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Cellular responses of the skin of carp *(Cyprinus carpio)* exposed to acidified water

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Abstract. The skin of carp was examined after exposure to acidified water. Degenerative cells were common in the upper epidermal layers.During the first days most of these cells exhibited signs of necrosis. Later on the incidence of necrosis decreased and that of apoptosis increased. In the acid-exposed fish, the upper filament cells and pavement cells produced secretory vesicles of high electron density, some of which showed peroxidase activity. This enzyme activity was also present in the glycocalyx covering these cells, and in the cytoplasm of apoptotic cells. Mitotic figures and newly differentiating mucous cells were common in the outer epidermal layers. Mucous cells became elongated and produced mucosomes of high electron density. Mucosomes with peroxidase activity were also found. Club cells increased in number. Chloride cells and solitary chemo-sensory cells, not seen in the controls, appeared in the upper epithelial layer. The skin was invaded by many leucocytes and by pigment-containing cytoplasmic extensions of melanocytes. Some leucocytes apparently penetrated into the club cells. These structural observations reflect the complexity of the physiological response of the skin to acid water.

Key words: Skin – Ultrastructure – Endogenous peroxidase – Water acidification – *Cyprinus carpio* (Teleostei)

Introduction

Anthropogenic acidification of fresh water has a serious impact on fish as well as other aquatic organisms. It causes disturbance of water and ion balance, reduced growth and reproduction, and may eventually lead to disappearance of fish populations (Fromm 1980; McDonald 1983; Wendelaar Bonga and Dederen 1986). The disturbance of the water and ion balance is mainly caused by damage inflicted upon the gills, and, as a consequence, many authors have examined the histopathological effects of acid water on the branchial epithelium (Wendelaar Bonga et al. 1990; Freda et al. 1991).

Contrastingly, the skin surface outside the branchial area has received little attention, although it is a metabolically active tissue that is important as a protective barrier between the water and the organism. It rapidly responds to external challenges such as pollutants or changes in the ionic content of the water (Whitear 1986).

The epidermis of teleost fish consists of several cell types: filament cells, mucous cells, club cells and sensory cells. In many species also chloride cells may be present (Whitear 1986). The epidermis is covered by a mucus layer attached to the tissue directly or via the glycocalyx (Iger et al. 1988). This layer forms an additional barrier to potentially harmful substances in the water. It contains proteases and phosphatases (Brown et al. 1990; Iger and Abraham 1990) and has also ion-concentrating capacities (Fromm 1980).

In this paper ultrastructural and cytochemical changes of both pathological and adaptive character are described in the epidermis and the underlying tissue of carp exposed for different periods to water of low pH. The results may contribute to our understanding of the cellular responses of the skin to stressors.

Materials and methods

Fifty-seven juvenile male and female carp, *Cyprinus carpio*, weighing 3–4 g, were kept in 3 groups for an acclimation period of 20 days in artificial tap water (demineralized water to which were added in mmol.l^{-1}: 3.8 NaCl; 0.8 $CaCl_2$; 0.2 $MgSO_4$; 0.335 $NaHCO_3$ and 0.06 KCl) at pH 7.5 and a temperature of 22° C. To reduce experimental disturbance, each group was subdivided and the fish were kept in different aquaria, with 3 fish per aquarium. One group of carp remained at this pH for the whole period. For the other 2 groups, the pH of the water was lowered gradually over a period of 2.5 h to pH 5 or pH 6, respectively, by adding continuously dilute H_2SO_4, under continuous aeration. During the experiment, the water pH was adjusted automatically with pH-stat equipment (Consort, Belgium). The water was continually changed (about 30% per day), well aerated, and filtered.

Skin biopsies (3 × 3 mm) of 3 fish from each group were taken 3 h, 24 h, and 3, 7, 14 and 23 days after reaching the final water pH.

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Each fish was sampled once and samples from the same fish were used for LM, TEM and SEM observation. The samples were taken from the dorsal part of the head of fish that were lightly anesthetized (Hypnocalmer, Jungle, Tex.), without killing them. The tissues were fixed in 3% glutaraldehyde in sodium cacodylate buffer (0.09 M, pH 7.3), washed in buffer and post-fixed in osmium tetroxide (1%) in the same buffer. Ethanol dehydrated tissues were embedded in Spurr's resin. Thick sections (1.5 μ m) stained with toluidine blue were used for LM study. Thin sections, collected on 150 mesh copper grids, were contrasted with uranyl acetate and lead citrate and were examined in a Jeol 100 CX transmission electron microscope. For SEM, ethanol dehydrated tissues were transferred into increasing concentrations of liquid CO_2, coated with gold in a Balzers coating unit (CPD 020, Balzers, Switzerland), and examined in a Jeol-JSM T 300 scanning electron microscope. The detection of endogenous peroxidase activity was carried out with the conventional diaminobenzidine technique followed by fixation with glutaraldehyde (Iger and Abraham 1990). Control specimens were incubated in the absence of H_2O_2. Six fish (2 from each group) were subcutaneously injected with horseradish peroxidase (HRP; Sigma, USA; 2.5 mg per 10 g body weight, dissolved in carp saline) for the detection of pinocytotic activity and the extent of the intercellular spaces. Fish were sampled 1 h after injection. HRP was visualised with the diaminobenzidine technique. The presence of mitotic cells was demonstrated in 3 fish from each group. These fish were subcutaneously injected with colchicine (Merck, Germany; 2 mg/100 g body weight, dissolved in carp saline). Fish were injected 20–22 h before dissection (total of 6 and 3 fish from the different groups, for days 14 and 23, respectively).

Morphometrical parameters (number of cells per 1 mm of section length and epidermal thickness) were obtained from sections of 1.5 μ m thickness and about 3 mm length. Data represent the mean ± S.D. of 6 sections per fish (3 fish per group), with 40–50 μm intervals between the sections. Control data were similar during the whole period and therefore were pooled ($n = 17$). The differences between experimental and control groups were tested for statistical significance with the Student t-test. For some morphometrical parameters, data represent the range of the values measured.

Results

Controls

Epidermis

The epidermis was 70–80 μm thick (Table 1) and composed mainly of several layers of filament-containing epithelial cells (filament cells). The upper layer of cells was flattened (pavement cells). In addition mucous cells and club cells were present.

Filament cells. The filament cells contained a web of microfilaments of about 8 nm in diameter, and were interconnected by desmosomes and cytoplasmic interdigitations. The basal layer of filament cells was attached to the underlying basal lamina and adjacent connective tissue by hemidesmosomes. The latter cells displayed intense endocytotic activity (Fig. 1) at the basal part of the outer cell membrane, as was reflected by the presence of several HRP-positive endosomes in fish injected with this marker (Fig. 2). HRP also penetrated freely between the filament cells (Fig. 3) and was found in the intercellular spaces throughout the epidermis. The permeation of HRP in the epidermis ended at the tight junctions that connect the pavement cells. The latter cells (Fig. 4) were characterised by folding of the apical cell membrane into microridges of about 0.4 μm high. The ridges contained a core of filaments that ran parallel to their long axis and that were integrated in the filaments of the terminal web. The pavement cells contained few electron-lucent vesicles. Occasionally the latter were seen to fuse with the apical cell membrane and to release their contents by exocytosis. We could not find any endogenous peroxidase activity in the pavement cells or at the epidermal surface (Fig. 5a). At the skin surface (Fig. 6) depressed areas, representing sites of sloughing of apoptotic and necrotic pavement cells, were only exceptionally found.

Mitotic cells were not observed in colchicine-untreated fish. Examination of colchicine-injected fish revealed 8–11 mitotic cells per 1 mm of epidermal cross-section. Mitosis occurred from the second layer from the basis to the middle layers of the epidermis.

Mucous cells. There were about 110 oval mucous cells per mm section length of the epidermis (Table 2). Differentiating mucous cells were connected by desmosomes to adjacent filament cells, and contained an extensive rER and well developed Golgi system. Small vesicles originat-

Table 1. Epidermal thickness of control fish and fish exposed for different periods to acidified water

Pooled controls ($n = 17$) 78.9 ± 6.16				
Time	pH 5 ($n = 3$)	P val.	pH 6 ($n = 3$)	P val.
3 h	73.5 ± 8.30	ns	75.2 ± 7.98	ns
24 h	64.8 ± 5.24	0.002	76.7 ± 8.26	ns
3 d	57.2 ± 8.66	0.0005	65.3 ± 6.88	0.003
7 d	82.4 ± 11.32	ns	74.9 ± 9.64	ns
14 d	94.6 ± 12.45	0.002	80.2 ± 11.50	ns
23 d	112.9 ± 14.76	0.0005	84.9 ± 12.74	ns

Fig. 1. Basal filament cell of a control fish. Endosomes (*arrows*) are seen along the basal membrane. *ct* Connective tissue. × 52 000

Fig. 2. Basal filament cell of a control fish. Endosomes (*arrows*) contain the HRP-marker that is present in the connective tissue. HRP-DAB reaction, otherwise unstained. × 52 000

Fig. 3. Basal filament cell of a control fish. Intercellular spaces are filled with HRP. HRP-DAB reaction. × 8100

Fig. 4. Pavement cells of a control fish. *G* Golgi system; *r* microridges; *s* secretory vesicles. × 27 500

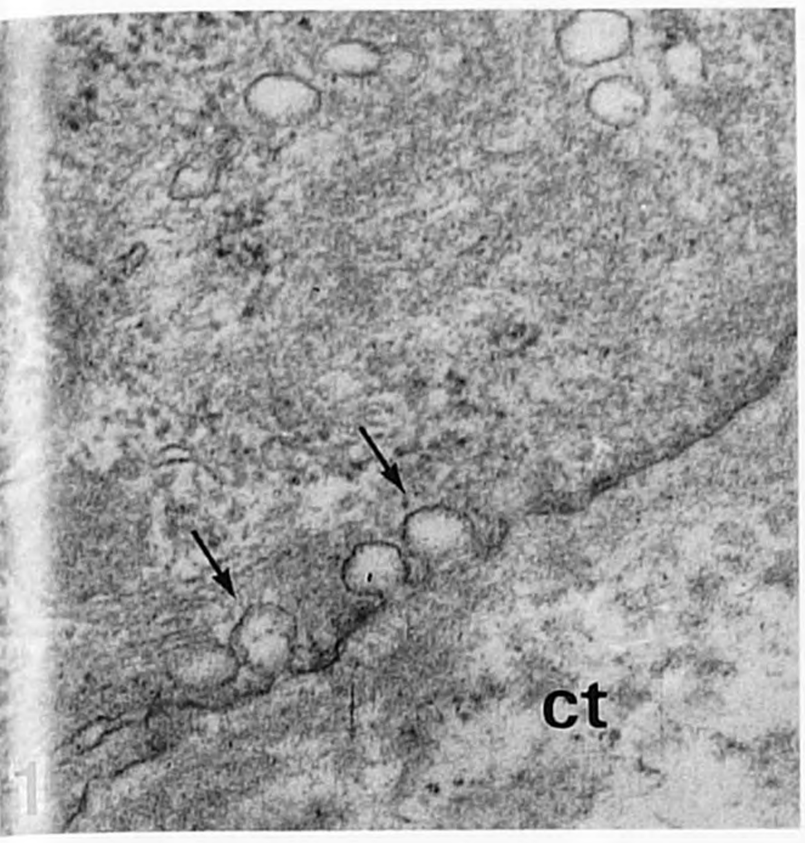
Fig. 5. Pavement cells with microridges, treated with DAB to reveal endogenous peroxidase activity, otherwise unstained. **a**. Control fish showing negative reaction × 18 000 **b**. Acid-exposed fish (pH 5, 7 days); the glycocalyx is strongly positive. × 24 800

Fig. 6. S.E.M. of pavement cells at the skin surface in control fish, showing microridges. × 6600

Fig. 7. S.E.M. showing normal pavement cells and dark areas (*arrows*) representing depressed sites where apoptotic and necrotic cells where rejected. pH 5, 7 days. × 4950

Fig. 8. Necrotic pavement cell; pH 5, 3 h. × 18 000

Fig. 9. Apoptotic pavement cell (*arrow*). pH 5, 7 days. × 31 800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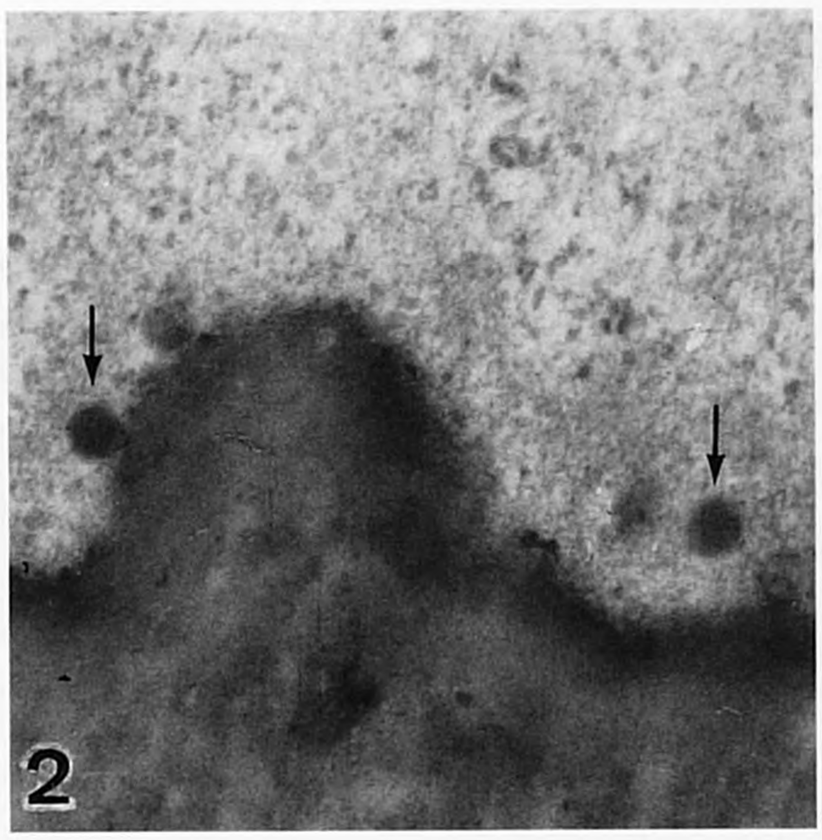
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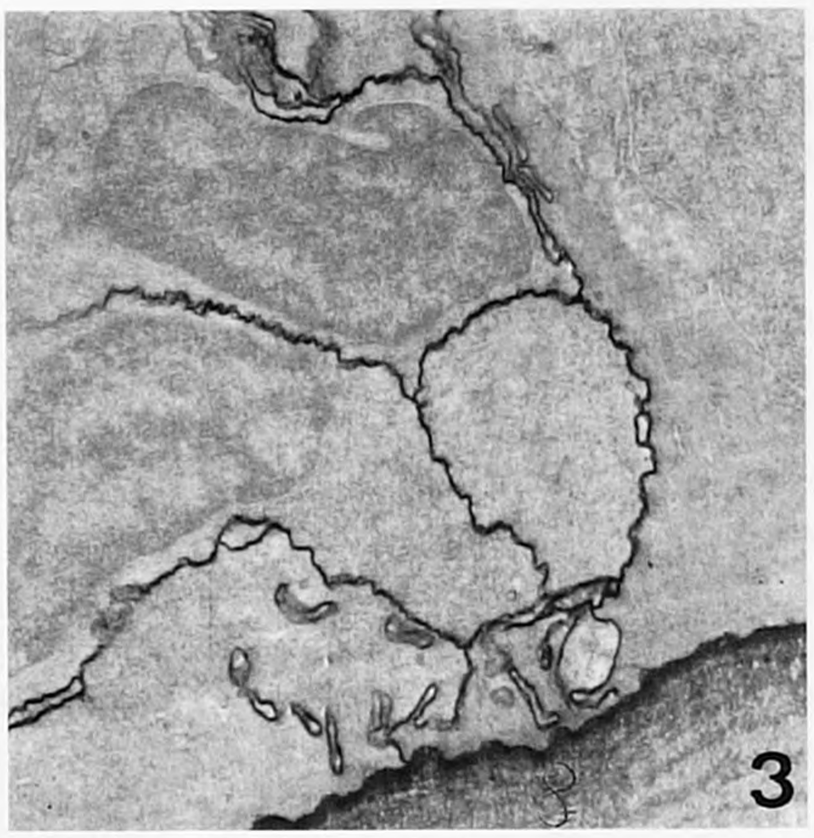
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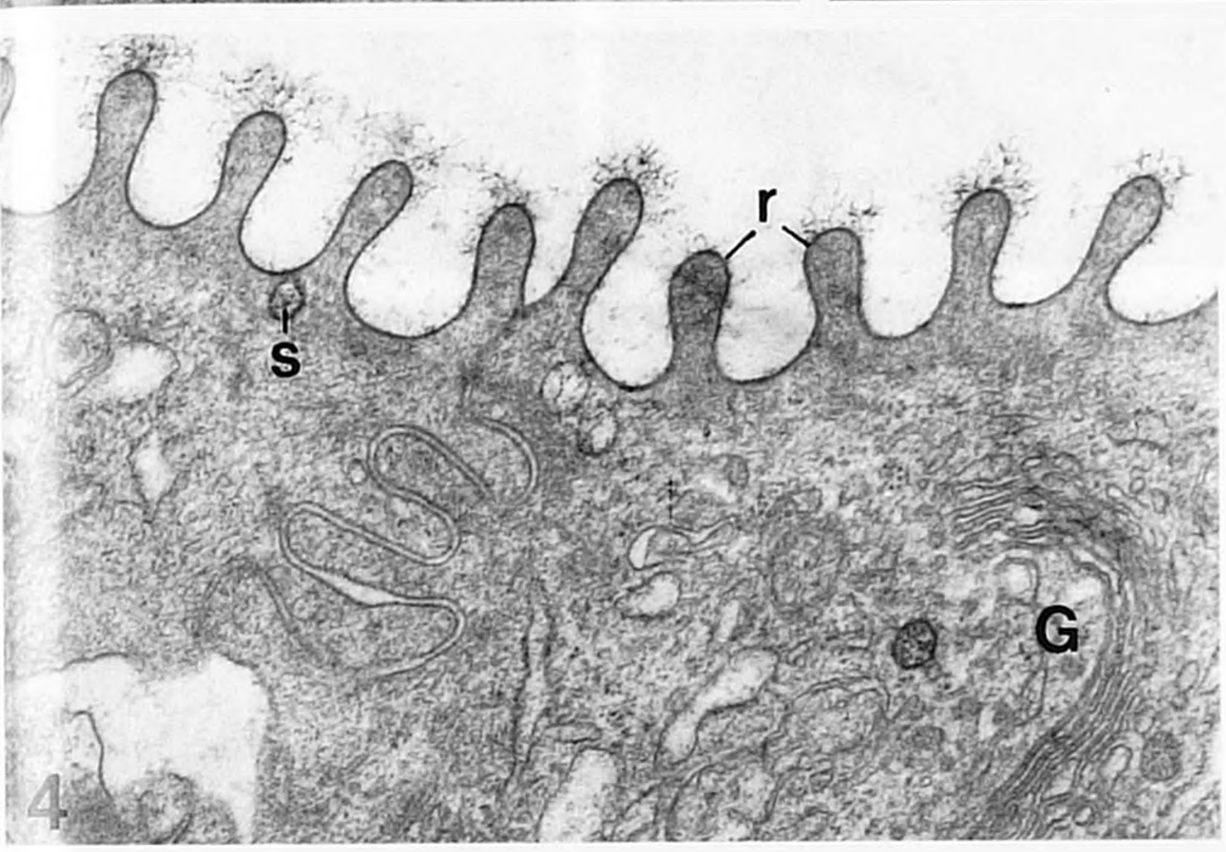
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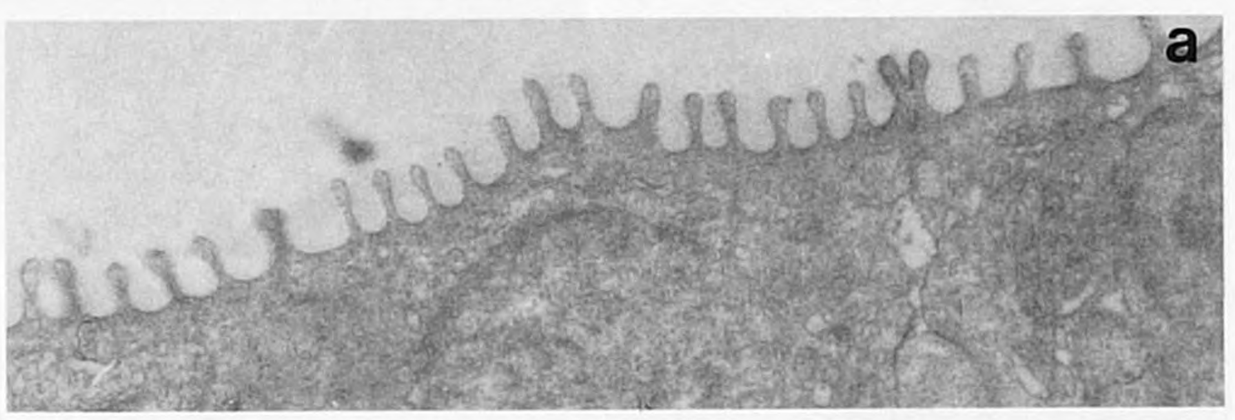
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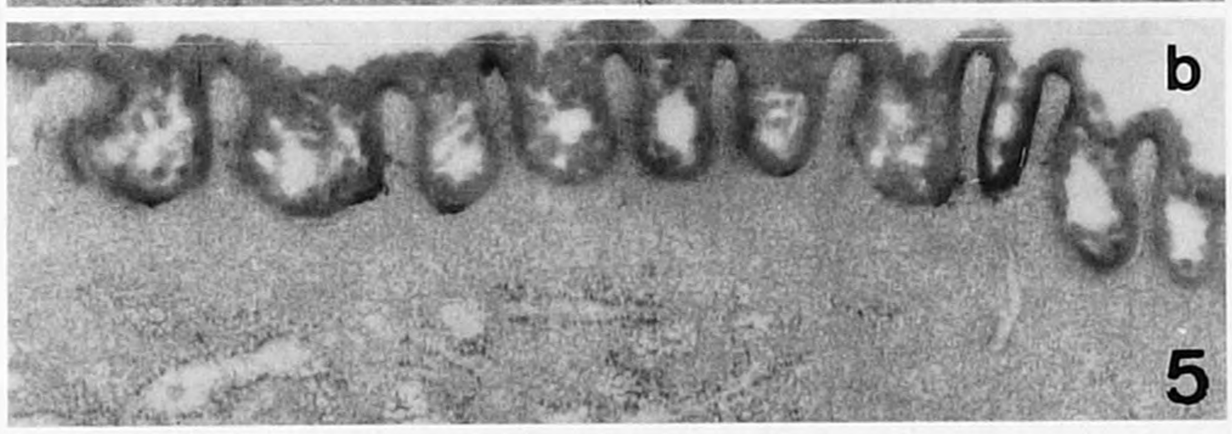
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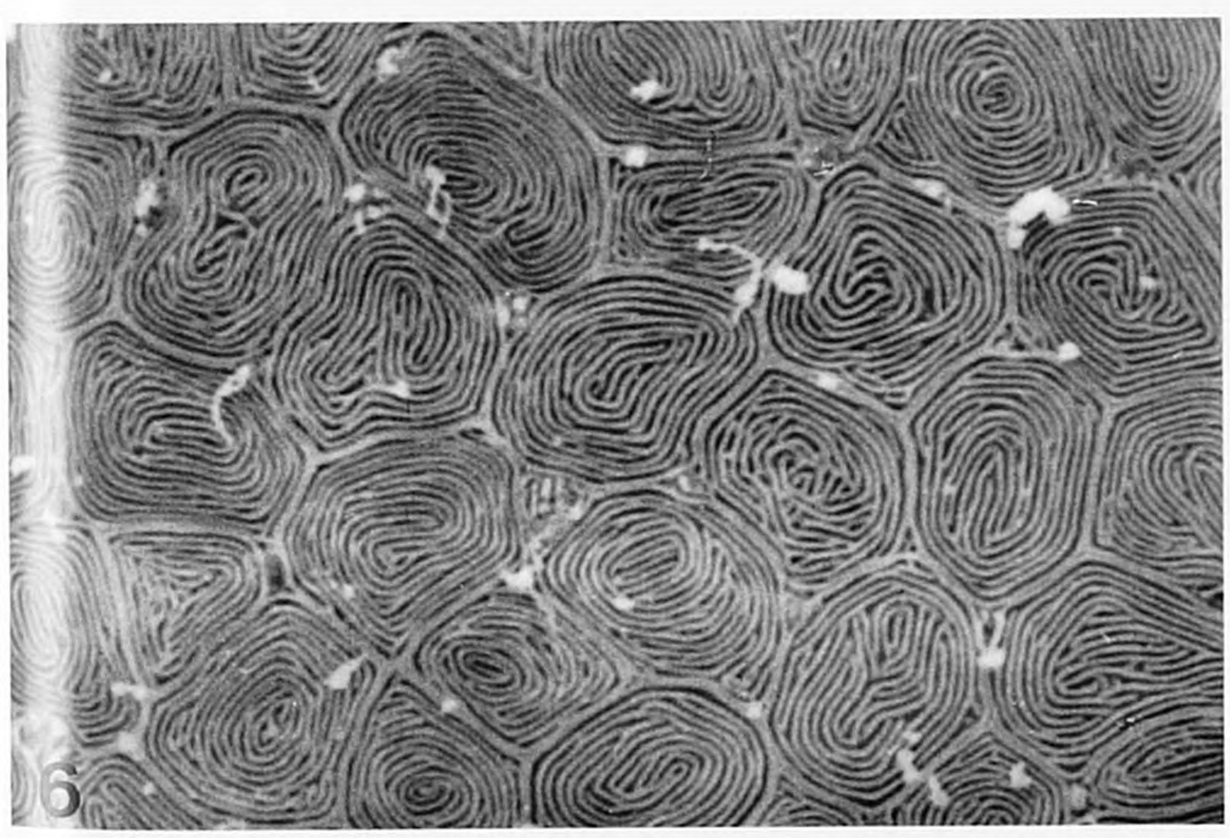
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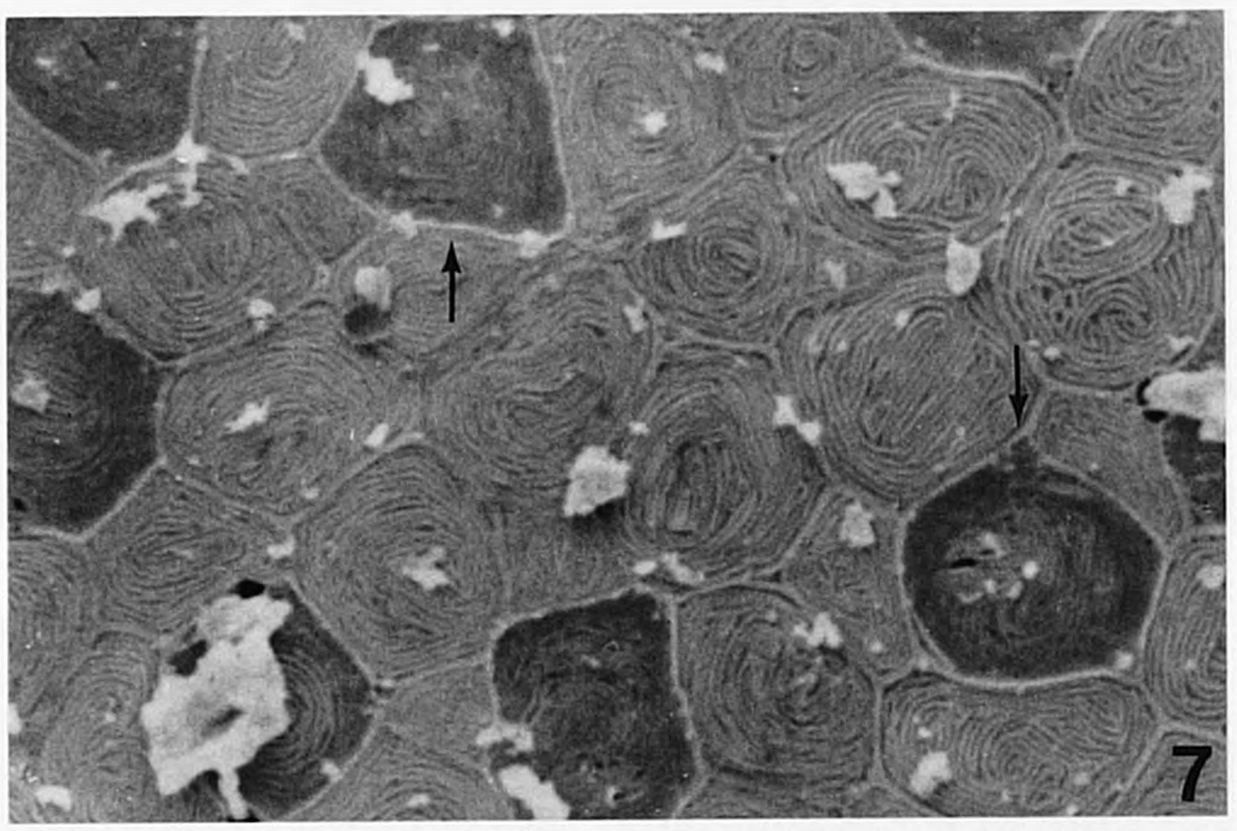
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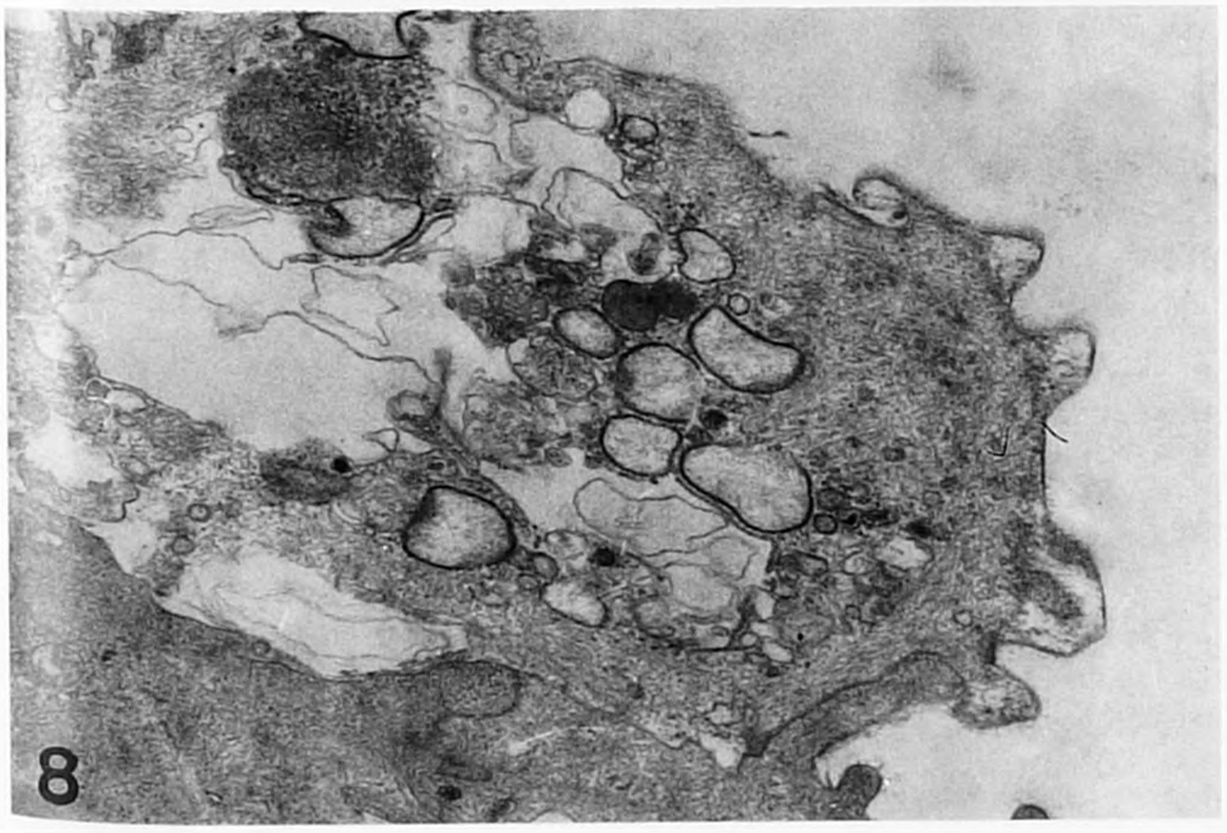
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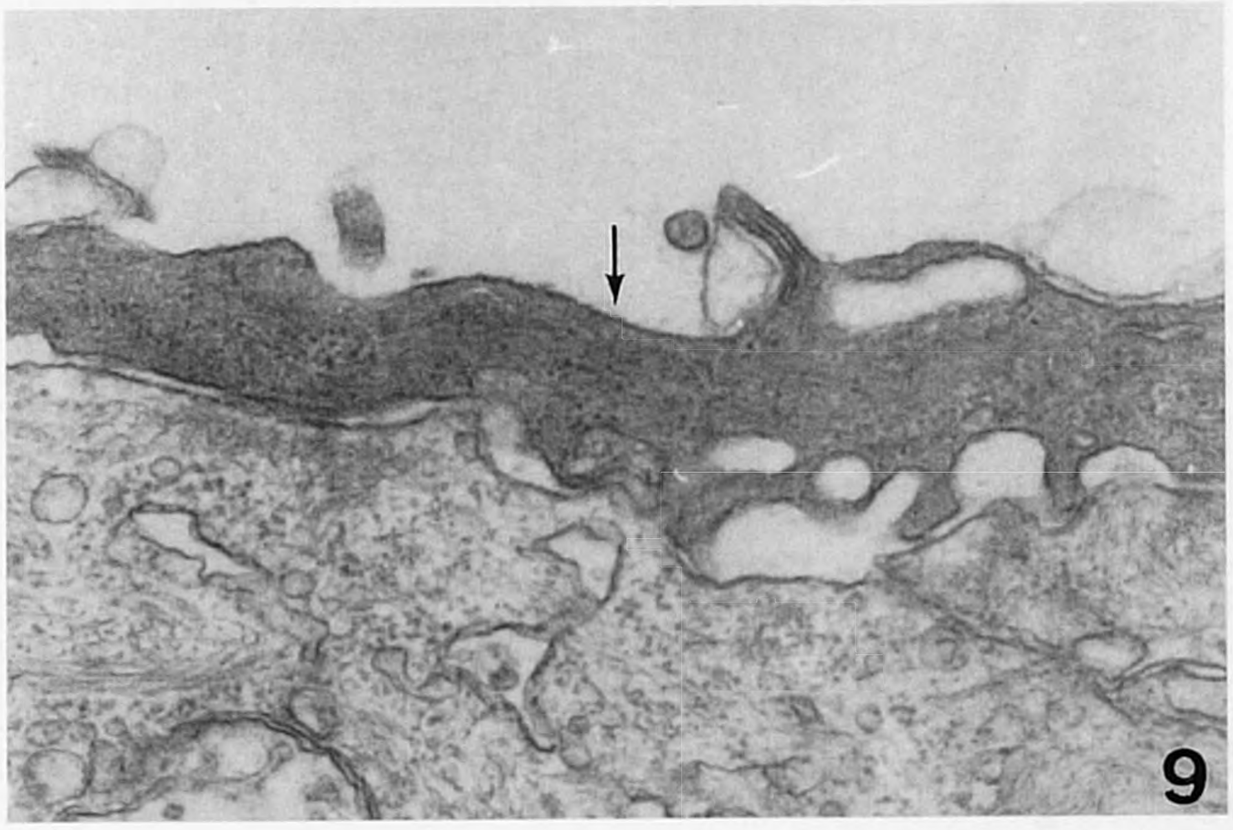
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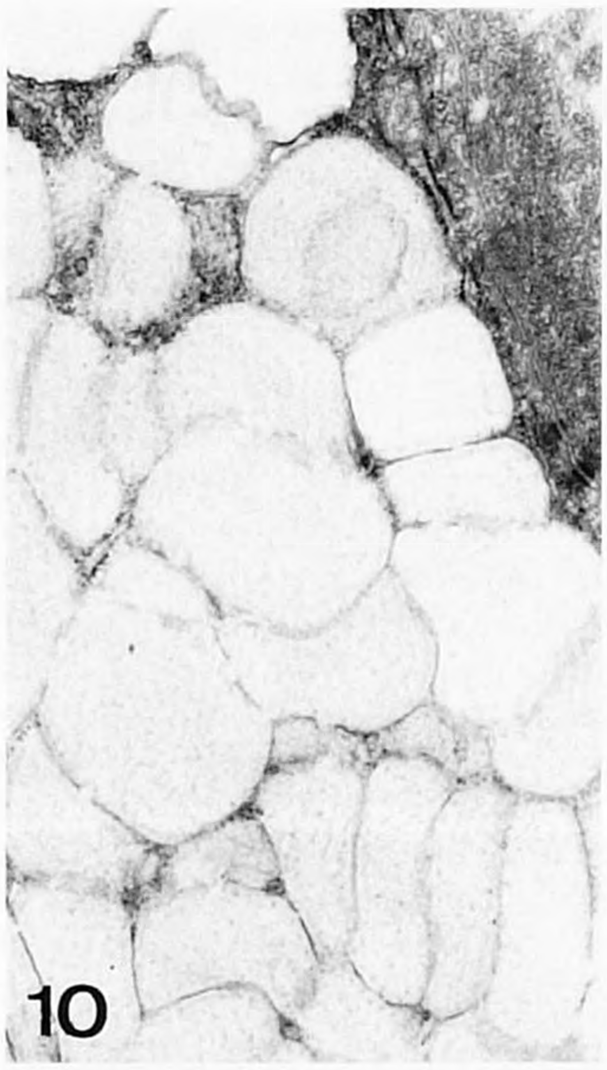

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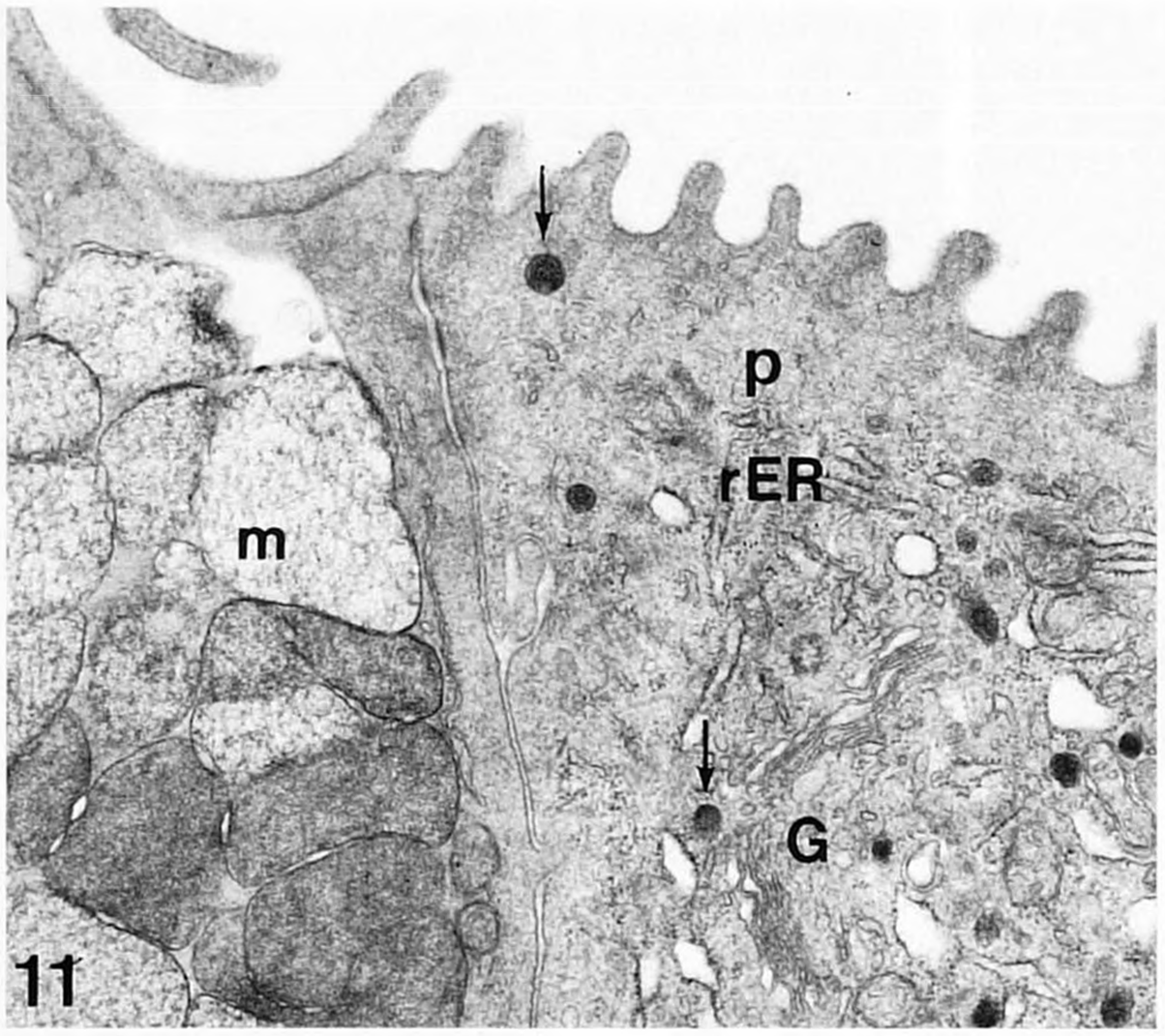

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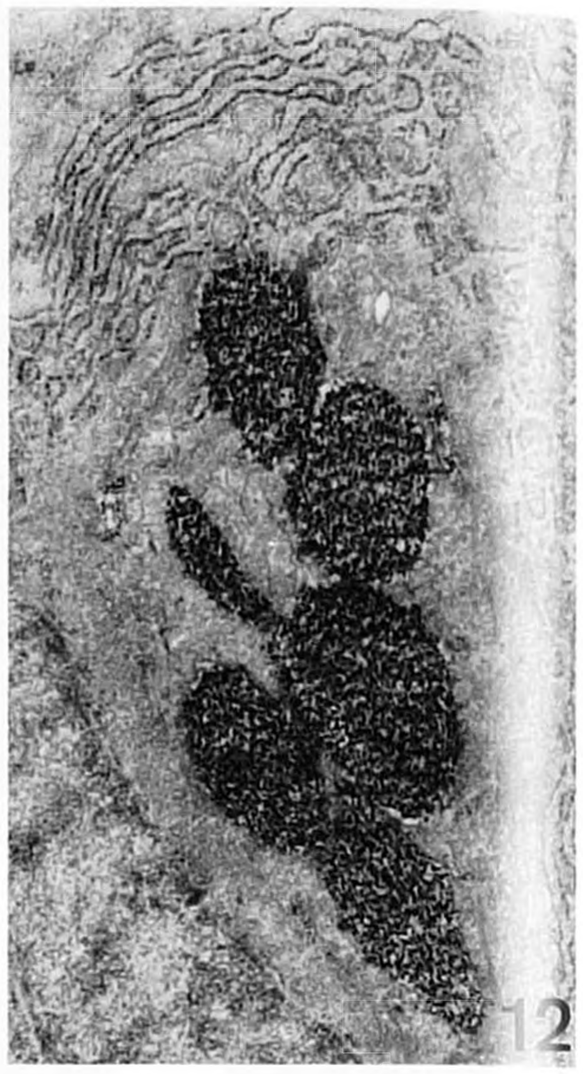

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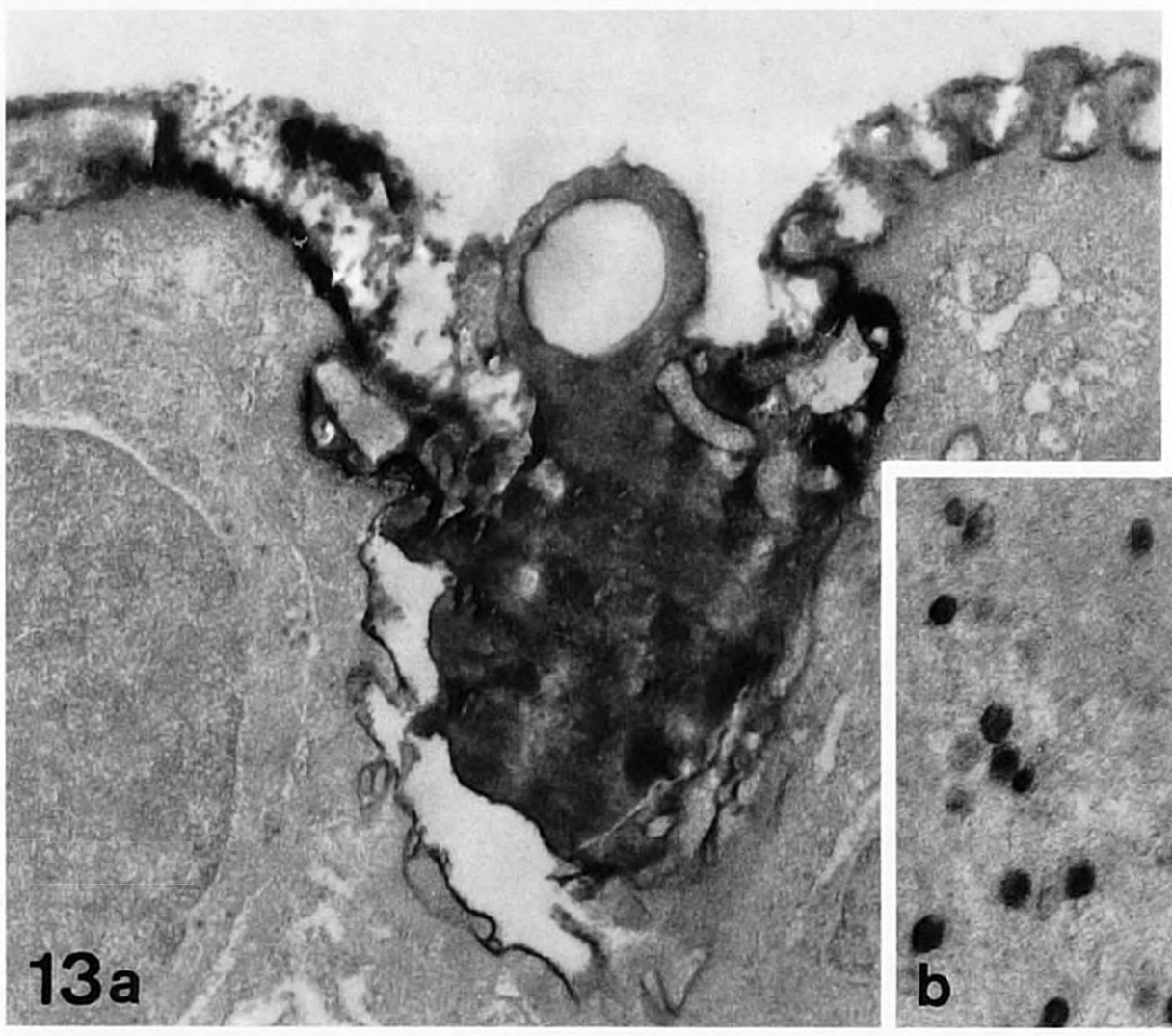

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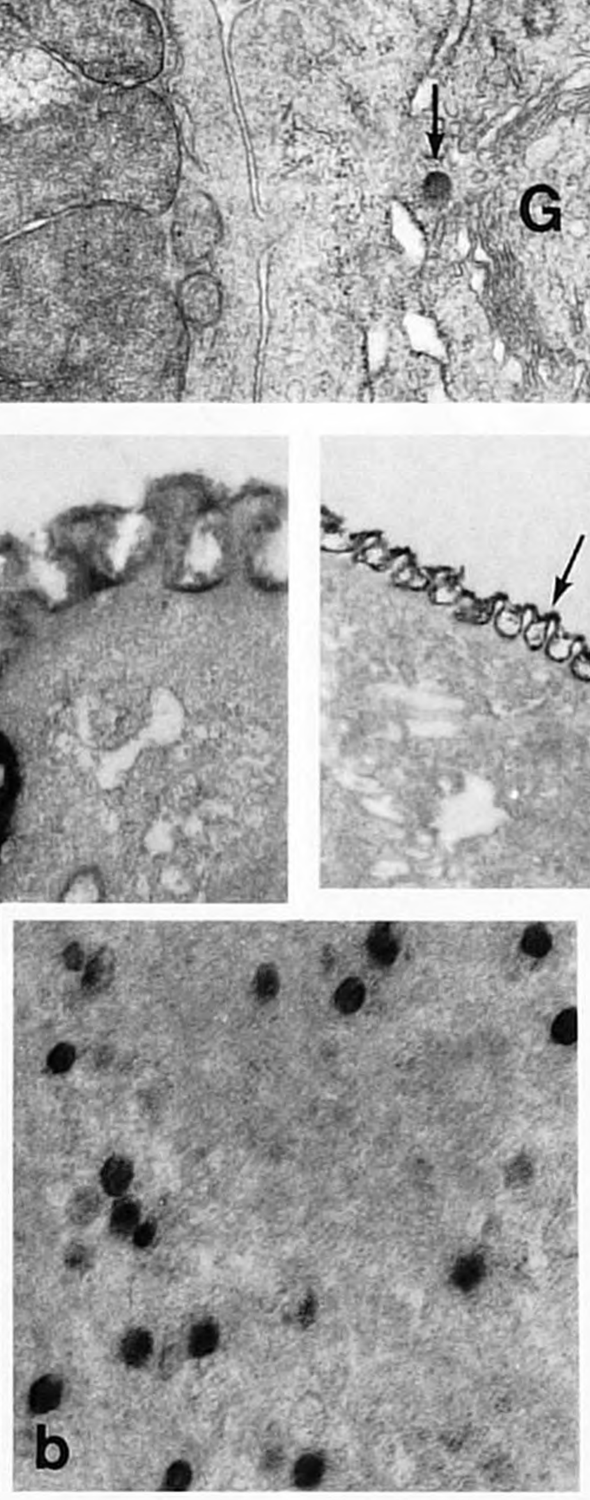

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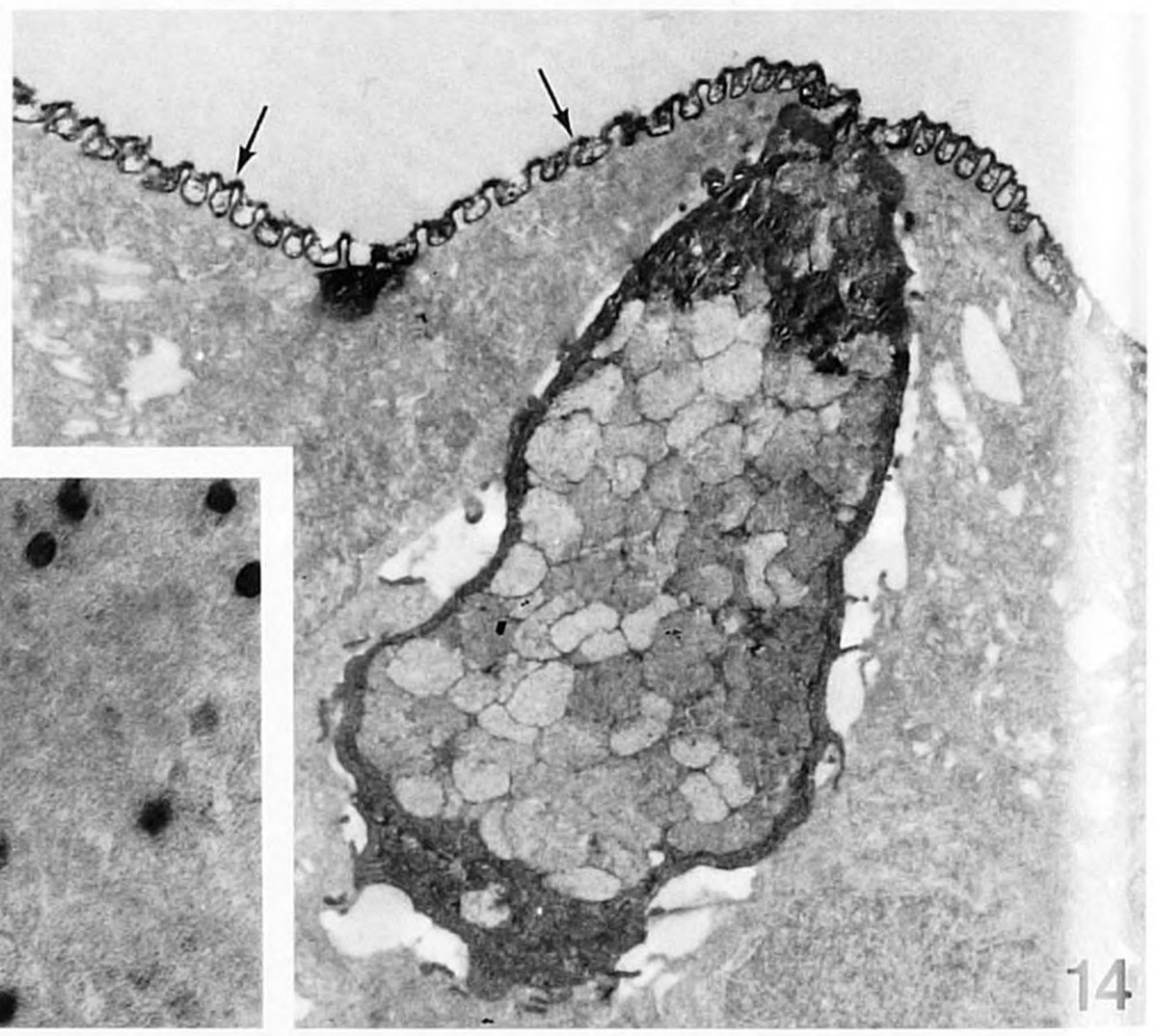

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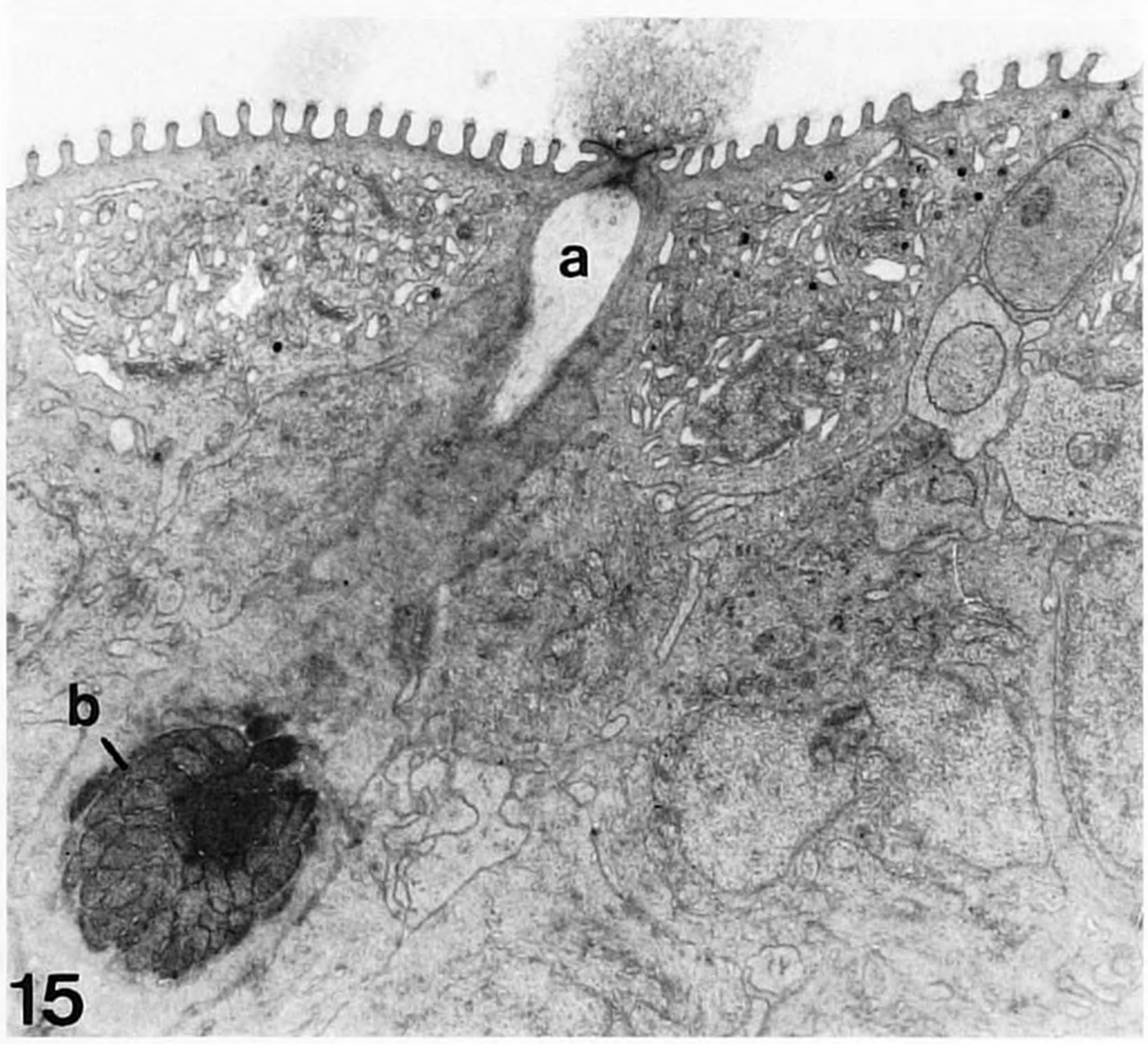

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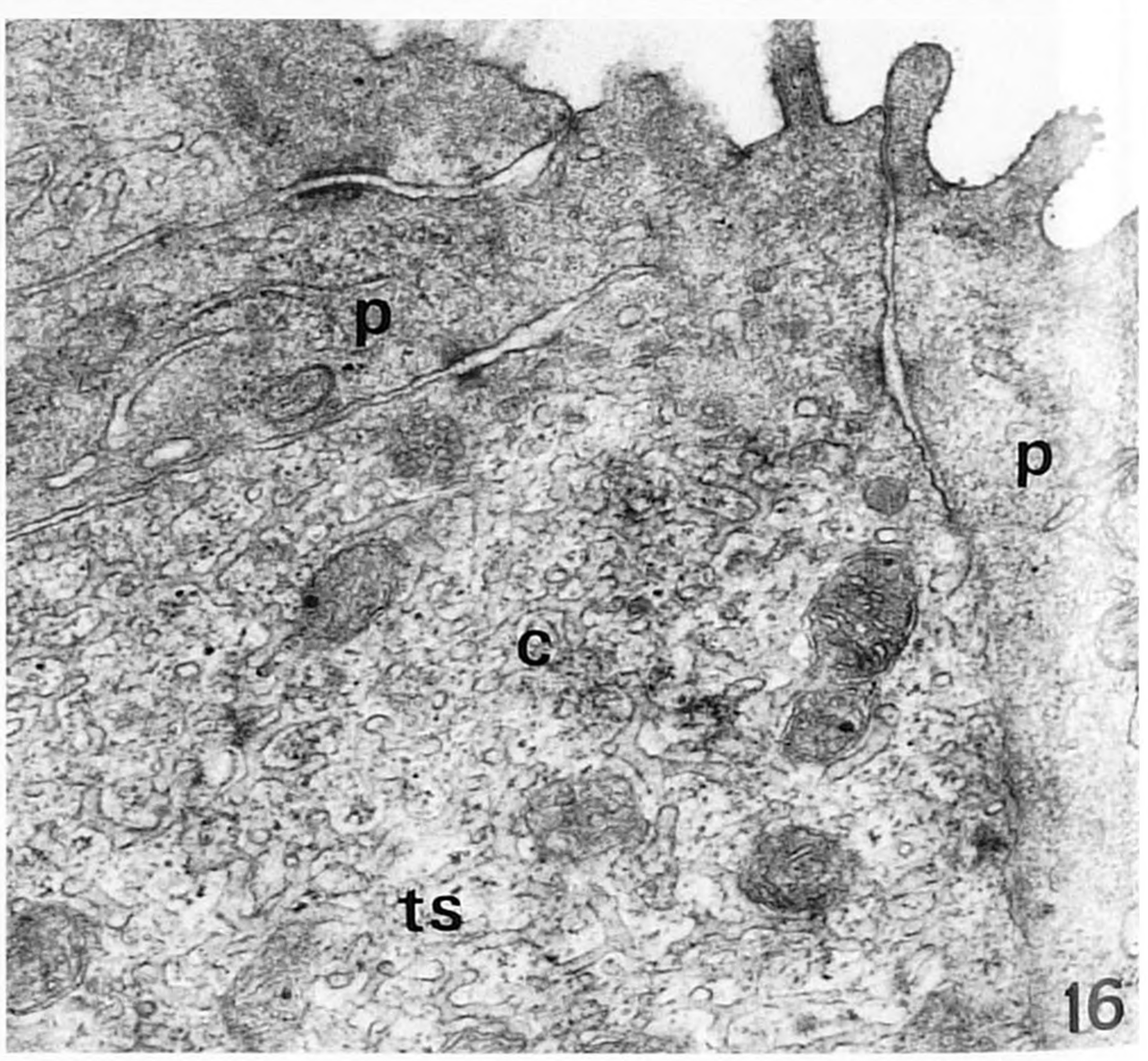

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Table 2. Number of mucous cells per 1 mm of epidermal section length in controls and fish exposed for different time periods to acidified water

Pooled controls ($n=17$) 112.4 ± 13.88				
Time	pH 5 ($n=3$)	P val.	pH 6 ($n=3$)	P val.
3 h	106.8 ± 10.54	ns	101.9 ± 11.76	ns
24 h	41.0 ± 5.93	0.0005	89.7 ± 8.34	0.014
3 d	26.8 ± 6.76	0.0005	68.4 ± 9.25	0.0005
7 d	93.7 ± 16.46	0.05	74.3 ± 11.36	0.0005
14 d	104.6 ± 12.43	ns	84.9 ± 9.70	0.004
23 d	42.7 ± 9.57	0.0005	83.6 ± 7.31	0.003

Table 3. Number of club cells per 1 mm of epidermal section length in controls and fish exposed for different time periods to acidified water

Pooled controls ($n=17$) 79.2 ± 8.97				
Time	pH 5 ($n=3$)	P val.	pH 6 ($n=3$)	P val.
3 h	82.6 ± 5.73	ns	81.4 ± 7.66	ns
24 h	76.4 ± 8.67	ns	78.5 ± 9.34	ns
3 d	34.7 ± 6.21	0.0005	72.6 ± 8.91	ns
7 d	74.3 ± 9.46	ns	71.3 ± 6.84	ns
14 d	104.6 ± 11.60	0.0005	80.9 ± 10.17	ns
23 d	152.8 ± 14.52	0.0005	98.1 ± 11.42	0.004

ed by budding from the Golgi membranes and coalesced to form electron-transparent mucosomes (Fig. 10) of about 1 μm in diameter. The cell nucleus was located basally in the cytoplasm. Cells filled with mucosomes had fewer and smaller desmosomes than filament cells. They migrated towards the epidermal surface where occasionally they were seen discharging their contents. The remaining cytoplasm of the mucous cells, usually not showing signs of apoptosis, was occasionally observed at the skin surface and most likely disappeared by sloughing.

Club cells. The club cells were less numerous than mucous cells (about 80 cells per mm section length; Table 3). Mature club cells were very large (about 27×20 μ m), and were located only in the middle of the epidermis. They were characterised by a centrally located multilobated nucleus surrounded by a thin rim of cytoplasm containing rER, some Golgi areas and mitochondria. Most of the cytoplasm was electron transparent and occupied by fine curved microfilaments, with a diameter of 4–5 nm.

Leucocytes. Lymphocytes were occasionally found in the epidermis. Basophilic granulocytes were rarely seen. Other types of leucocytes were not seen.

Sensory elements. Merkel cells, each of them containing 10 to 15 small granules per cross-section, as well as taste buds, were occasionally observed. Solitary chemo-sensory cells (Whitear and Kotrschal 1988) were not found, reflecting their rarity in the areas examined.

Dermis

In the dermis capillaries were never found within 60 μm from the basal lamina. Endothelial cells showed many small transparent vesicles at the luminal and the peripheral side of the endothelial cells. These vesicles were indicative of endo- and exocytosis, respectively. Pigment granules were located in the cell bodies of melanocytes, and were only occasionally found in the cytoplasmic extensions of these cells. Dermal papillae, containing axons and capillaries, were scarce. Lymphocytes and basophilic granulocytes were rarely found.

A diagram of the epidermis and the underlying dermal connective tissue of control fish is presented in Fig. 24.

Acid water - pH 5.0

Epidermis

The thickness of the epidermis significantly ($P<0.01$) decreased after 24 h and 3 days of acid water exposure, when compared to control fish. Later, the thickness was restored and even significantly ($P<0.01$) increased on days 14 and 23 (Table 1). The epidermal surface had a wavy appearance throughout the experiment. This was pronounced during the first 3 days and after 23 days. The decrease in epidermal thickness was associated with signs of necrosis (swelling of the cells, disruption of membranes) of many filament cells and pavement cells (Figs. 7, 8).

Filament cells. The incidence of necrosis decreased during the first days, and more and more of the degenerative

ig. **10.** Part of mucous cell of control fish, containing electron-ransparent mucosomes. × 19 000

ig. **11.** Pavement cell (*p*) with secretory vesicles (*arrows*) and mu-ous cell (*m*) with mucosomes of increased electron density. *rER* ough endoplasmic reticulum; *G* Golgi area. pH 5, 14 days. 20 500

ig. **12.** Part of a mucous cell with mucosomes showing endogenous eroxidase activity; DAB reaction, otherwise unstained. pH 5, 7 ays. × 21 200

ig. **13. a**. Apoptotic pavement cell, showing endogenous peroxi-se activity; DAB-reaction; otherwise unstained. pH 5, 14 days x 800. **b** Cytoplasm of normal pavement cell with vesicles showing roxidase activity. pH 5, 7 days. × 28 800

ig. **14.** Mucous cell at an early stage of apoptosis. The shrunken toplasm of the mucous cell, as well as the cover of the microridges the adjacent filament cells (*arrows*), show endogenous peroxidase tivity; DAB reaction, otherwise unstained. pH 5, 3 days. × 6500

g. **15.** Elongated mucous cell releasing mucus at its apical pole (*a*) d synthesizing electron-dense mucosomes at its basal pole (*b*). 5, 7 days. × 5100

g. **16.** Apical part of chloride cell (*c*) between pavement cells (*p*). *ts* bular system. pH 5, 3 days. × 23 500

pavement cells were apoptotic (cellular shrinkage, condensation of cellular components, loss of junctional complexes; Fig. 9). On days 14 and 23 the apoptosis prevailed among the degenerative pavement cells. The outline in the section of the non-degenerative pavement cells changed from flattened to oval, and showed signs of increased secretory activity: well developed rER and Golgi areas. Already after 3 h small vesicles had appeared, with a diameter of 150–200 nm and a finely granular or homogeneous matrix of high electron density (Fig. 11). Their number increased rapidly and from 24 h onwards, they also appeared in the upper 2 or 3 layers of the filament cells. Most of the high electron-dense vesicles (about 25–35 per cross-section of these cells) were observed on days 7 and 14. Some of these vesicles gave a positive response in the peroxidase test (Fig. 13b). Such vesicles were located mainly in cells lacking the glycocalyx cover. Peroxidase activity was prominent in the cytoplasm of apoptotic cells (Fig. 13a) and in the glycocalyx cover of the skin surface (Figs. 5b, 14), especially in the glycocalyx of normal, rather than of apoptotic pavement cells. The glycocalyx itself was more pronounced than in the controls (Fig 5b). The distribution of injected HRP in the intercellular spaces was similar to that in the controls. The number of desmosomes decreased. The terminal web in these cells became more pronounced from day 3 onwards, and the ridges became slightly higher (up to 0.5 μm). In colchicine-treated fish from acid water, mitosis of filament cells was frequently observed (about 25–30 per mm of epidermal length in cross sections). It occurred in all layers except in the uppermost and the innermost layer of the epidermis. Mitosis was also observed in colchicine-untreated fish. Filament-containing cells differentiating into either mucous cells or club cells were found in the same areas as the mitotic cells. The endocytotic activity of the basal filament cells was suppressed almost completely after 3 h, and was restored at day 1. The basal lamina became highly undulating, and this lasted throughout the experiment.

Mucous cells. Mucus secretion was stimulated at the beginning of the exposure period. It was reflected by the presence of most mucous cells close to the epidermal surface and by a significant ($P<0.001$) decline of the total number of mucous cells (Table 2) after 24 h and 3 days. The number of mucous cells was restored later, and declined significantly ($P<0.001$) again after 23 days. In the first 14 days of exposure to the acidified water, the newly synthesized mucosomes - of about 0.4–0.6 μm in diameter - showed a high electron density (Figs. 11,15), which became visible already after 24 h. Fused mucosomes of high electron density, as well as apoptotic newly differentiated mucous cells, were commonly found. Most mucous cells were elongated rather than oval shaped, with a length up to 20 μm. Such cells showed synthesising activity (abundant rER and many Golgi systems from which new mucosomes budded) at their basal pole, close to the nucleus. This part was connected with a strand of cytoplasm to the apical pole where release took place (Fig. 15). At day 23 dense mucosomes became less common. Whereas in the controls the mucous layer usually disappeared during fixation, in acid-exposed fish a mucous layer of 7–9 μm thickness frequently remained attached to the epidermal surface (Fig. 20). This layer, as well as some of the mucosomes (Fig. 12) and the cytoplasm of apoptotic mucous cells (Fig. 14), showed a positive reaction in the peroxidase test.

Chloride cells. Clusters of 4–6 chloride cells, absent in the controls, were commonly seen from day 3 onwards in the upper epidermal cell layers (Fig. 16). They were characterised by an extensive tubular system and many mitochondria, and were connected by desmosomes to adjacent pavement cells. Most chloride cells showed an apical crypt. Accessory chloride cells were also present. Occasionally chloride cells with the high electron density of apoptotic cells (Wendelaar Bonga and Van der Meij 1989) were observed. Some of the latter cells were engulfed by macrophages. At the end of the experiment (day 23), chloride cells were only occasionaly seen.

Club cells. The cytoplasm around the nucleus contained extensive rER, Golgi systems, and many free ribosomes. Small vesicles, possibly primary lysosomes, were found in the Golgi areas and in the cell periphery, close to the outer cell membrane. Some of the club cells had large phagosomes. After 3 h and until day 14, many club cells were located close to the skin surface. At day 3 the number of club cells was significantly ($P<0.001$) reduced, when compared to control fish (Table 3). At day 7, however, their number was back to control values and at days 14 and 23 was significantly ($P<0.001$) higher than in the controls.

Leucocytes. Already after 3 h, macrophages (Fig. 18), as well as lymphocytes, had penetrated the epidermis. From day 1 onward, also many eosinophilic and basophilic granulocytes extravasated and appeared in the dermal connective tissue and in the epidermis (Fig. 19). Macrophages and basophilic granulocytes appeared very active, with well developed rER and Golgi areas, and

Fig. 17. Club cell containing a leucocyte (*le*); a lymphocyte (*ly*) possibly penetrates this cell. pH 5, 3 days. × 6500

Fig. 18. Macrophage containing a phagosome (*p*) and a lysosome (*l*), in the epidermis. pH 5, 14 days. × 9000

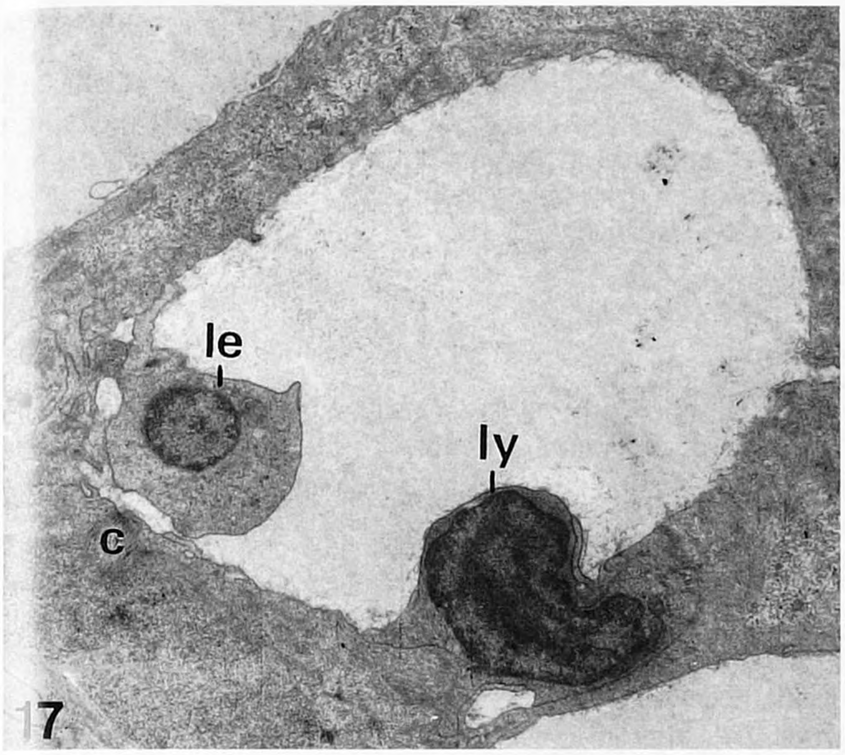
Fig. 19. Eosinophilic granulocyte (*g*) between filament cells (*f*) of the epidermis; pH 5, 3 days. × 14 800

Fig. 20. Sensory process of solitary chemo-sensory cell (*c*) protruding at the skin surface; *m* part of the mucus layer. pH 5, 14 days. × 14 200

Fig. 21. A Merkel cell (*M*) at early stages of necrosis, showing swollen cytoplasm and electron-dense secretory granules (*arrows*). pH 5, 24 h. × 20 500

Fig. 22. A Merkel cell (*M*) with many secretory granules and otherwise normal structure, located close to the skin surface. pH 5, 3 days. × 18 000

Fig. 23. Remnants of melanocyte extensions (*arrows*) in the epidermis close to the skin surface. pH 5, 14 days. × 5200



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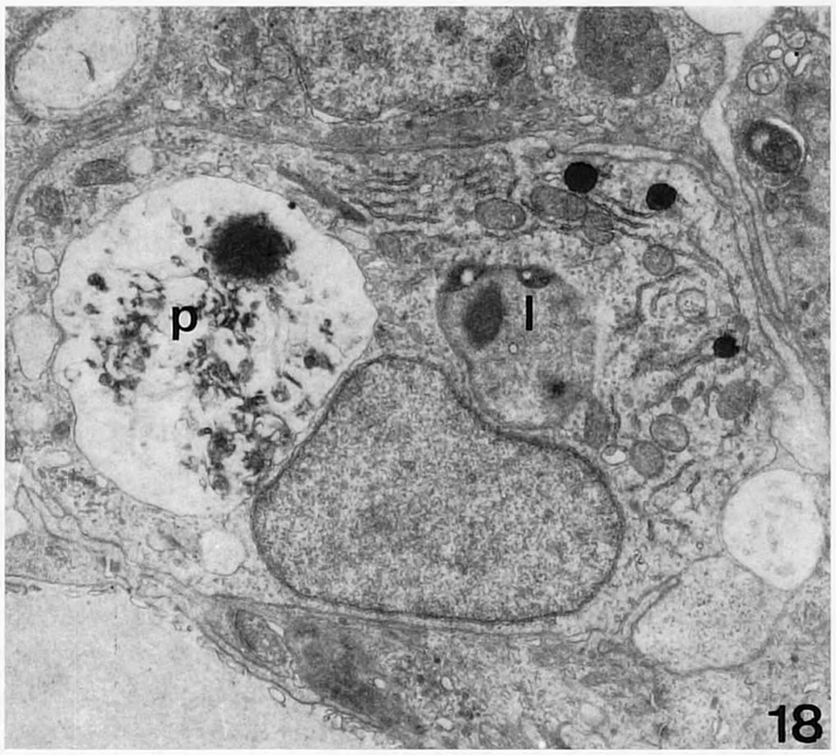

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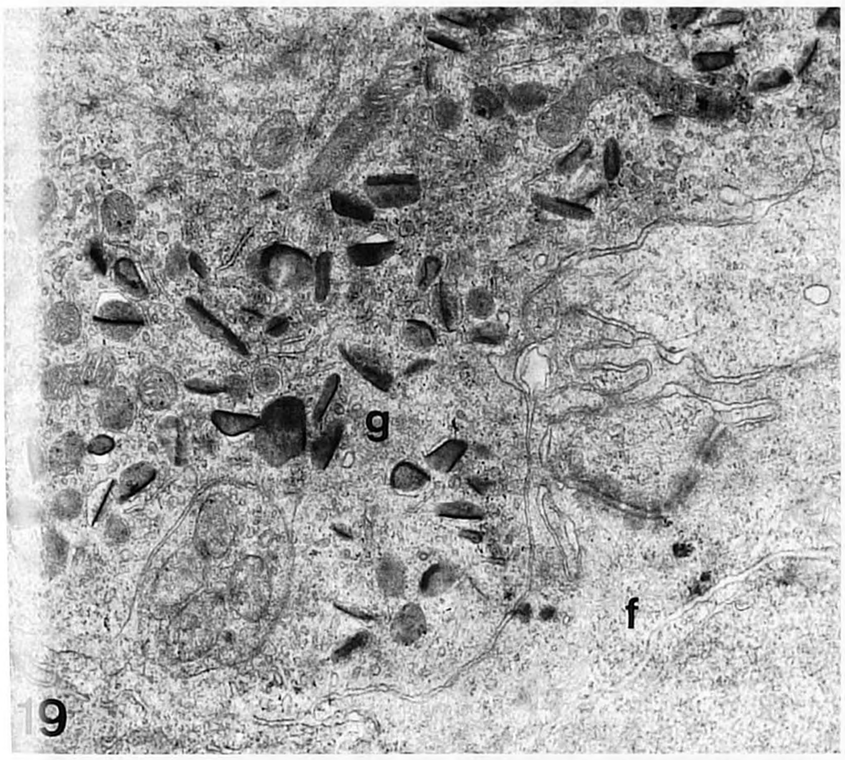

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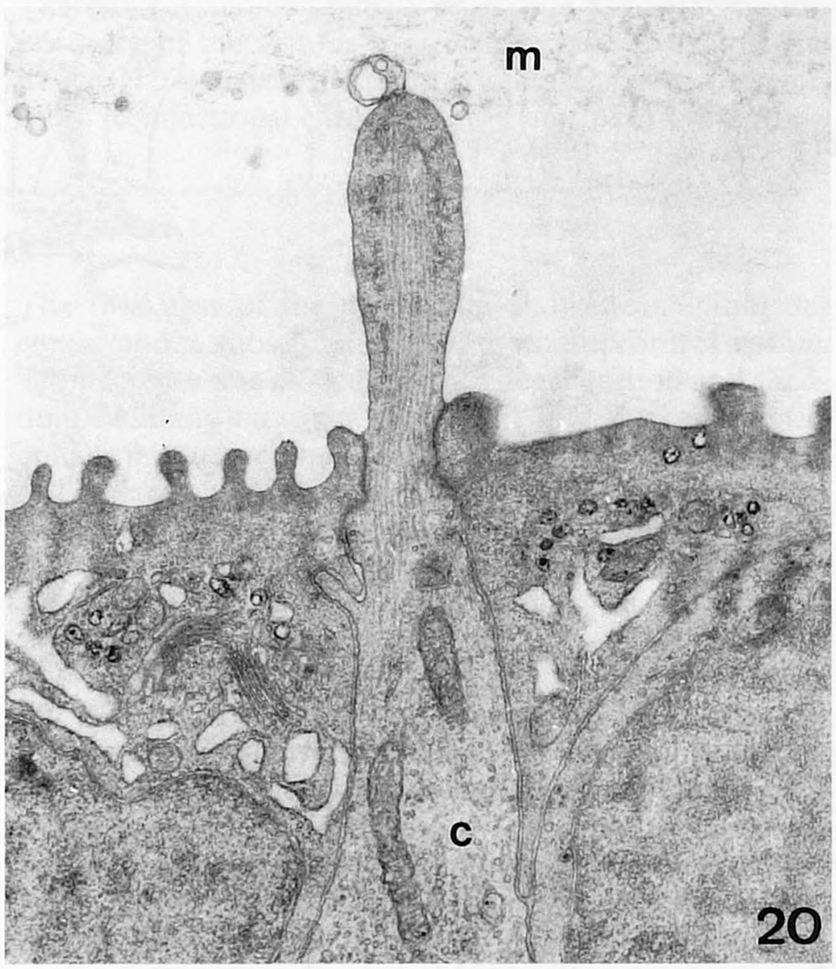

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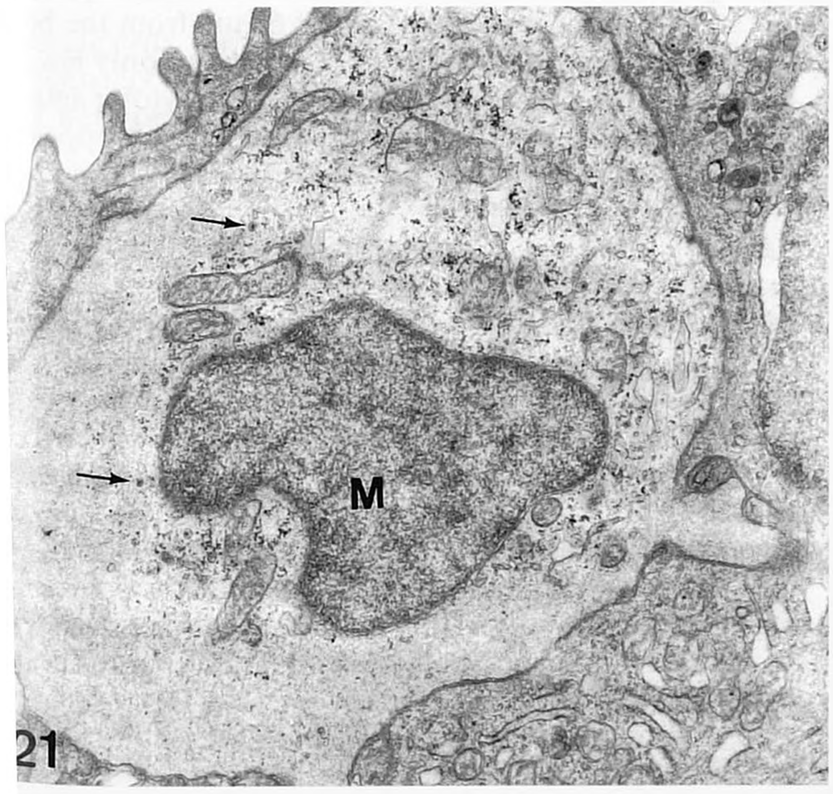

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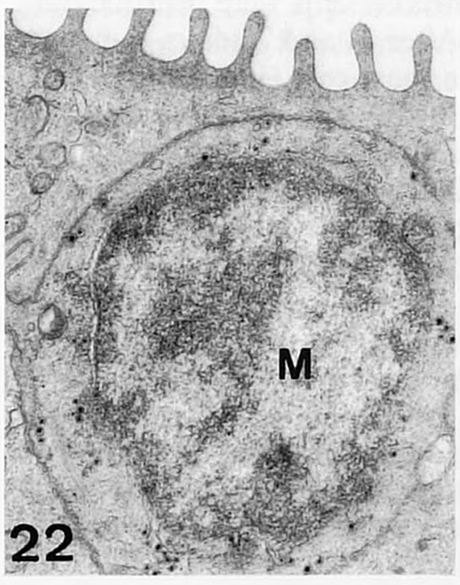

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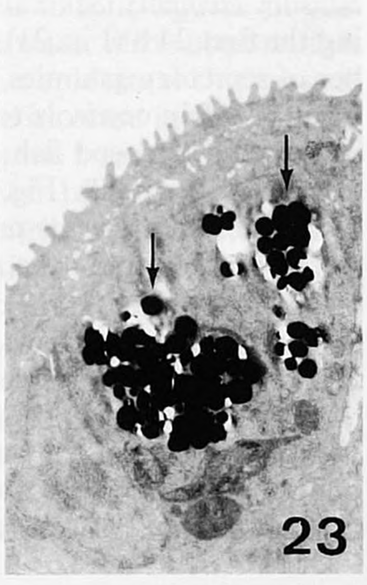

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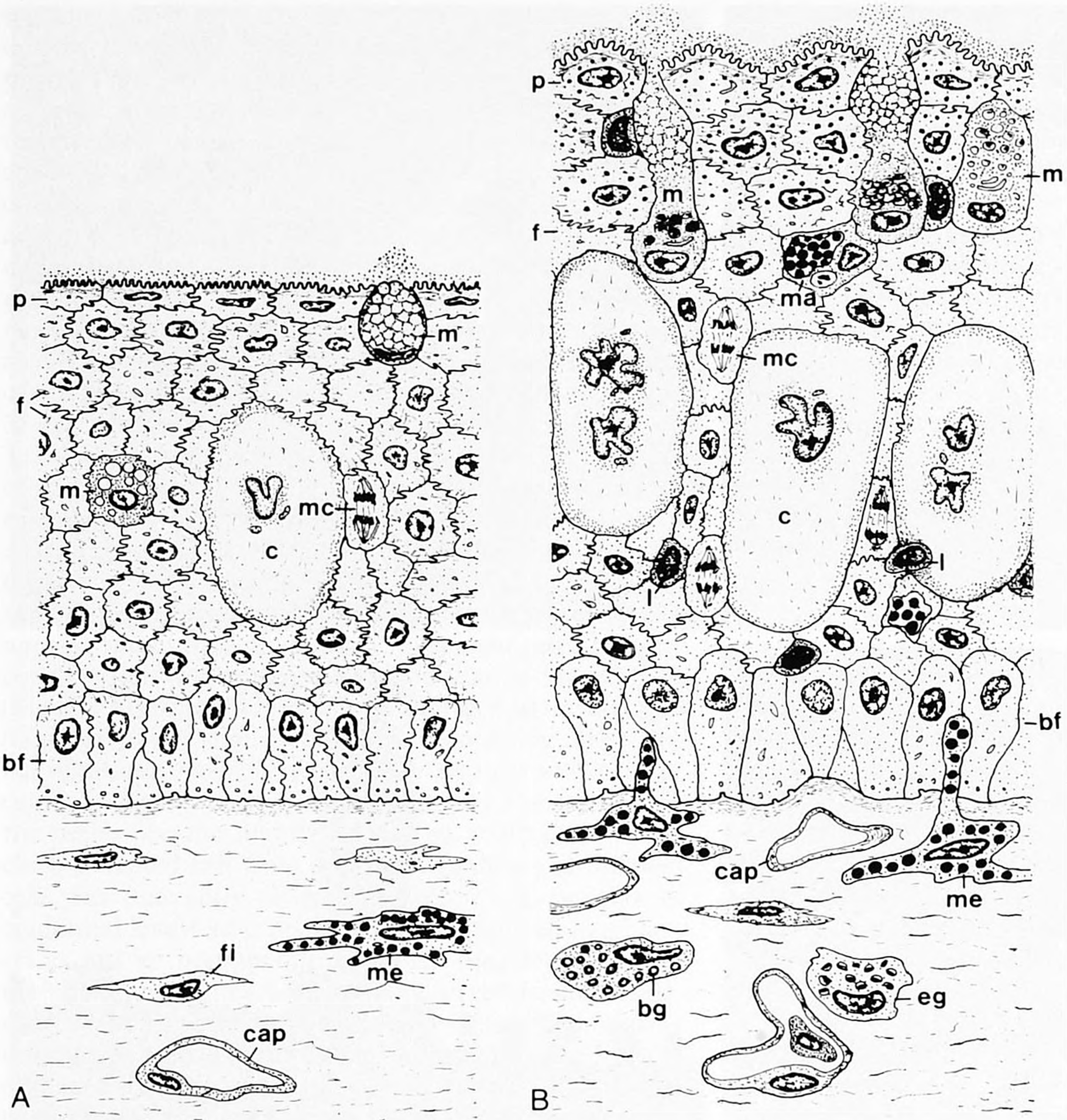

Fig. 24A,B. Schematic diagram of cross-sections of the skin of control carp (**A**) and carp exposed for 23 days to water of pH 5 (**B**). *p* Pavement cells with microridges and a thin (**A**) or thick (**B**) mucous layer. *f* Filament cells; *m* mucous cells; *c* club cells; *mc* mitotic cells; *ma* macrophage, containing phagosome with pigment granules; *l* lymphocyte; *bf* basal filament cells; *me* melanocytes; *fi* fibroblasts; *cap* blood capillaries; *bg* basophilic granulocyte; *eg* eosinophilic granulocyte

several phagosomes. Several lymphocytes and granulocytes were found adjacent to club cells and the impression was that they had entered club cells (Fig. 17). The latter apparently had an intact outer membrane and did not show signs of degeneration.

Sensory elements. Several Merkel cells were necrotic during the first 24 h (Fig. 21). After 14 and 23 days, the number of secretory granules in these cells (Fig. 22) increased from 10–15 in controls to 30–40 granules per cell profile in the acid exposed fish. From day 7 onwards, solitary chemo-sensory cells (Fig. 20), with extensions containing a core of microtubules projecting into the mucous layer, were observed in all the experimental fish.

Dermis

Dermal papillae were very common from the third day onwards. Shortly after water acidification the endothelial cells of the capillaries became extended in the direction of the epidermis. Whereas in the controls the capillaries were located at a distance of about 60 µm from the basal lamina, this distance gradually decreased to only few µm at day 3 in acid water. The endo- and exocytotic activity of the endothelial cells almost completely disappeared within 3 h after the start of the experiment. This activity was resumed already at day 1 and was back to normal at day 3.

Melanocytes. The skin of experimental fish was darker than that of control fish. The pigment granules were located in the cytoplasmic extensions of the melanocytes rather than in the cell bodies. These cytoplasmic extensions were no longer restricted to the dermal connective tissue: already after 3 h they penetrated into the basal layers of the epidermis. Within 3 days and until the end of the experiment they were observed all over the epidermis, up to one layer from the epidermal surface (Fig. 23). Some of the melanocyte processes were apoptotic and confined within macrophages.

Acid water – pH 6.0

The effects of exposure to pH 6.0 were in many respects similar to those of exposure to pH 5, although from a quantitative point of view intermediate between those observed in the latter group and the controls, or occurred later than in pH 5.

Epidermis

The thickness of the epidermis was significantly ($P < 0.01$) reduced at day 3 (Table 1), but it increased later and stabilised at control levels.

Filament cells. After 24 h onwards, pavement cells were actively synthesising and secreting small secretory vesicles that reacted positively with the peroxidase test. The vesicles were less abundant than at pH 5. The glycocalyx also reacted positively. The tight junctions between the pavement cells were intact. The height of the microridges increased to about 0.45 μ m. Apoptotic cells were only occasionally observed. Their cytoplasm showed peroxidase activity. Necrosis was rarely found. The number of mitotic cells, mostly in the neighbourhood of club cells, was 35–40 per mm of skin section. The endocytotic activity in the basal layer of filament cells slightly diminished during the first day. The basal lamina became slightly undulating.

Mucous cells. From 24 h onwards the number of mucous cells was significantly ($P < 0.01$) lower when compared to controls (Table 2). The lowest number was found on day 3 but it remained below control values throughout the experiment. Differentiating mucous cells were found only in the middle and upper epidermal layers. The mucous vesicles were smaller than in the controls. Elongated cells connected by desmosomes, showing mucosome release at the top and synthesis at the basal pole, were common. After fixation a mucous layer of about 1–2 μm thickness remained attached to the epidermal surface. It showed some peroxidase activity.

Chloride cells. Single chloride cells appeared after 3 days and clusters of 2–3 cells after 7 days. On day 23, chloride cells were rarely seen.

Club cells. The number of club cells was only slightly decreased during the first week, but significantly ($P < 0.01$) increased later on when compared to control levels (Table 3). Club cells were found close to the skin surface as well as at their normal location (e.g., in mid-epidermal layers). Only a few club cells contained leucocytes.

Sensory elements. Projections of solitary chemo-sensory cells were common from day 7 onwards.

Dermis

The endocytotic activity of the endothelial cells of the blood capillaries diminished slightly during the first day. At the same time the endothelial cells became extended in the direction of the basal lamina, and after 7 days capillaries were present very close to this lamina.

Melanocytes. The skin was slightly darker than in the controls. Pigment granules were located in the periphery of the melanocytes. Penetration of cytoplasmic extensions of these cells into the epidermis was restricted to the first 3 days. Afterwards, in the epidermis only remnants were observed, mostly within macrophages.

A diagram of the epidermis and the underlying dermis of acid-exposed fish is presented in Fig. 24.

Discussion

The results of this study show that exposure of fish to acid water had pronounced effects on the epidermal and dermal skin layers. These effects are for a small part degenerative. Most effects represent adaptive changes. Their extent and complexity reflect the physiological importance of the skin as a protective barrier against the direct (H^+-penetration) and indirect (increased susceptibility to infections) effects of low water pH.

Epidermis

The thickness of the epithelium showed an initial decrease and a subsequent increase above control values. The decrease was associated with degeneration and shedding of many pavement cells and mucous cells at the epidermal surface, which apparently surpassed the rate of cell replacement. Necrosis, a process reflecting accidental cell death (Wyllie 1981), and perhaps a direct effect of reduction of the water pH, was prominent during the first days. Degeneration by apoptosis, which is the conventional, physiologically controlled cell death (Wyllie 1981) increased during the first week and dominated later. This reflects accelerated ageing of the cells, caused by their increased activity or, directly, by the low water pH. Necrosis and apoptosis were also reported for the gill epithelium of acid-exposed tilapia, *Oreochromis mossambicus* (Wendelaar Bonga et al. 1990).

Our observations of colchicine-treated fish indicate an increase of mitotic activity of the epidermal cells in acid-exposed fish. The appearance of mitotic figures in colchicine-untreated fish from acid water also supports this view. The subsequent increase in epidermal thickness is therefore probably caused by an increase in the number of filament cells and later also by the increase in the number of club cells. The infiltration of the epidermis by many leucocytes may further contribute to the increase in skin thickness. The higher mitotic rate, together with the appearance of many apoptotic cells, indicates that at this pH the turnover rate of the cells was increased. This conclusion was also drawn for epidermal cells and gill cells of tilapia exposed to acid water (Wendelaar Bonga et al. 1990). The increased mitotic rate in fish at pH 6, in combination with a skin thickness that is hardly increased, also reflects increased cell turnover. An increase in epi-

dermal thickness as found here for fish at pH 5 has been reported earlier in our studies on the effects of manure (Iger et al. 1988) and wounding (Iger and Abraham 1990) on carp, and of acid water on tilapia (Wendelaar Bonga et al. 1990).

The regulation of epidermal cell proliferation is under endocrine control, and cortisol as well as prolactin have been implicated (Wendelaar Bonga and Meis 1981; Iger 1992). Whether this also holds for apoptosis is unknown. In addition to endocrine factors distributed by the blood circulation, local factors produced in the epidermis itself may be involved. Budtz and Spies (1989) noted that in the skin of the toad *Bufo bufo* apoptotic cells were often found in the presence of Merkel cells and suggested that the latter cells produced apoptosis-stimulating substances. Both Merkel cells and club cells contain serotonin (Fujita et al. 1988; Zaccone et al. 1990)

The endocytotic activity normally observed basally in the basal layer of filament cells was reduced on the first day at both pH 5 and 6. This was also observed after wounding in carp (Iger and Abraham 1990). Through this process metabolites may pass from the dermis to these cells. Thus, shortly after water-acidification the active uptake of metabolites seems to be reduced transiently.

The tight junctions between the pavement cells remained intact and unchanged. In the gill epithelium of trout (*Oncocorhynchus mykiss*), Freda et al. (1991) observed that the tight junctions between pavement cells and chloride cells were less extensive in acid water than in neutral water.

The high secretory activity of the pavement cells in acid water was associated with a more elaborate cytoskeleton, including the terminal web. Also the microridges were slightly higher in the fish from acid water. This phenomenon has been reported earlier, e.g., for fish in water with organic manure (Iger et al. 1988). Increase of intercellular spaces, which frequently occurs in the epidermis of stressed fish (Whitear 1986; Wendelaar Bonga and Lock 1992) did not occur.

The pavement cells produced electron-dense secretory vesicles. Similar vesicles have been described before in the skin of other fish species (e.g., Wendelaar Bonga and Meis 1981) and their appearance has been correlated with copious secretion from the surface cells (Whitear and Mittal 1986). This is supported by our findings, showing a considerable increase in the formation, storage, and release of these vesicles in the pavement cells of carp exposed to acid water. Surprisingly, some of these secretory vesicles displayed peroxidase activity. This enzyme activity was also found in the glycocalyx covering these cells, indicating that it was released from these vesicles to the cell surface. On top of apoptotic cells, which showed a strongly diminished secretory activity, the glycocalyx displayed only a low peroxidase activity. Since peroxidase activity was detected mainly in vesicles of cells lacking cover of glycocalyx at skin surface, we assume that the massive polymerization of DAB at the skin surface, in particular at the surface of intact cells, reduced its penetration into these cells. The enzyme activity may have an antibacterial function.

Mucous cells. The secretion of mucus by fish skin is greatly stimulated in acid water. This has been reported earlier for fish in acid water (e.g., Daye and Garside 1976; Wendelaar Bonga et al. 1990), as well as for fish exposed to pollutants (Iger 1992; Benedetti et al. 1989). The relatively thick layer of mucus attached to the epidermal surface in fish from low pH may have been formed by the coagulation of mucus under this condition (Fromm 1980). Greater adherence to the skin may also be caused by a change in chemical composition of mucus (Gona 1979) or by alteration in the binding capacity of the skin surface (higher microridges, more elaborate glycocalyx). In our experiment changes in mucus composition are indicated by the higher electron density of the mucosomes and by their peroxidase activity. The smaller size of the mucosomes may also reflect a higher viscosity of the mucus (Lewis 1976). To the best of our knowledge, the appearance of newly differentiated mucous cells close to the skin surface, as we found in acid water, has not been previously reported.

Acid water had also an effect on the secretory process of the mucous cells. In the controls the mucus was released from rounded goblet-shaped cells, which displayed very little synthetic activity. At low water pH, however, the cells became more elongated and in their basal part the synthesis of mucus vesicles apparently continued during the release of mucus from the apex of the cells. Apparently, the secretory potential of the mucous cells was exploited more fully in acid water than under control conditions.

Chloride cells. In control fish chloride cells were found only in the gills and in the epithelium covering the inner side of the operculum of these fish (Wendelaar Bonga, unpublished). Within 3 days in acid water they also appeared in the skin covering the head, indicating that the time of differentiation of these cells was less than the 4 days suggested by Chretien and Pisam (1986) for *Lebistes reticulatus*. Following the description of chloride cells in tilapia (Wendelaar Bonga et al. 1990), we identified accessory (replacement), immature, and apoptotic chloride cells. The appearance of these ion transporting cells may be connected with disturbance of ionic regulation in acid-exposed fish, and indicates that the ion-regulatory capacity of the gill area is insufficient during the first weeks of acid stress. Apoptotic chloride cells were phagocytosed by macrophages, in contrast to the apoptotic remnants of pavement and mucous cells, which were shed. This confirms our observations on pavement cells and chloride cells in the gills of tilapia (Wendelaar Bonga and Van der Meij 1989; Wendelaar Bonga et al. 1990).

Club cells. An increase in club cell numbers as a response to a stressor, in the present experiment water acidification, has not been previously reported. These cells are characteristic for the epidermis of several orders of teleost fish and have been suggested to be the source of a specific alarm substance (at least in ostariophysans, Pfeiffer 1977; Whitear 1986) and other bioactive substances, including serotonin (Zaccone et al. 1990).

Leucocytes. Reduction of water pH immediately evoked infiltration of the epidermis by different types of leucocytes. This phenomenon has commonly been described for the epithelium covering in the gills, as a response to a variety of stressors such as water pollutants, or handling of the fish (for reviews see Mallatt 1985; Wendelaar Bonga and Lock 1992). We have reported leucocyte infiltration in the skin of carp after treatment of the water with manure (Iger et al. 1988), lead (Iger 1992) and after wounding of the fish (Iger and Abraham 1990). This infiltration may at least partially explain the leucopenia that is characteristic for stressed fish (Pickering and Pottinger 1987). Surprisingly, leucocytes seemed to penetrate non-degenerative club cells. The same phenomenon was observed in fish kept in water containing heavy metals such as lead and cadmium (Iger 1992). Whether or not the club cells act as "killer cells" for the leucocytes is unclear; it is known that club cells contain toxins (Al-Hassan et al. 1987).

Sensory elements. The Merkel cells were among the first to degenerate in acid water. Degeneration of sensory elements in response to environmental changes has been reported before, for instance after exposure to copper (Benedetti et al. 1989) or mercury (Pevzner et al. 1986). The appearance, later on, of many solitary chemo-sensory cells in the epidermis of acid exposed fish, may be either a response associated with the thick and viscous mucous cover that may hamper sensory perception or a direct effect of the drop in water pH.

Dermis

Exposure to acid water induced extension of endothelial cells in the direction of the basal lamina, followed by angiogenesis. This may be connected with greater nutritional requirements of the epidermis caused by the increased mitosis and cellular activity. In the dermis of fish kept at pH 5, it may also be connected with the increased thickness of the epithelium: because of the outgrowth of the capillaries, the diffusional distance between the blood and the upper epidermal layer remained about the same.

Melanocytes. In acid-exposed fish we observed increased pigmentation of the skin and the appearance of pigment-containing cytoplasmic extensions of these cells in the epidermis. We have reported similar phenomena for carp from water polluted with manure (Iger et al. 1988) or heavy metals (Iger 1992), and they may be a general response to stressors. The significance of the penetration of pigment containing processes in the epidermis is unclear. It does not seem to contribute noticeably to the coloration of the skin. This is eventually affected by the redistribution of melanosomes between melanocyte cell-bodies and the cellular processes. It may represent some form of defense mechanism, because intermediates of the melanin synthetic pathways have bactericidal capacities as a result of hydrogen peroxide generation through NADH oxidation (Edelstein 1971; Ellis 1977).

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