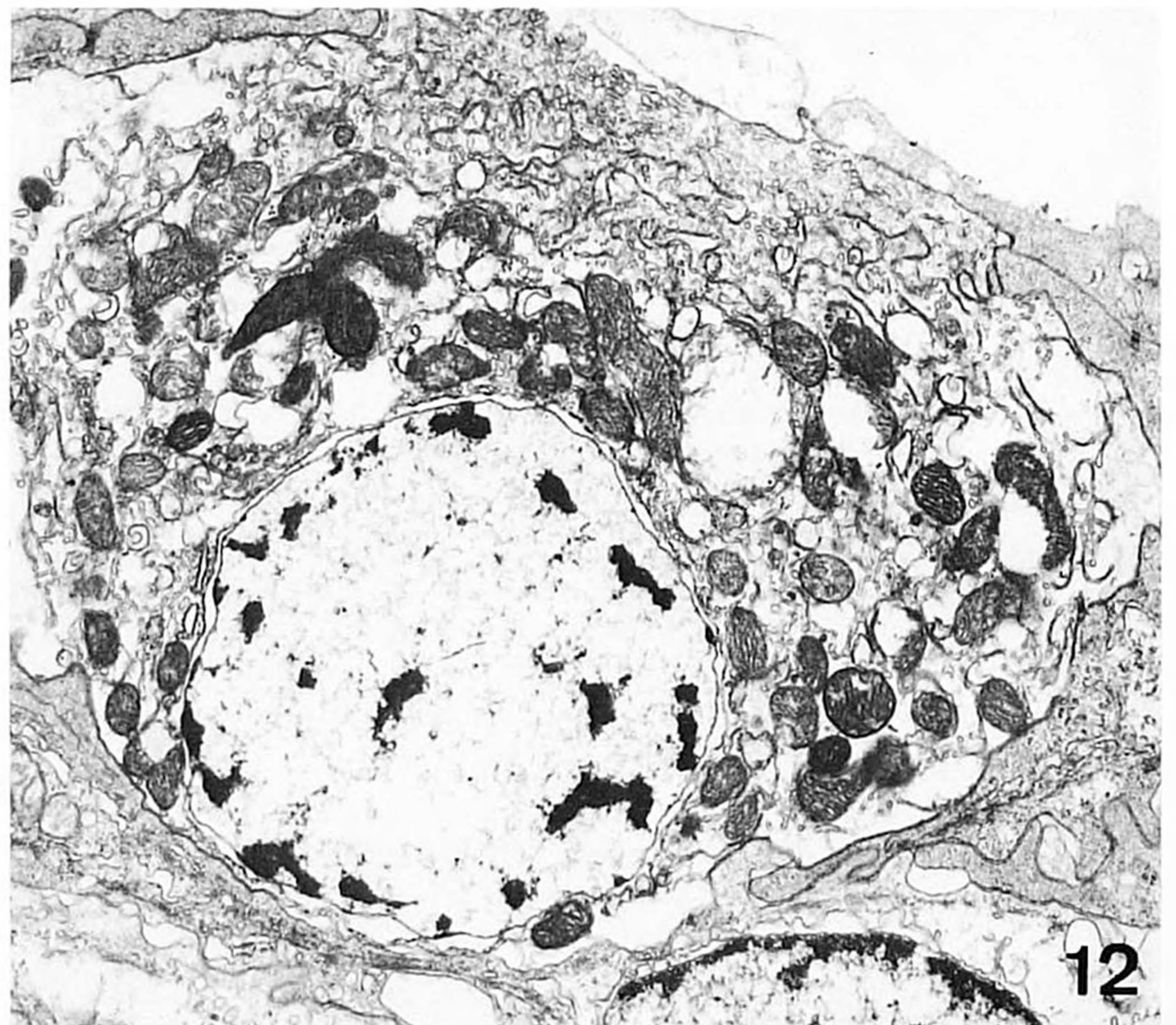
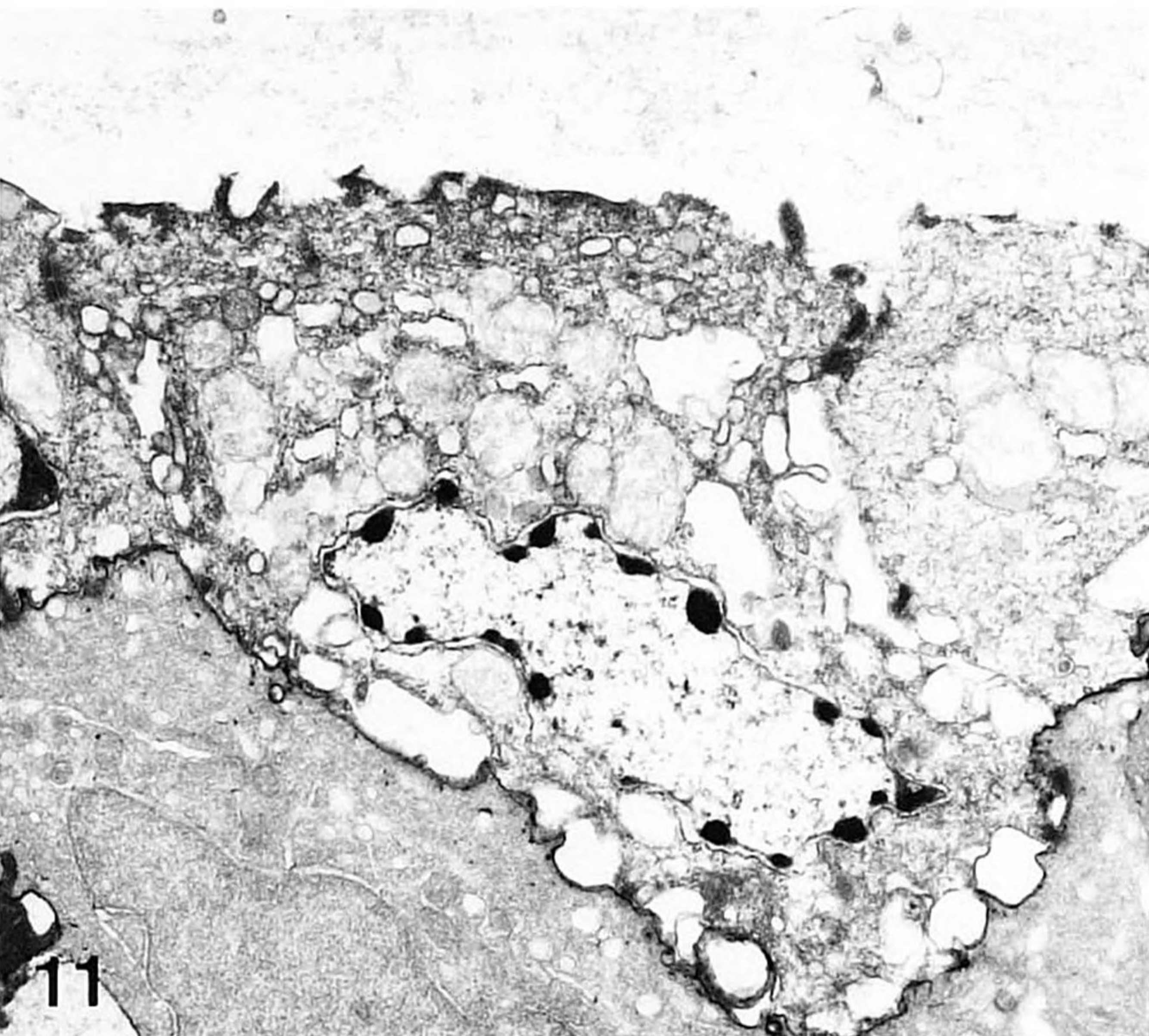
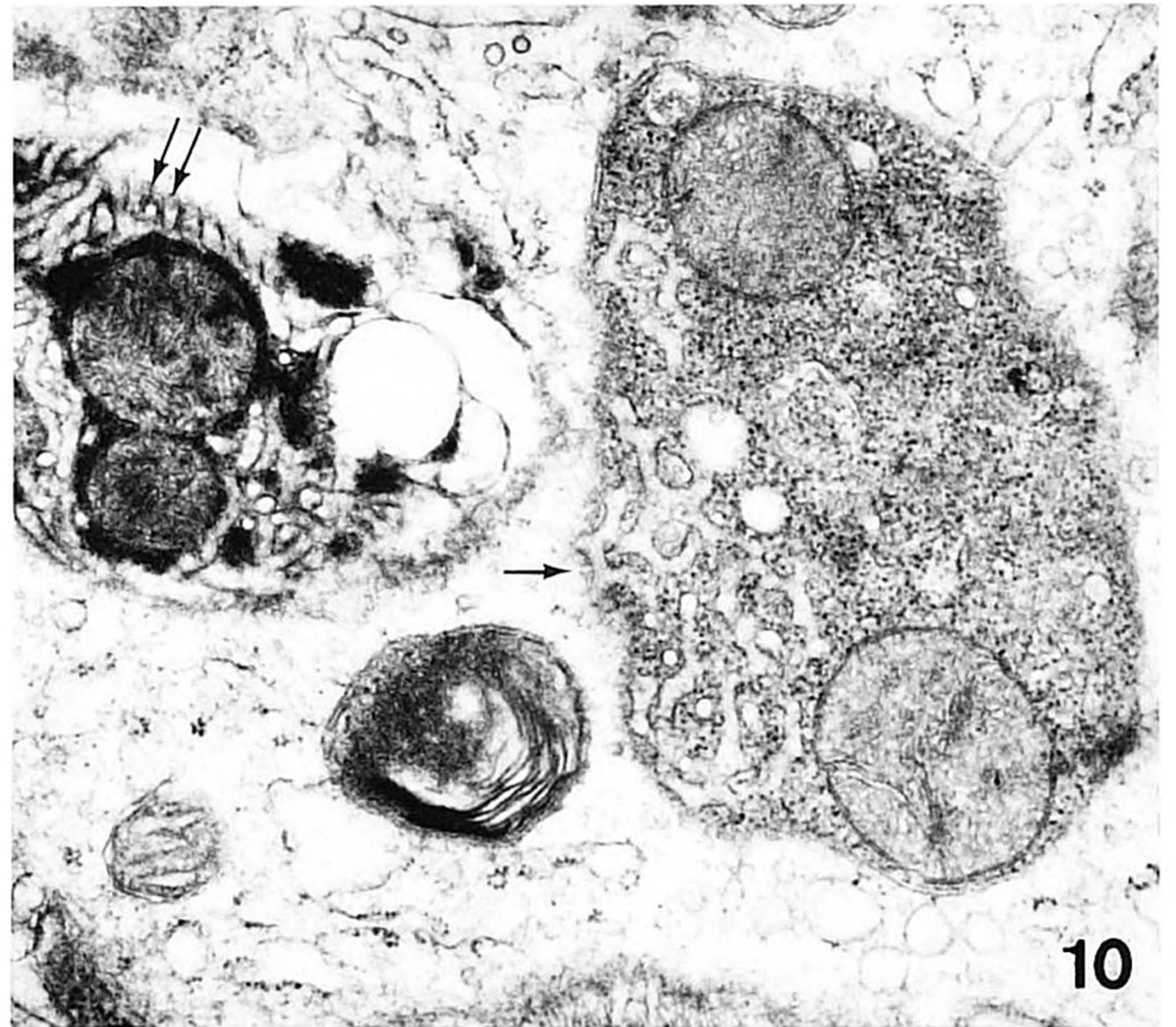
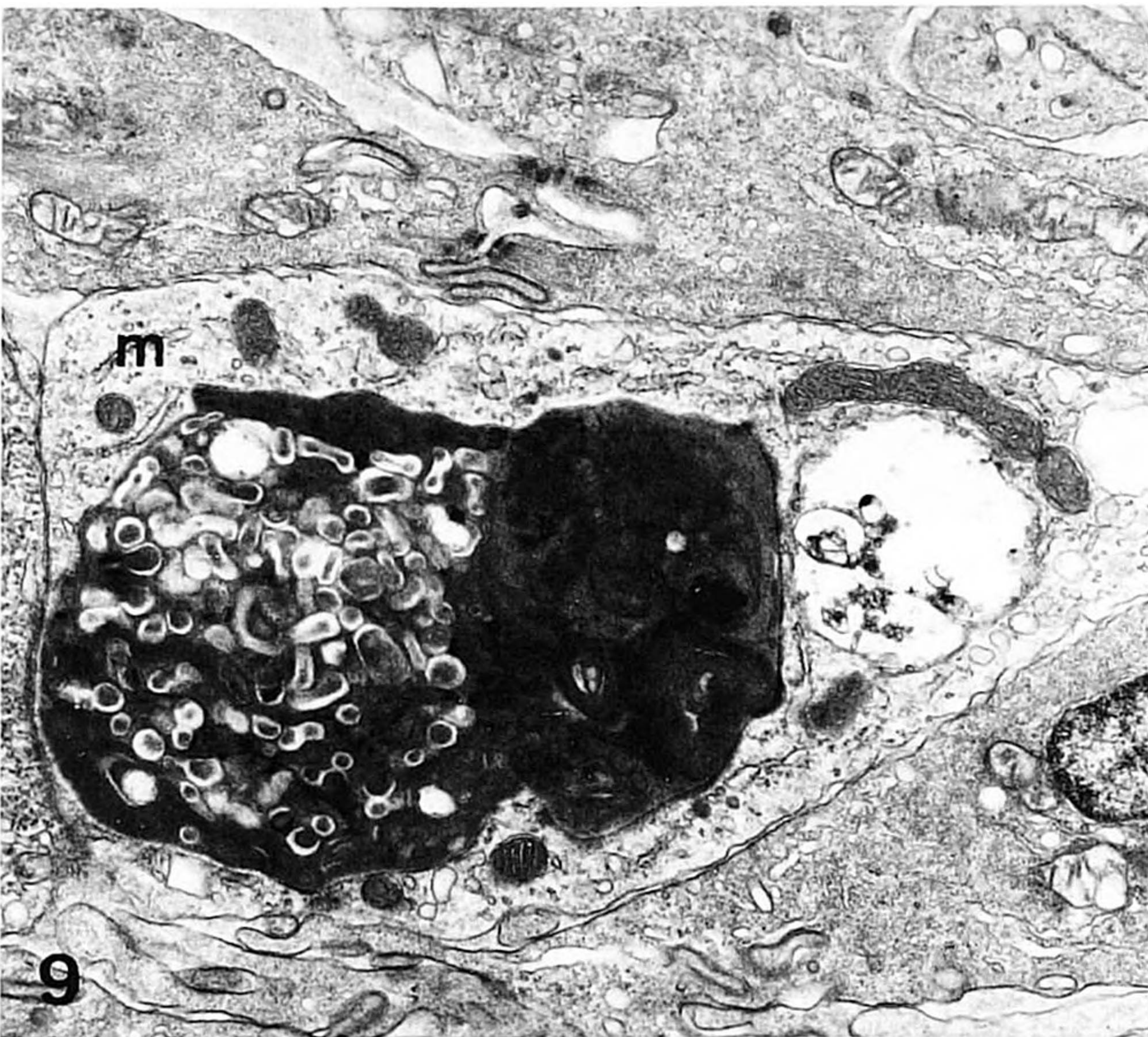
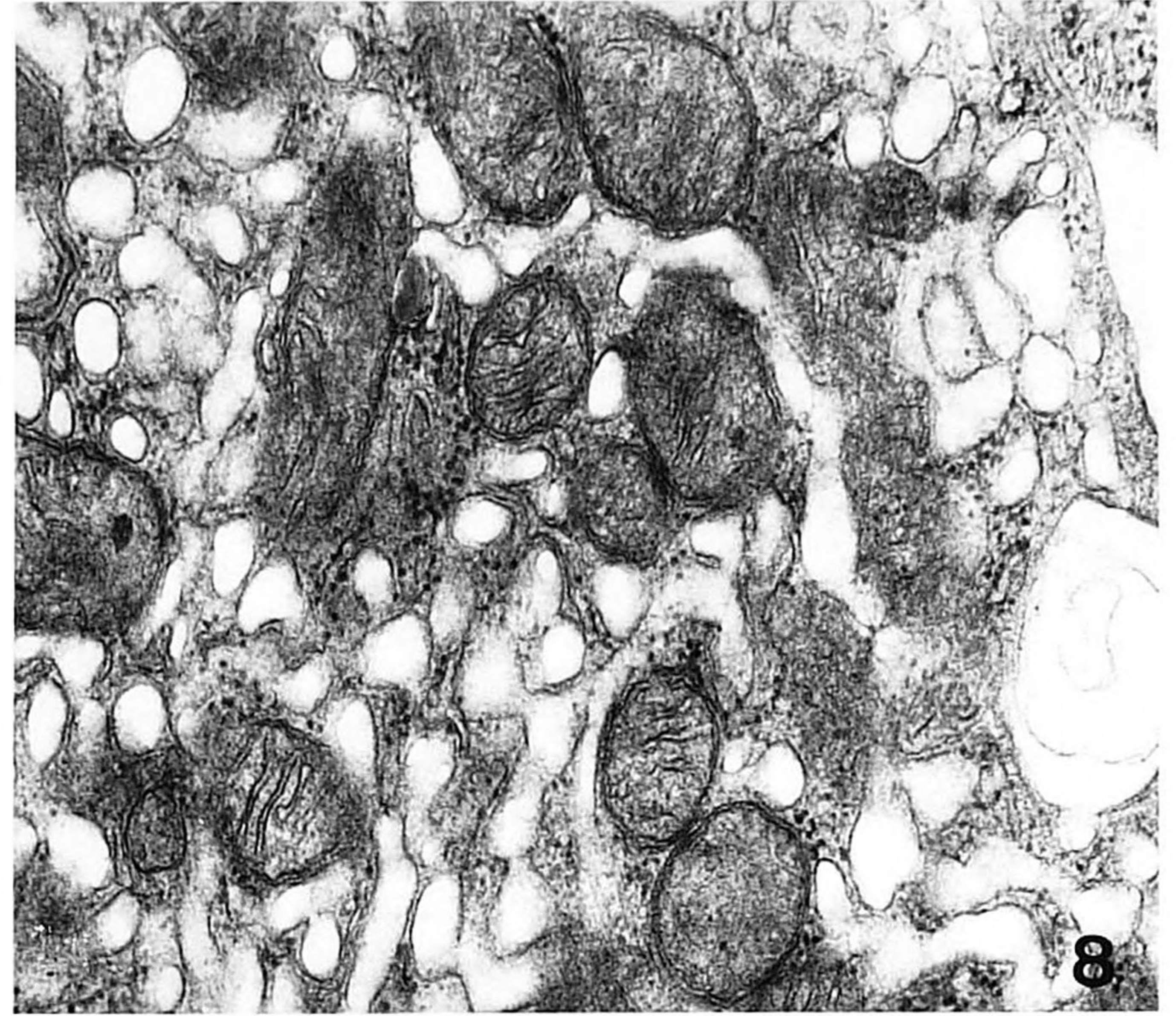
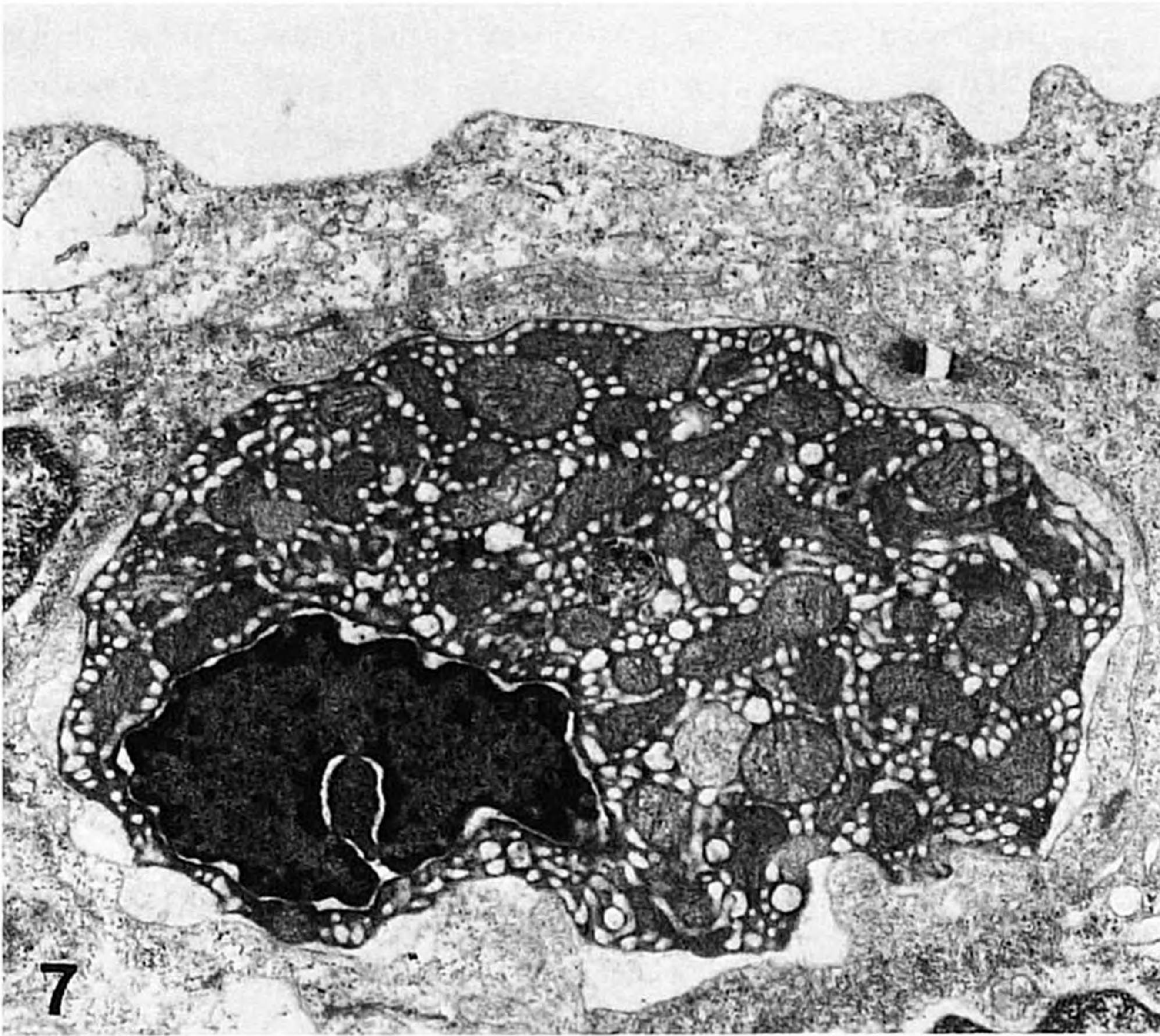
**Fig. 5.** Mature chloride cell accompanied by a small accessory cell (*ac*); the mature chloride cell shows an apical cavity with villous cytoplasmic projections; *arrows* tight junctions between chloride cell and pavement cells. × 10000

**Fig. 6.** Group of three chloride cells; the left and right cells are mature chloride cells; the slender cell in the middle shows cell shrinkage, cytoplasmic condensation, and distension of the tubular system typical for apoptosis. × 12000











extensive tubular system and a more developed granular endoplasmic reticulum (Fig. 5). Chloride cells and accessory cells are interconnected by desmosomes and, when the apex of the accessory cells penetrates towards the apical cavity, by tight junctions. Desmosomes between chloride cells and pavement cells are also found.

Occasionally, chloride cells are present that show signs of degeneration. Most of these cells are distinguished from the mature cells by a higher electron density of the cytoplasm and a variable shrinkage of the cell volume. We interpret these cells as representing different phases of apoptosis. In cells with only a slight degree of cytoplasmic condensation, the lumen of the tubular system is distended and apical specializations disappear (Figs. 4, 6, 8). In other cells, these phenomena are more pronounced and, in addition, the mitochondria become more electron dense, whereas the nuclear envelope shows indentations and the chromatin condenses. Such cells are globular and are usually separated from the ambient water by cytoplasmic extensions of pavement cells (Fig. 7). Cells that are even more dense form a compact mass of distended tubules, mitochondria, and contain a small multilobular and heterochromatic nucleus, similar in appearance to the apoptotic bodies described in other tissues (Wyllie et al. 1980; Schoots et al. 1983). These bodies are usually found in the cytoplasm of cells that are tentatively identified as macrophages because of the electron-transparency of their cytoplasm and the occasional presence of lysosomes. These cells lack the network of filaments and the desmosomes characteristic of the pavement cells, and apparently remove the apoptotic bodies by lysosomal digestion (Figs. 9, 10).

### Seawater fish

After transfer to seawater, most areas of the filament epithelium remain structurally intact, but in some areas substantial necrotic damage to the superficially located pave-

ment cell layers is noticeable in the first days following transfer. Mitochondria are swollen and their matrix is electron transparent. The matrix of the nuclei also becomes transparent, with electron-dense accumulations of chromatin concentrated at the periphery (Fig. 11). In more progressive stages of necrosis, the cell boundary may rupture and the cytoplasmic architecture is destroyed. These phenomena are rarely found in fish examined four weeks after transfer. In these fish, the pavement cells are more flattened than in freshwater fish, in particular in the epithelium covering the edges of the filaments. The frequency of pavement cell apoptosis is not noticeably different from that of freshwater fish.

Three and five days after transfer to seawater, the chloride cells are more frequent than in freshwater fish. They usually occur in groups of two to four cells, i.e. as accessory cells, mature chloride cells and degenerating chloride cells. The accessory cells increase in size and number after transfer. As in fresh water, they are characterised by an extensive endoplasmic reticulum and an underdeveloped tubular system (Figs. 13, 16). Some of these cells seem structurally intermediate between accessory cells and mature chloride cells (Fig. 16). Cytoplasmic arms of accessory cells that interdigitate with the apical membranes of mature chloride cells, as described in other seawater-adapted fish (Laurent 1985), are absent in *O. mossambicus* (Fig. 13). The mature chloride cells have the same structure as those in freshwater fish, although the apical cavities may be larger, the tubular system more intensely anastomosing and the apical vesicles more numerous after transfer to seawater. These characteristics are also typical for the chloride cells of fish examined four weeks after transfer.

Degenerating cells are common. Three days after transfer, some chloride cells exhibit signs of necrosis, i.e. electron transparent cytoplasm, swollen mitochondria (often with fragmented cristae), electron-transparent nuclei with dense patches of chromatin, and occasionally disrupted nuclear and cellular membranes (Fig. 12). Necrotic chloride cells are rarely observed three weeks after transfer. Apoptotic chloride cells are very common after three days in seawater (Figs. 14, 15). A conservative estimate, based on the analysis of hundreds of electron micrographs, points to a four-to five-fold increase in the number of these cells when compared with freshwater fish. The frequency of these cells is still high three weeks after transfer. This is true for all stages of apoptosis. As in freshwater fish, we interpret condensation of the cytoplasm and distension of the tubular system (Fig. 13) as the first stage of this process. The nuclei become more electron-dense, the cell bodies may become concave and the intercellular spaces around these cells may enlarge, probably as a result of cellular shrinkage (Fig. 14). Finally, the cells may become globular (Fig. 15) and engulfed by macrophages. Macrophages containing large lysosomal bodies that are recognisable as apoptotic remnants of chloride cells are more common than in freshwater fish, in particular during the first days after transfer (Fig. 16).

## Discussion

### Differentiation and degeneration

Our results show that degeneration and death of the epithelial cells of filaments and lamellae mainly occur via apopto-

**Figs. 7–12.** Branchial epithelium of freshwater fish (Figs. 7–10) and of fish exposed for 3 days to seawater (Figs. 11–12)

**Fig. 7.** Apoptotic chloride cell separated from the ambient water by pavement cells; the nucleus is electron-dense and indented; the tubular system is distended; *d* desmosome between pavement cells; trailing edge of filament.  $\times 11\,000$

**Fig. 8.** Interlamellar epithelium of seawater fish; cytoplasm of chloride cell showing distension of the tubular system and condensation of the cytoplasm, typical for the first stage of apoptosis.  $\times 36\,000$

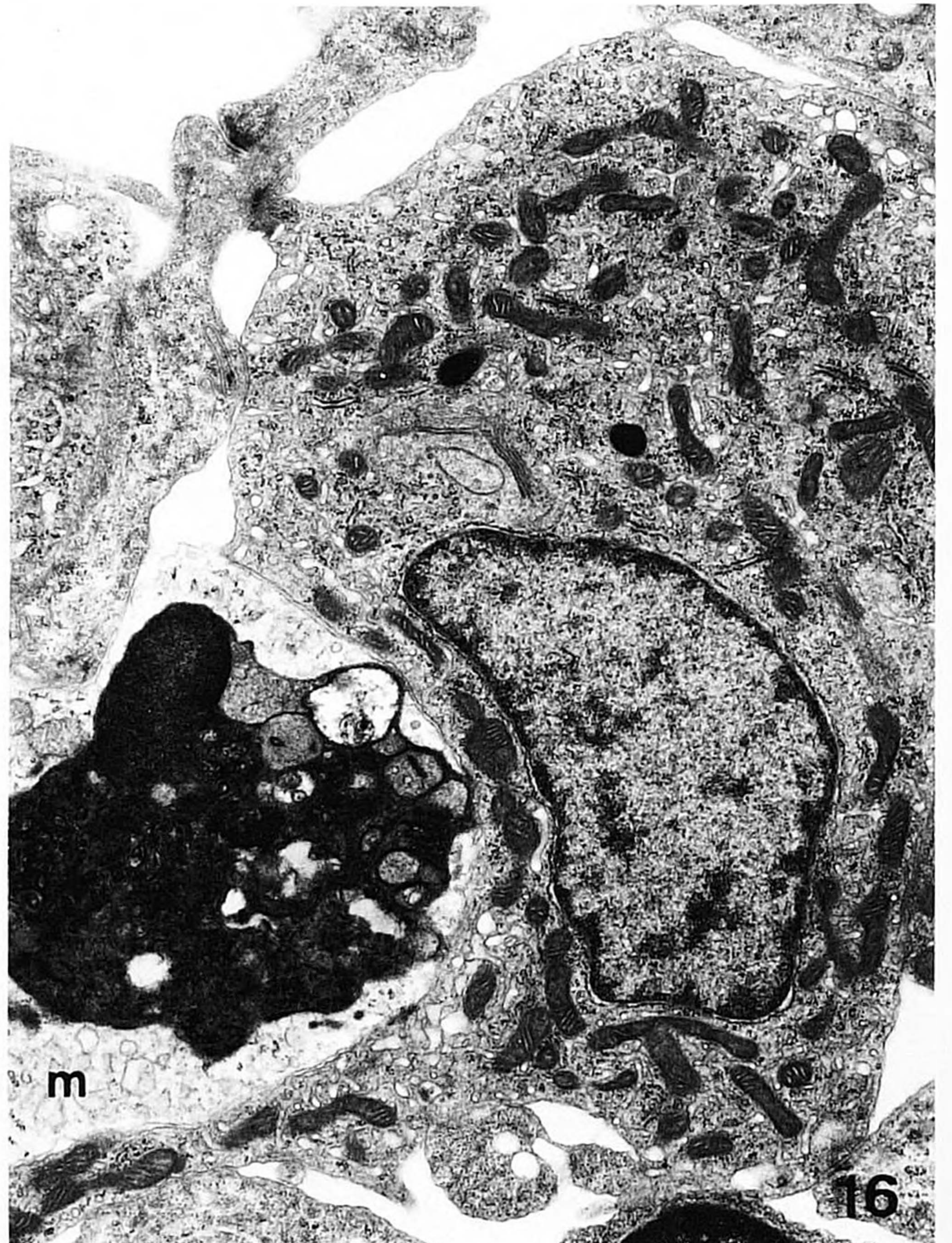
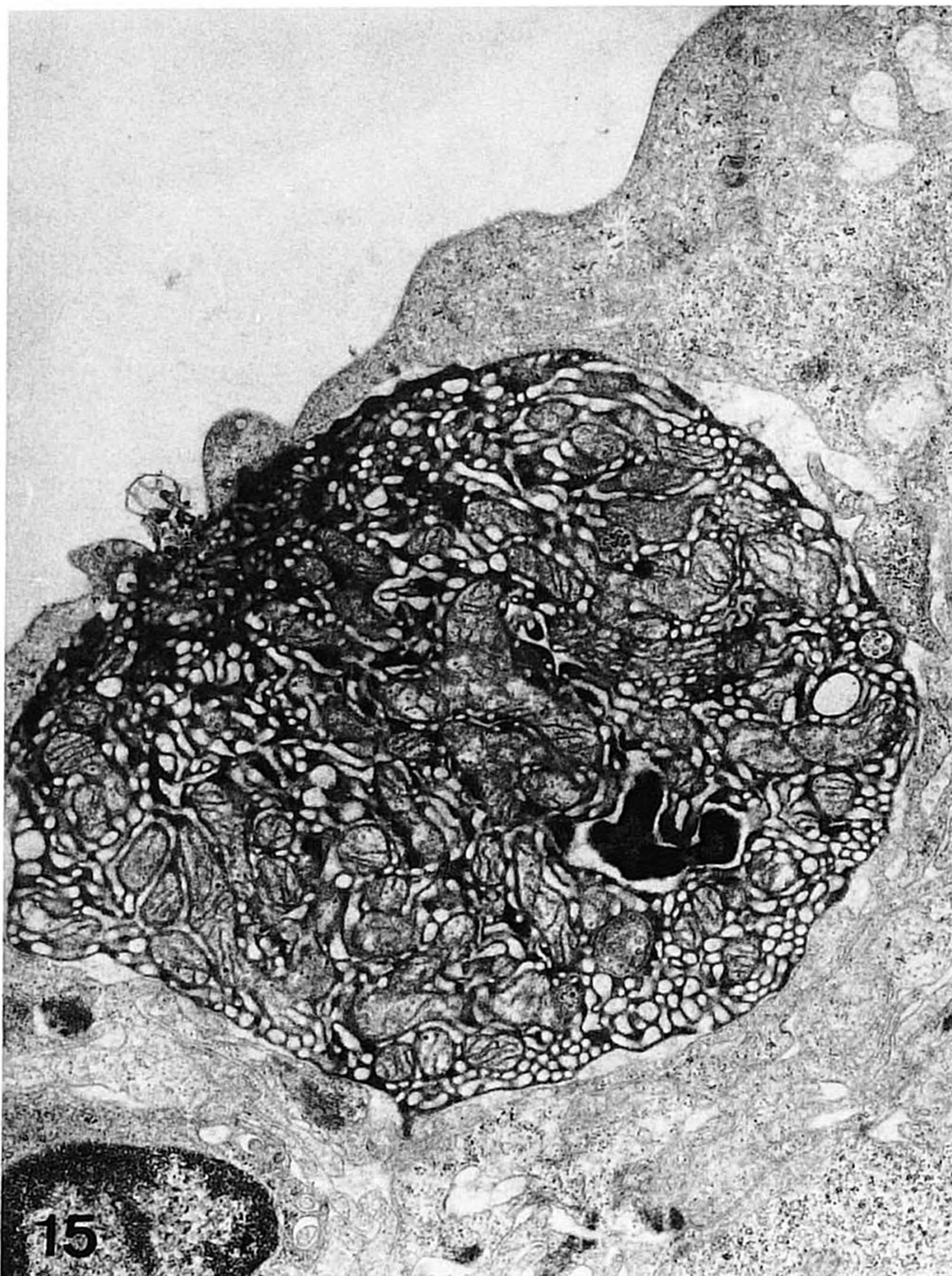
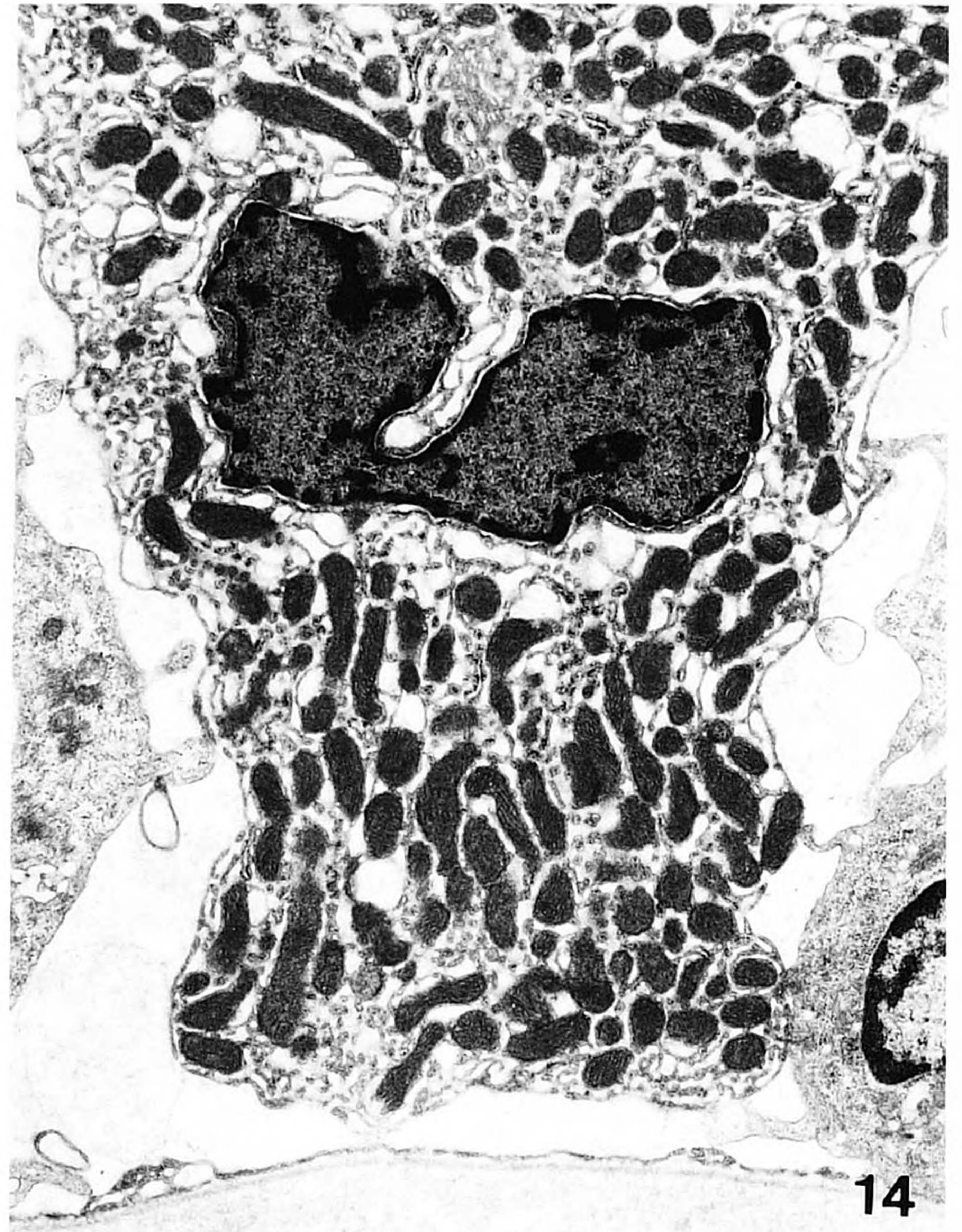
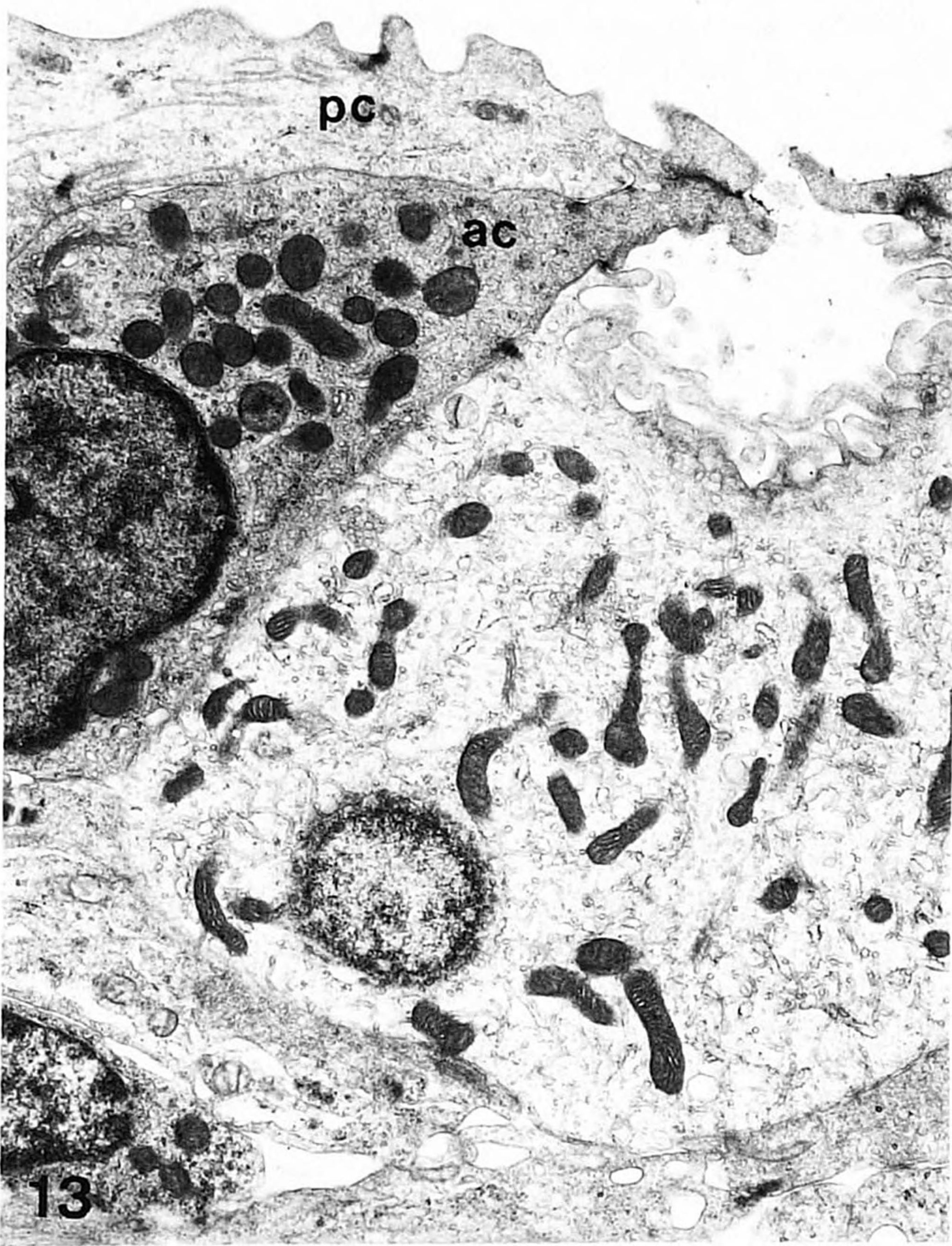
**Fig. 9.** Interlamellar epithelium showing a macrophage (*m*) surrounded by pavement cells; the macrophage contains a large lysosome-like body containing remnants probably representing apoptotic bodies of chloride cells.  $\times 11\,000$

**Fig. 10.** Interlamellar epithelium of seawater fish; part of a macrophage showing lysosome-like bodies containing remnants of an apoptotic chloride cell (*double arrow*) and a seemingly intact tubular system (*single arrow*).  $\times 23\,000$

**Fig. 11.** Trailing edge of seawater fish; pavement cell showing necrotic degeneration.  $\times 9\,200$

**Fig. 12.** Necrotic chloride cell in the interlamellar epithelium of a seawater fish.  $\times 9\,600$







sis, in freshwater- and seawater-adapted fishes. This indicates that cell death in the branchial epithelium is mostly physiologically controlled, and only indirectly influenced by external factors (Wyllie et al. 1980; Bowen 1981). Accidental cell death, structurally reflected by necrosis, is mainly observed immediately after abrupt freshwater to seawater transfer, but rarely in fully adapted freshwater or seawater *O. mossambicus*.

Cell degeneration has often been observed in branchial epithelia although the descriptions are often incomplete. With respect to fish gills, necrosis has been reported in several studies on the effects of water pollutants (Gaino et al. 1984; Mallatt 1985). To the best of our knowledge, apoptosis in gill tissue has not been mentioned before, although it has been described for hatching gland cells in pike embryos (Schoots et al. 1983) and for endocrine cells of the Stannius bodies in some teleosts (Wendelaar Bonga and Pang 1986).

#### *Pavement cells*

The structure of the pavement cells covering the branchial filaments is similar to that of the homologous cell type forming the epithelium covering the skin outside the gill area, the filament containing cells (Whitaker 1977; Wendelaar Bonga and Meis 1981). We have shown for the skin of *O. mossambicus* that these cells arise in the basal cell layers and subsequently develop a dense network of microfilaments and desmosomes that interconnect these cells. When they arrive at the epithelial surface, they form micro-ridges and tight junctions. They finally disappear by sloughing (Wendelaar Bonga and Meis 1981). In the gills, sloughing occurs on filaments as well as on the lamellae. The present observations show that sloughing is preceded by a process of cellular shrinkage and condensation that is related to apoptosis. The final stages of apoptotic cell death, namely formation of one or more rounded apoptotic bodies and phagocytosis of these bodies by adjacent cells (Wyllie et al. 1980), are absent in the pavement cells: they are apparently shed into the water. This type of degeneration is not limited to the cells of the branchial epithelium. It also occurs in the pavement cells of the skin epithelium (our unpublished observations; Fig. 9 in Wendelaar Bonga and Meis 1981). No clear difference in the frequency of these

phenomena is observed between freshwater- and seawater-adapted fish. This indicates that the rate of renewal of the pavement cells is similar in freshwater and seawater, a conclusion in agreement with data obtained in eels and guppies following [<sup>3</sup>H]thymidine labelling of the pavement cells (Tondeur and Sargent 1979; Chrétien and Pisam 1986).

#### *Chloride cells*

*Accessory chloride cells.* Accessory cells are found in freshwater and seawater *O. mossambicus*. They are considered to be developing stages of chloride cells (Hootman and Philpott 1980; Pisam 1981), or a specific cell type (Laurent and Dunel 1980; Chrétien and Pisam 1986). Hootman and Philpott (1980) have not ruled out the possibility that the accessory cells might represent degenerate stages of chloride cells, because some cells contained large lysosome-like bodies. We favour the interpretation that they are young stages of chloride cells, and not degenerating chloride cells. Accessory cells usually have a granular endoplasmic reticulum that is more developed than that of mature chloride cells whereas the tubular system is less developed; in addition, their ultrastructure is clearly different from either apoptotic or necrotic chloride cells.

*Mature chloride cells.* The mature chloride cells of *O. mossambicus* are structurally similar to those described in many other species of fish. The differences observed between freshwater and seawater fish are, however, less pronounced than reported by others (Doyle and Epstein 1972; Sardet et al. 1979; Laurent 1984). Clusters of chloride cells are common in both freshwater and seawater *O. mossambicus*. Chloride cell clusters have been reported as typical of fish in seawater, whereas single cells predominate in freshwater fish (Laurent 1984) although chloride cell groups have been reported before, e.g. in freshwater eels (Laurent and Dunel 1980). Apical cavities, for long considered a seawater characteristic but recently also reported for freshwater fish (Fishelson 1980; Leino et al. 1987), are found in freshwater and seawater *O. mossambicus*, although their size and frequency increases after seawater transfer. Leino et al. (1987) have shown that apical cavities become more prominent under conditions of osmoregulatory stress induced by acid water, and conclude that the cavities reflect increased chloride cell activity. Other structural features described as typical of fish transferred to seawater are an increase in the number of mitochondria, a more densely anastomosing tubular system, the presence of more apical vesicles, and apical associations of chloride cells with accessory cells (Shirai and Utida 1970; Doyle and Epstein 1972; Sardet et al. 1979; Philpott 1980; Pisam 1981; Laurent 1984). With the exception of the apical associations, these phenomena are also observed in our study.

*Degenerative chloride cells.* Many chloride cells show condensation of the cellular membranes and compartments that can be interpreted as various stages of apoptosis. Whereas cells and nuclei show shrinkage, the lumen of the tubular cells increases in diameter. This is understandable as this lumen is in open connection, via pores in the basolateral cell membranes, with the intercellular space. The progressive condensation of the cells leads to the formation of globules with the characteristic apoptotic bodies. These are apparently engulfed by macrophage-like cells and digested

**Figs. 13–16.** Interlamellar epithelium of fully adapted seawater fish (Fig. 13) and of fish 3 days after transfer to seawater (Figs. 14–16)

**Fig. 13.** Mature chloride cell, with an apical cavity and villous cytoplasmic projections; *ac* accessory cell; *pc* pavement cell.  $\times 10000$

**Fig. 14.** Chloride cell showing cellular shrinkage, distension of the tubular system, and condensation and indentation of the nucleus, typical for apoptosis.  $\times 10000$

**Fig. 15.** Progressive stage of apoptosis of a chloride cell, characterised by distension of the tubular system, condensation of the cytoplasm, and condensation and indentation of the nucleus.  $\times 12000$

**Fig. 16.** Accessory chloride cell, showing extensive endoplasmic reticulum and poor development of the tubular system (*right*), and a macrophage (*m*) with a large lysosome-like body, possibly an apoptotic body of a chloride cell.  $\times 17000$



in their lysosomal system. This kind of cell deletion has been reported for many tissues of the higher vertebrates (Wyllie 1980; Oates et al. 1986) and, in fish, for the hatching gland cells of pike larvae (Schoots et al. 1983). Phagocytosis of the bodies by adjacent epithelial cells, as often occurs in epithelia, is not observed in *O. mossambicus*. Macrophage-like cells or structurally similar cells described as leucocytes or wandering cells have been observed in the intercellular or lymphoid spaces of several other species (Hughes and Wright 1970; Morgan and Tovell 1973; Laurent 1984). Our study shows that one function of these cells is the removal of aged chloride cells.

Degenerative chloride cells have been described in the gills of other species. However, this description usually concerns cells showing the fragmentation and swelling typical of cell death by necrosis (Morgan and Tovell 1973; Laurent et al. 1985), for example in gills exposed to water pollutants (Mallatt 1985). In *O. mossambicus*, it is common in the first days after transfer to seawater, but disappears later on, indicating that it is caused by the rapid change in water composition rather than by seawater itself.

The occurrence of apoptotic chloride cells increases after transfer of the fish to seawater. Together with the higher frequency of differentiating chloride cells, this is further evidence for the increased turnover of chloride cells in seawater when compared with those from freshwater fish, as demonstrated for other species (Tondeur and Sargent 1979; Chrétien and Pisam 1986).

Although to our knowledge apoptosis in the branchial epithelium has not been mentioned in the literature, the degeneration of chloride cells in lampreys (Morris and Pickering 1976; Pickering and Morris 1976; Peek and Youson 1979), shows various characteristics of apoptotic cell death. We have found apoptotic cell death in chloride cells of trout, carp and eel (unpublished observations). Using the concept of apoptosis, much of the structural variation in the branchial epithelium in *O. mossambicus* can be explained, in particular that seen during stimulated cell turnover induced by transfer to seawater.

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