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Histology and Ultrastructure of the Gut Epithelium of the Neotenic Cave Salamander, *Proteus anguinus* (Amphibia, Caudata)

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ABSTRACT Histological, histochemical, and ultrastructural features of the gut of the European endemic cave salamander *Proteus anguinus* were studied. The gut is a relatively undifferentiated muscular tube lined with a simple columnar epithelium containing numerous goblet cells. The mucosa and underlying lamina propria/submucosa are elevated into a number of high longitudinal folds projecting into the lumen. The enterocytes are covered apically with uniform microvilli. Irregularity in the arrangement of microvilli was observed. Occasionally, irregular protrusions of the cytoplasm appear between groups of microvilli. Pinocytotic activity occurs at the bases of the intermicrovillous space. Mitochondria are numerous in the apical cytoplasm and basally beneath the nuclei. The supranuclear cytoplasm contains most of the cell organelles. The lateral plasma membranes of adjacent cells interdigitate and are joined by junctional complexes. The periodic acid-Schiff (PAS) reaction, indicating neutral mucosubstances, is positive only in the apical brush border of enterocytes and in goblet cells. The goblet cells also stained with Alcian blue (AB), at pH 2.5, thus revealing the presence of carboxylated glycosaminoglycans. Compact aggregations of AB- and PAS-negative cells are situated directly below the epithelium. Mitotic figures are present in individual clusters of cells. The fine structure of cells in these clusters indicated that these cells could be responsible for renewal of intestinal epithelium. Numerous endocrine-like cells could also be seen. The closely packed smooth muscle cells and amorphous extracellular material with collagen fibrils constitute a net-like structure under the basal lamina that is very closely associated with the epithelium. There are numerous acidophilic granular cells between epithelial cells, in the lamina propria/submucosa, and between cells aggregations. They seem to be associated with nematode infections and possibly constitute a humoral defense mechanism. J. Morphol. 259:82–89, 2004.

KEY WORDS: digestive tract; *Proteus anguinus;* light and electron microscopy

Proteus anguinus is the only species of the European cave salamander and the most remarkable troglobiont in the underground waters of Slovenia's Dinaric karst region. Slow metabolism and starvation resistance (Vandel, 1965; Hüppop, 1985; Hervant et al., 2000, 2001), among other general troglomorphic characteristics, focused our attention and first led to our investigation of the morphology of its digestive system. Because of the temporal and spatial patchiness of food availability in most cave biotopes, periods of prolonged starvation are common events in the life of subterranean organisms (Vandel, 1965; Hüppop, 1985). *Proteus anguinus* is able to survive for long periods without food, ranging from 18–96 months or more, with no signs of illness (Vandel, 1965; Bulog et al., 2000; Hervant et al., 2001). We are interested in how starvation influences the morphology and histochemistry of the digestive system in *P. anguinus.*

The ultrastructure of *Proteus anguinus* gut mucosa has not been described previously. Earlier studies of the histology of its gut were based only on light microscopy (Noble, 1955). The purpose of our study was to analyze the ultrastructure of the anterior part of the small intestine of *P. anguinus*. The presence of mucosubstances is also established and the cytological features of the epithelial cells are described. The results provide a morphological basis for further research on the influence of food deprivation on the ultrastructure of the gut mucosa and certainly provide groundwork for future research on the very low metabolism of *P. anguinus*.

MATERIALS AND METHODS

Specimens of the neotenic cave salamander *Proteus anguinus* (Amphibia, Caudata) were collected in the Planina Cave, Slovenia, but we could only use a small number due to the very strict enforcement of nature conservation laws. For this study we had four animals, each with a body length of about 220 mm. They were narcotized with MS 222 (ethyl m-aminobenzoate methane sulphonic acid salt) and decapitated. Animals were collected with the permission of the Ministry of the Environmental and Spatial Planning of the Republic of Slovenia.

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For light microscopy, the segment of small intestine just posterior to the entrance of the hepatopancreatic duct was removed, fixed in Carnoy's solution, and embedded in Paraplast. Fixative was injected into the gut lumen before removal of tissue from the animal. Sections 5 μm thick were stained by standard hematoxylin-eosin methods and according to the Pantin or Mallory-Heidenhain method (Humason, 1967). For the detection of mucopolysacharides, sections were treated with the periodic acid-Schiff procedure (PAS) and with Alcian blue (AB) at pH 2.5 (Pearse, 1968). The control sections for PAS were treated with saliva at 37°C (Pearse, 1968).

For transmission electron microscopy, small, 1-mm thick samples of tissue were fixed in ice-cold 1.25% glutaraldehyde and 0.5% paraformaldehyde, buffered at pH 7.4 with 0.05 M sodium cacodylate for 2 h at 4°C. The tissue was washed in 0.05 M cacodylate buffer with 0.25 M sucrose at room temperature overnight and postfixed in 1.0% osmium tetroxide for 2 h at 4°C, then dehydrated in an ethanol series, infiltrated, and embedded in Epon 812. Sections were cut on an LKB Broma ultramicrotome, stained with uranyl acetate and lead citrate, and then examined with a JEOL 8T electron microscope.

Semithin sections for light microscopy were stained with 0.1% Toluidine blue in 2.5% borate buffer (Trump et al., 1961) or with Richardson blue (Richardson et al., 1960).

RESULTS

Light Microscopy

The anterior portion of the gut of *Proteus anguinus* exhibits the structural arrangement typical of tubular organs: an epithelial mucosa overlying a lamina propria/submucosa of loose connective tissue, and a muscularis externa composed of inner circular and outer longitudinal layers of smooth muscle. Externally there is a very thin serosal layer, the visceral pleuroperitoneum.

The mucosal surface and the underlying connective tissue of the lamina propria/submucosa create longitudinal folds (Fig. 1a). The absence of a muscularis mucosa prevents the distinct separation of lamina propria from submucosa. The lamina propria/submucosa contain a large number of blood vessels, many of which are closely associated with the epithelium (Fig. 1b). The epithelium is composed of simple columnar enterocytes, with numerous interspersed goblet cells (Figs. 1a, 2a). Enterocytes exhibit round nuclei basally; their cytoplasm is PAS-negative and only the apical brush borders gave a distinct PAS-positive reaction (Fig. 3a). Goblet cells were stained strongly by the PAS reaction as well as by AB at pH 2.5 (Fig. 3a,b). The coexistence of acid mucosubstances and neutral mucopolysaccharides is shown by purple staining with PAS-AB at pH 2.5. Enterocytes rest on the infolding basal lamina (Fig. 1b).

Compact aggregations of cells are situated directly beneath the epithelium, in the connective tissue of the lamina propria/submucosa (Figs. 1a,b, 2a), lying parallel to the surface epithelium and connecting to it. Their number varies considerably from one cluster to another, from 3–8 cells per cluster, sometimes more. The cytoplasm of these cells is PAS-AB-negative. In individual cells of the clusters mitotic figures are present that generally were not seen in the surface epithelium.

In the connective tissue of the lamina propria/submucosa as well as in the epithelium and between cell aggregates, cells with granular, acidophilic cytoplasm are present (Fig. 2a). These cells are round and have eccentric nuclei. They are particularly numerous in animals infected by parasitic nematodes. Nematodes were found in the lumens or attached to the epithelium in the intestines (Fig. 2b); sometimes nematode instars encysted in the intestinal tissues.

Transmission Electron Microscopy

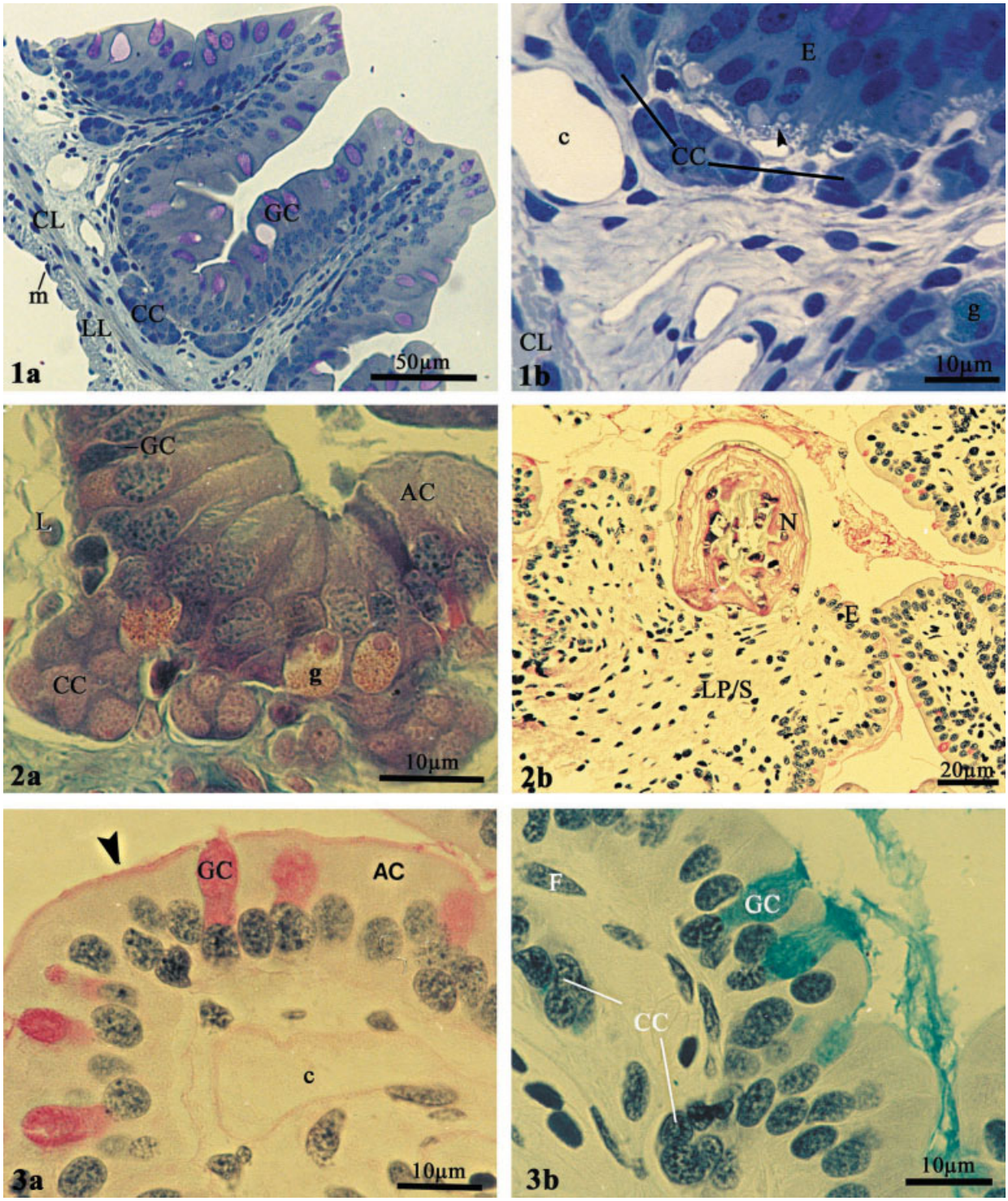
The apical surface of the epithelial cells is characterized by the presence of regular microvilli (Fig. 4a). The core of each microvillus is filled with fine filaments that extend from the dense tip of the microvilli into the terminal web underlying the striated border. There are frequent invaginations of the plasma membrane at the base of microvilli (Fig. 4b). Beneath the terminal web, numerous mitochondria, electron-dense granules, small smooth vesicles, and, rarely, multivesicular bodies are apparent (Fig. 4a). Their nuclei are round and euchromatic. The supranuclear cytoplasm contains short cisternae and vesicles of rough endoplasmic reticulum, scattered granules, mitochondria, and Golgi complexes (Fig. 4c).

Junctional complexes composed of an occluding junction, a desmosome, and a gap junction were seen between neighboring epithelial cells (Fig. 4b). Many desmosomes are present along the remainder of the lateral plasma membrane; the tonofilaments extend into each side of the cytoplasm (Fig. 5a). In the deeper portions the adjacent lateral plasma membranes interdigitate to a considerable degree (Fig. 5b).

Occasionally, irregular protrusions of the cytoplasm appeared between groups of microvilli (Fig. 6a). These apical bulges often include ribosomes, vesicles, and small dark granules (Fig. 6b) that sometimes appeared to be pinched off from the apical surface. Occasionally, membrane-bound cytoplasmic fragments appeared free in the lumen.

The basal portions of enterocytes rest on a distinct, amorphous basal lamina that separates the epithelium from the lamina propria/submucosa. The basal region of the cytoplasm is filled with mitochondria (Fig. 7a,b).

Beneath the basal lamina, we very often found closely packed smooth muscle cells (Fig. 7a,b). The cross-sectioned muscle cells and amorphous extracellular material with collagen fibrils constitute a net-like structure (Fig. 7b). There are fine filaments and sparse mitochondria in the cytoplasm of these muscle cells. Pinocytic vesicles appeared in association with the cellular plasma membrane or in the cytoplasm itself (Fig. 7b, insert). The nuclei are small with pale nucleoplasm.

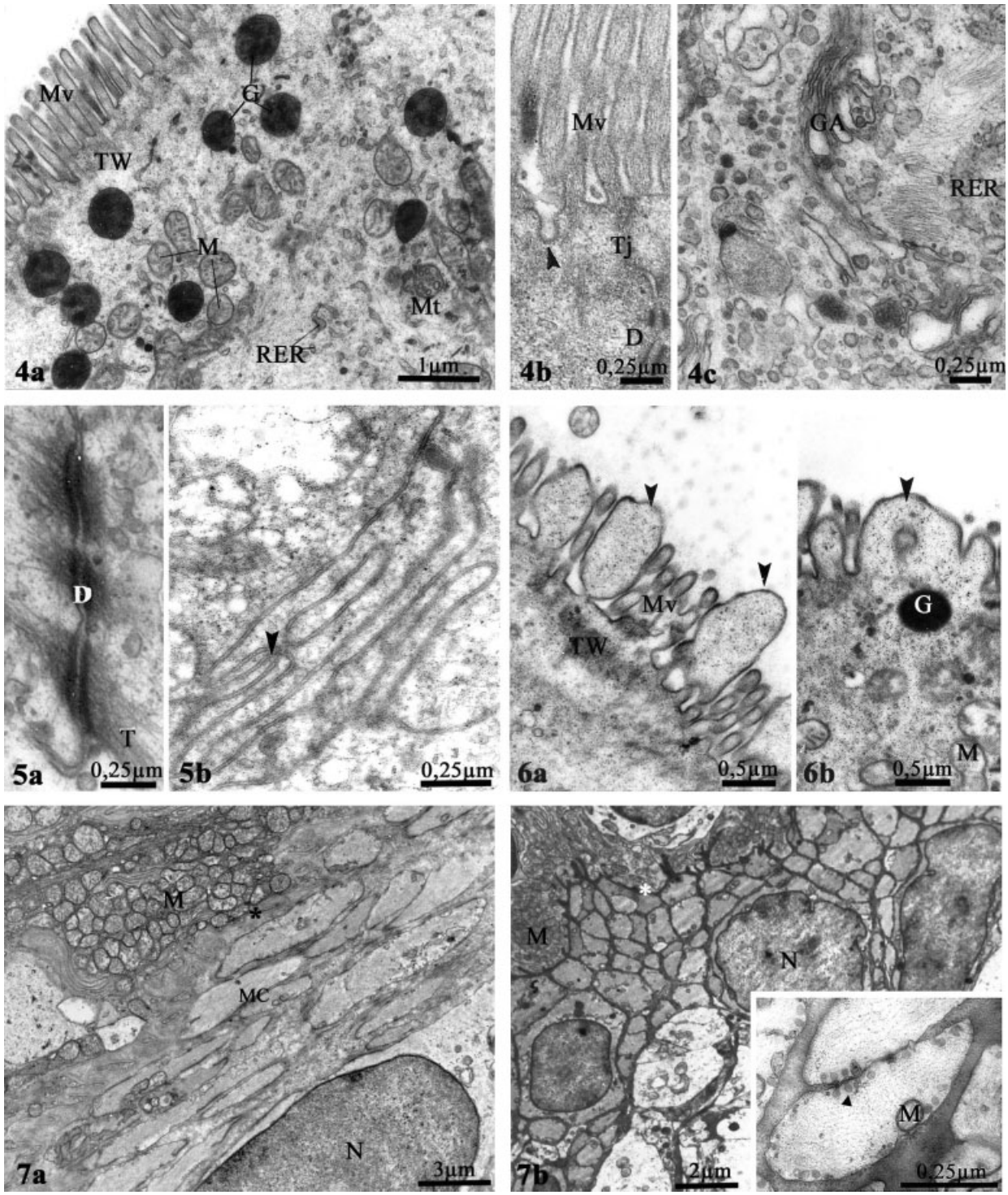


Figs. 1–3. Anterior portion of the small intestine of *Proteus anguinus.* Light micrographs.

Fig. 1. **a:** Goblet cells (GC) are scattered through the epithelium. The mucosal surface is thrown into folds. The epithelial sheet covers the folds and is underlain by a lamina propria/submucosa of loose connective tissue, which continues into the folds. There is no muscularis mucosae. The tunica muscularis is arranged as inner circular (CL) and outer longitudinal layers (LL). CC, cell cluster; m, nucleus of mesothelial cell. **b:** The lamina propria/submucosa contains many blood vessels (c). Some capillaries are closely associated with epithelium (E) arrowhead, basal lamina; CC, cell cluster; CL, inner circular layers of tunica muscularis; g, granular cell. **a,b**: Semithin section, 0.1% Toluidine blue.

Fig. 2. *Proteus anguinus.* **a:** The surface epithelium with interspersed goblet cells (GC). Scattered granular cells (g) and compact cell aggregations (CC) are also visible. AC, absorptive cell; L, lymphocyte. Pantin method. **b:** Nematodes (N) attached to the intestinal epithelium (E). LP/S, lamina propria/submucosa. Periodic acid-Schiff counterstained with hematoxylin.

Fig. 3. *Proteus anguinus.* **a:** The PAS reaction demonstrating neutral mucin is strongly positive in the enterocyte brush border (arrowhead) and goblet cells (GC). AC, absorptive cell; c, capillary. Periodic acid-Schiff counterstained with hematoxylin. **b:** Goblet cells (GC) contain carboxylated mucin. CC, cell clusters; F, fibroblast. Alcian blue, pH 2.5, counterstained with hematoxylin.



Figs. 4–7. Anterior portion of small intestine of *Proteus anguinos*. TEM micrographs.

Fig. 4. *Proteus anguinus.* The enterocyte. TEM. **a:** Apical cytoplasm with uniform microvilli (Mv) and numerous mitochondria (M), electron-dense granules (G), vesiculated rough endoplasmic reticulum (RER), and rare multivesicular bodies (Mt) beneath the terminal web (TW). **b:** Frequent invaginations of the plasma membrane at microvillar bases (arrowhead). The lateral junctional complex joining neighboring cells is visible. D, desmosomes; Tj, tight junction. **c:** Detail of the supranuclear cytoplasm with short cisternae and vesicles of rough endoplasmic reticulum (RER), scattered granules, and Golgi apparatus (GA).

Fig. 5. *Proteus anguinus.* **a:** Desmosomes (D) along the lateral plasma membranes of two adjacent enterocytes. T, tonofilaments. **b:** A considerable degree of interdigitation of lateral plasma membranes (arrowhead) occurs beneath the junctional complex.

Fig. 6. *Proteus anguinus.* Irregular bulges of the cytoplasm between groups of microvilli. **a:** Detail of the apical bulges (arrowheads). Some appear pinched off from the surface. At the upper left corner of the figure a membrane-bound cytoplasmic fragment is visible in the lumen. Mv, microvilli; TW, terminal web. **b:** The apical bulge (arrowhead) often includes vesicles and small dark granules (G). M, mitochondria.

Fig. 7. *Proteus anguinus.* Smooth muscle cells (MC) deep to the basal lamina (*). **a:** Longitudinally sectioned smooth muscle cells. **b:** Cross-sectioned smooth muscle cells. Dark, amorphous extracellular material with collagen fibrils separates individual muscle cells. M, mitochondria of enterocyte; N, nucleus of muscle cell. Insert: High magnification of **b**. Collagen fibrils at the upper left corner are seen as small dots between cells. Arrow, pinocytotic vesicles; M, mitochondria.

Goblet cells are numerous and are scattered throughout the gut epithelium. The nuclei of the cells are elliptical and lie basally in the epithelium. On the apical surface, irregular microvilli are present. The cytoplasm between the nucleus and the apical border is distended with mucous granules (Fig. 8a) that are enveloped by a very fine membrane that frequently appeared fragmented. Towards the apical surface of the cells, some of the mucous granules are larger. Many of them coalesce and lose their surrounding membranes. Apical mucus appeared to extrude into the lumen and remnants of the plasma membranes are visible above it (Fig. 8a). The lamellae of the Golgi apparatus and rough endoplasmic reticulum are located between the bulging masses of mucus and the nucleus (Fig. 8b). Other cytoplasmic organelles, including mitochondria and free ribosomes, are located at the lateral and inferior cell margins and between mucous granules. The junctional complexes between goblet cells and absorbing cells do not differ from those found between columnar absorbing cells.

Compact cellular aggregations that are present under the epithelial lining (Figs. 1a,b, 2a, 9a) generally have elliptical nuclei and, frequently, a well-developed nucleolus. The nuclear surface seemed somewhat irregular. In the cytoplasm, numerous mitochondria and many free ribosomes are present (Fig. 9b). Cytoskeleton bands of microfilaments are scattered across the cytoplasm. Multivesicular bodies occur between mitochondria. The short cisternae of rough endoplasmic reticulum are located in the basal cytoplasm and around the nuclei (Fig. 9b,c). The Golgi apparatus is not prominent; it forms a short lamellar structure and vesicles are present in the vicinity of the plasma membrane. The lateral plasma membrane of adjacent cells interdigitates. Desmosomes along the lateral plasma membrane are numerous. Cell aggregates are lined by a thin, wavy basal lamina (Fig. 9a,c)

The intestinal mucosa also contains numerous endocrine-like cells scattered among the enterocytes close to the basal lamina. They are characterized by a clear cytoplasm, numerous electron-dense granules, large granules, and a few lipid droplets (Fig. 10).

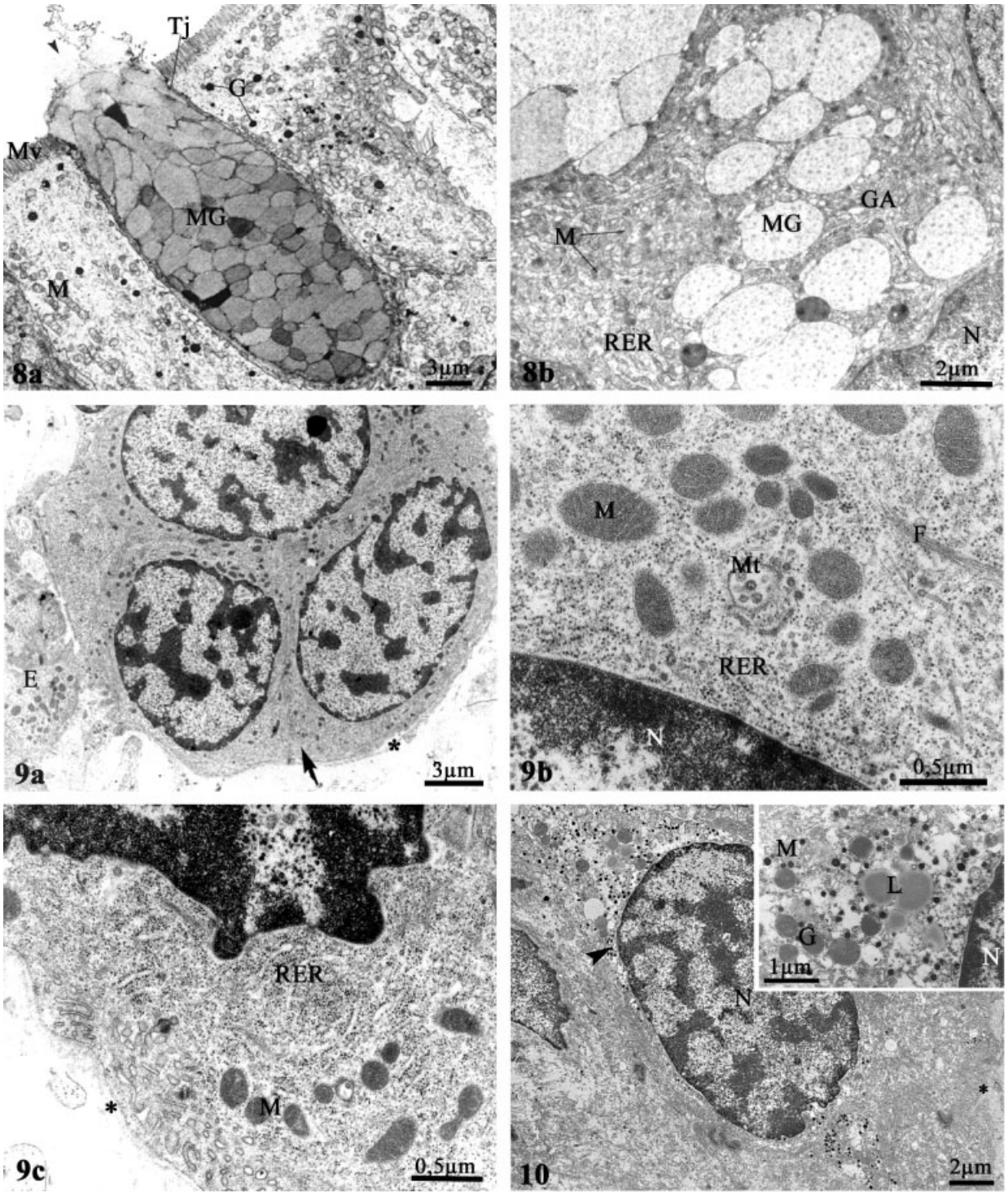
DISCUSSION

The intestine of *Proteus anguinus* is anatomically and histologically simply organized when compared to that of mammals and resembles that described in fish and other amphibians (Noble, 1955; Trier, 1963; Trier et al., 1965; Bonneville and Weinstock, 1970; Duellman and Trueb, 1986; Caceci and Hrubec, 1990; Stevens, 1990; Gargiulo et al., 1998).

The study of the *Proteus anguinus* gut morphology by light and transmission electron microscopy disclosed the presence of compact cellular aggregates situated under the epithelial lining. Among vertebrates such intestinal cell aggregates are characteristic only in urodele amphibians (Dawson, 1927; Noble, 1955; Andrew, 1963). Early authors believed they were secretory glands discharging into the gut lumen, while others proposed a replacement function for the surface epithelium. These reports were based only on light microscopy. We describe their fine structure in *P. anguinus* for the first time. Their histology is very similar to those in *Necturus maculosus* (Dawson, 1927), with only a few differences. No lumen or secretory granules as described in *N. maculosus* are present. Also, the PAS- or AB-positive reactions did not occur. Furthermore, we did not observe ultrastructural characteristics of secretory activity in cluster cells. The cluster cell fine structure of *P. anguinus* resembles the undifferentiated cells in human small intestinal crypts (Trier, 1963) and the basal cells of frog esophageal epithelium (Gallego-Huidobro et al., 1992) and could be responsible for the renewal of intestinal epithelium. Mitotic figures which occur very rarely in the *P. anguinus* clusters could be justified by replacement needs of the intestinal epithelium. A slow proliferation rate is expected due to the well-known inverse relationship between genome size and cell proliferation rate, and also an inverse relationship between genome size/cell size and metabolic rate (Licht and Lowcock, 1991; Gregory, 2001). *Proteus anguinus* has very large genome size (cit. in Licht and Lowcock, 1991), enormous cell size, and also extraordinarily low metabolism (Vandel, 1965; Hüppop, 1985; Hervant et al., 2000, 2001). In general, the vertebrate intestinal epithelium has a rapid renewal rate (Fawcett, 1986).

Another feature of the intestinal mucosa of *Proteus anguinus* is the presence of many granular cells in the lamina propria/submucosa, within the epithelium, and between cell aggregations. They seem similar to eosinophilic granular cells described in the helminth-infected intestines of pike and in different salmonides (Reite, 1996, 1997). The presence of eosinophilic granular cells also occurred in other organs, e.g., the spleen, liver, and kidney of the affected fish (Kent et al., 1993). A large number of similar cells have also been observed in the epidermis of brown trout with ectoparasitic infections (Blackstock and Pickering, 1980). Fish eosinophilic granular cells show cytochemical and functional similarities to mammalian mast cells (Kent et al., 1993; Reite, 1997). The presence of large numbers of granular cells in the *P. anguinus* intestine seems to be associated with nematode infection and possibly constitutes a humoral defense mechanism.

The histochemical AB-PAS studies of neutral and carboxylated acid mucopolysacharides in the *Proteus anguinus* intestinal mucosa revealed localization only in the apical brush border of enterocytes and in goblet cells. Goblet cells also stained strongly with AB at pH 2.5, thus revealing the presence of acid-carboxylated MS and neutral MS. The presence



Figs. 8–10. Anterior protions of small intestine of *Proteus anguinus*. TEM micrographs.

Fig. 8. *Proteus anguinus.* Goblet cell. **a:** Discharging goblet cell between enterocytes. Its content is extruded after bursting of the apical plasma membrane (arrowhead). G, electron-dense granules; M, mitochondria; MG, mucous granules; Mv, microvilli; Tj, tight junction. **b:** Supranuclear region of a goblet cell. Rough endoplasmic reticulum (RER) and Golgi lamellae (GA) are located between the bulging masses of mucus and the nucleus (N). The nucleus of an enterocyte is to the right. M, mitochondria, MG, mucous granules.

Fig. 9. *Proteus anguinus.* Aggregations of cells under the epithelial lining. **a:** The three cells constitute a cell cluster (arrow). The nuclei frequently contain well-developed nucleoli. *, basal lamina; E, enterocyte. **b:** High magnification of the cytoplasm of the cluster cells. Numerous mitochondria (M) and many free ribosomes are present. F, microfilaments; Mt, multivesicular body; N, nucleus; RER, rough endoplasmic reticulum. **c:** The basal part of a cell cluster with rough endoplasmic reticulum (RER) and mitochondria (M). Cell cluster is lined by basal lamina (*).

Fig. 10. *Proteus anguinus.* Endocrine cell. Small electron-dense granules (arrowhead) are present in the cytoplasm. N, nucleus; *, basal lamina. Insert shows numerous small electron-dense granules, large granules (G), and lipid droplets (L). M, mitochondria; N, nucleus.

of mucosubstances in the *P. anguinus* intestinal mucosa is comparable with those described in amphibians and other groups of animals (Suganuma et al., 1981). In some fishes a PAS-positive reaction in the supranuclear zone of enterocytes has been described (Caceci and Hrubec, 1990), but in *P. anguinus* it is present only on the glycocalyx.

Electron microscopy revealed an irregular arrangement of absorptive cell microvilli with irregular cytoplasmic protrusions between groups of microvilli. These protrusions often include ribosomes, vesicles, and occasionally dark granules, and sometimes appeared to be pinched off from the apical surface. Furthermore, the membrane-bound cytoplasmic fragments occasionally appeared free in the intestinal lumen. The apical budding of cytoplasmic fragments has been described in the crypt epithelium of the human intestine, where it may represent a special type of apocrine secretion (Trier, 1963). Apocrine secretion is also known in the intestine of some insects (Welsch and Storch, 1976). However, further studies are needed to clarify the significance and functional role of these apical protrusions in *Proteus anguinus* intestine.

The ultrastructure of the goblet cells in *Proteus anguinus* corresponds to that of goblet cells in other vertebrates (Trier, 1963; Hollman, 1963; Freeman, 1965; Trier et al., 1965; Bonneville and Weinstock, 1970; Caceci and Hrubec, 1990). The mechanism of extrusion of goblet cells has been repeatedly debated. Many researchers have stated that the rupture of the surface plasma membrane is the mechanism of mucus discharge (Hollmann, 1963), but they did not refer to details in the morphological events at discharge, especially the fate of the membrane surrounding the mucus droplets and their relationship with the cell surface membrane. Freeman (1965) reported that in apocrine secretions, some cytoplasmic organelles and cytoplasmic ground substances are expelled among the mucous droplets. Kurosumi et al. (1981) discussed the possibility of different mechanisms of secretion discharge in the rat jejunum. Besides exocytosis, they found an apocrine mechanism for extrusion of mucous substances in goblet cells that might possibly occur simultaneously in goblet cells. In *P. anguinus*, the cytoplasm between the nucleus and the apical border was distended with mucous granules that appeared to coalesce and swell prior to secretion. After bursting, the plasma membrane apical mucus seems to be extruded by exocytosis.

In *Proteus anguinus* the arrangement of the lateral plasma membranes between enterocytes was comparable to that described in other amphibians and mammals (Trier, 1963; Trier et al., 1965; Bonneville and Weinstock, 1970; Ross et al., 1989; Caceci and Hrubec, 1990). The typical junctional complex region of the cell is situated just below the free surface. Along the remainder of the lateral plasma membrane many desmosomes are present. In the deep portion the lateral plasma membranes interdigitate to a considerable degree. These infoldings increase the lateral surface of the cell and are particularly prominent where there is rapid fluid transport and, together with numerous desmosomes, maintain epithelial mechanical integrity (Bonneville and Weinstock, 1970).

In *Proteus anguinus*, like in lampreys, teleosts, and anurans (Welsch and Storch, 1976), a typical muscularis mucosae is absent. A layer of closely packed smooth muscle cells may frequently be found under the epithelium of *P. anguinus*. In cross-sections these muscle cells with amorphous extracellular material and collagen fibrils appear to constitute a net-like structure beneath the basal lamina of enterocytes. This net-like structure seemed to be very closely associated with the epithelium and may play supporting and strengthening roles in the gut wall and could also serve a contractile function.

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