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CYTOLOGY AND POLYSACCHARIDE CYTOCHEMISTRY OF THE GILL OF THE AMERICAN EEL, *ANGUILLA ROSTRATA*

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Much work has been published on the gills of teleosts, (Vickers, 1961; Munshi, 1964; Steen and Kruysse, 1964; Hughes and Grimstone, 1965; Newstead, 1967; Hughes and Wright, 1970). Morgan and Tovell (1973) and Morgan (1974) described the structure and development of secondary lamellae in gills of trout. Work on eel gills has included cytological studies, (Ogawa, 1962; Yamada and Yokote, 1975), electron microscopic investigations of gill fine structure (Mizuhira, Amakawa, Yamashina, Shirai, and Utida, 1969), and studies of osmotic adaptation of eels to freshwater and seawater for the Japanese eel, *Anguilla japonica*. Keys and Willmer (1932) described chloride-secreting cells in the common eel, *Anguilla vulgaris;* Getman (1950) and Doyle and Epstein (1972) reported on osmotic effects and adaptive changes of chloride cells in the American eel, *Anguilla rostrata*.

The present study describes cytological details of gill filaments and secondary lamellae of freshwater-adapted early juvenile and adult American eels. Arrangement and morphology of epithelium, gill rays, mucous-secreting cells, pillar cells, and blood spaces within secondary lamellae are described and illustrated. Polysaccharide cytochemistry as revealed by periodic-acid–Schiff (PAS) staining as well as Alcian blue reactions at various pH values and with increasing concentrations of $MgCl_2$ of all cell types and connective tissue are described; results are compared with similar tissues in the *Anguilla* species, and in teleosts in general.

Materials and Methods

Fresh water-adapted juvenile and adult eels were obtained from the Aquaculture Laboratories, Mercer Generating Station, Trenton, New Jersey. Animals were killed by decapitation and gills were excised and fixed in Davidson's solution at room temperature for 24 hr. Tissues were processed and embedded in paraffin (Humanson, 1972); sections were cut at 5 μm.

Stains used were hematoxylin and eosin, periodic-acid–Schiff (PAS) (McManus, 1948), Feulgen Picro-Amido-black (modified from Farley, 1969), Alcian blue 8GX (Gurr, London) at pH 0.5 (Lev and Spicer, 1964), Alcian blue pH 2.6, and Alcian blue pH 5.7 with post-treatment in ascending concentrations of $MgCl_2$ (Scott and Dorling, 1965; Mowry, 1970).

Slides were examined and photographed with the Zeiss Photomicroscope II using Kodak SO-410 Monochrome Photomicrography film with a Kodak #66 Wratten gelatin filter.

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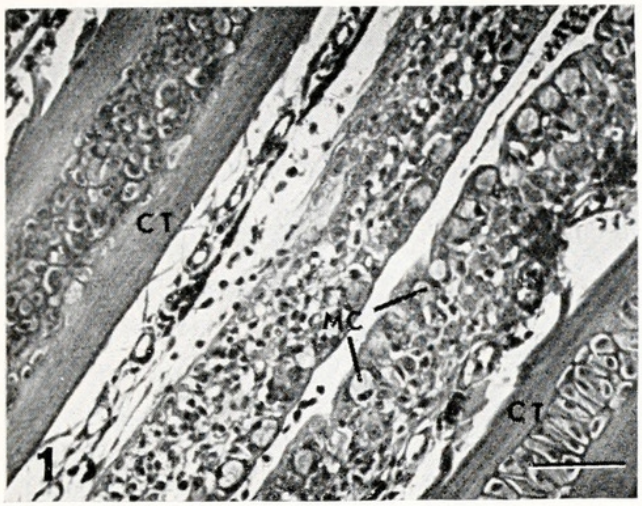

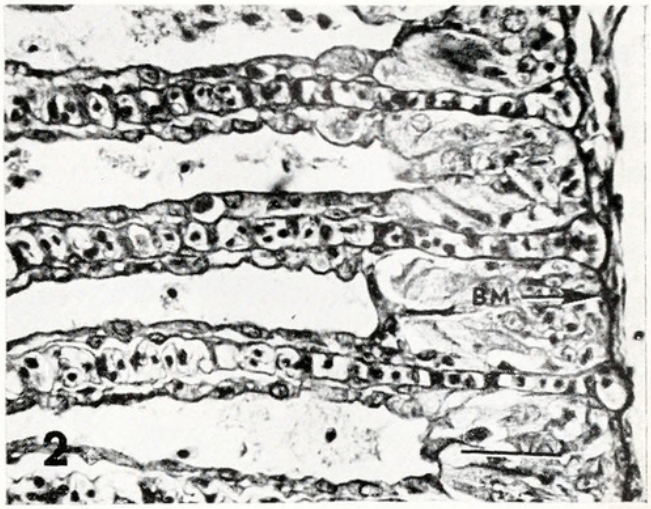

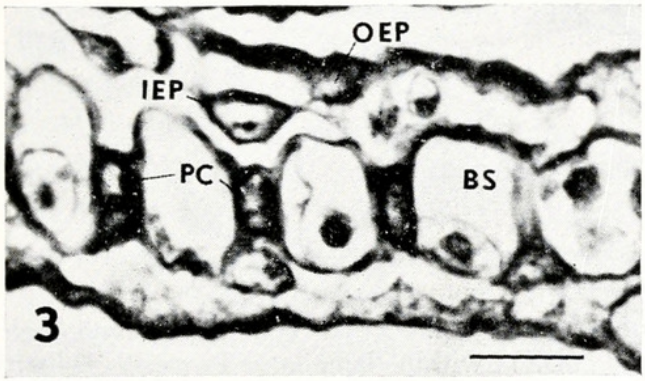

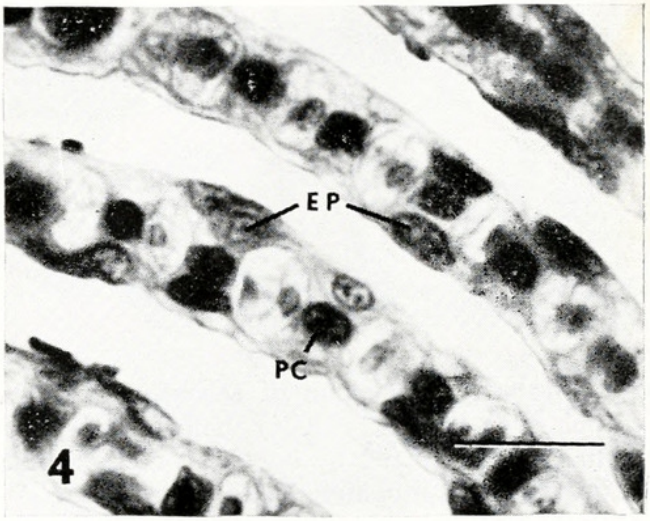

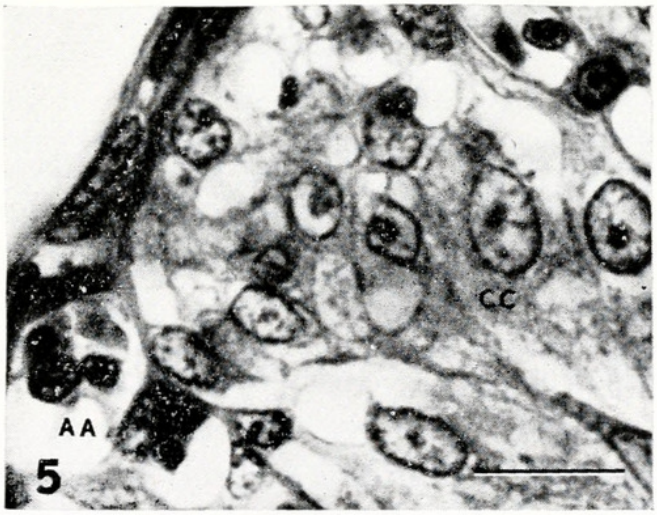

FIGURE 1. Cartilaginous gill rays (CT) support filaments of the eel gill. Mucus-secreting cells (MC) appear at bases of filaments. Hematoxylin and eosin stained; scale bar is 25 μm.

FIGURE 2. Basement membrane (BM) in gill filament stained by PAS. Scale bar is 25 μm.

FIGURE 3. Two-celled layered epithelium of secondary lamella of adult eel. Nuclei of inner cell layer (IEP) shown over pillar cell body (PC); pillar cells delimit blood spaces (BS) in lamellae. Note nucleus of outer layer of epithelium (OEP); scale bar is 10 μm.

FIGURE 4. Secondary lamellae of early juvenile eel, showing single epithelial layer (EP) with nuclei over pillar cells (PC). Blood cells crowd blood spaces between pillar cells. Hematoxylin and eosin stained; scale bar is 10 μm.

FIGURE 5. Chloride cells (CC) at the base of lamellae near afferent lamellar artery (AA). Note the prominent nucleolus, visible with Feulgen Picro-Amido-Black stain; scale bar is 10 μm.

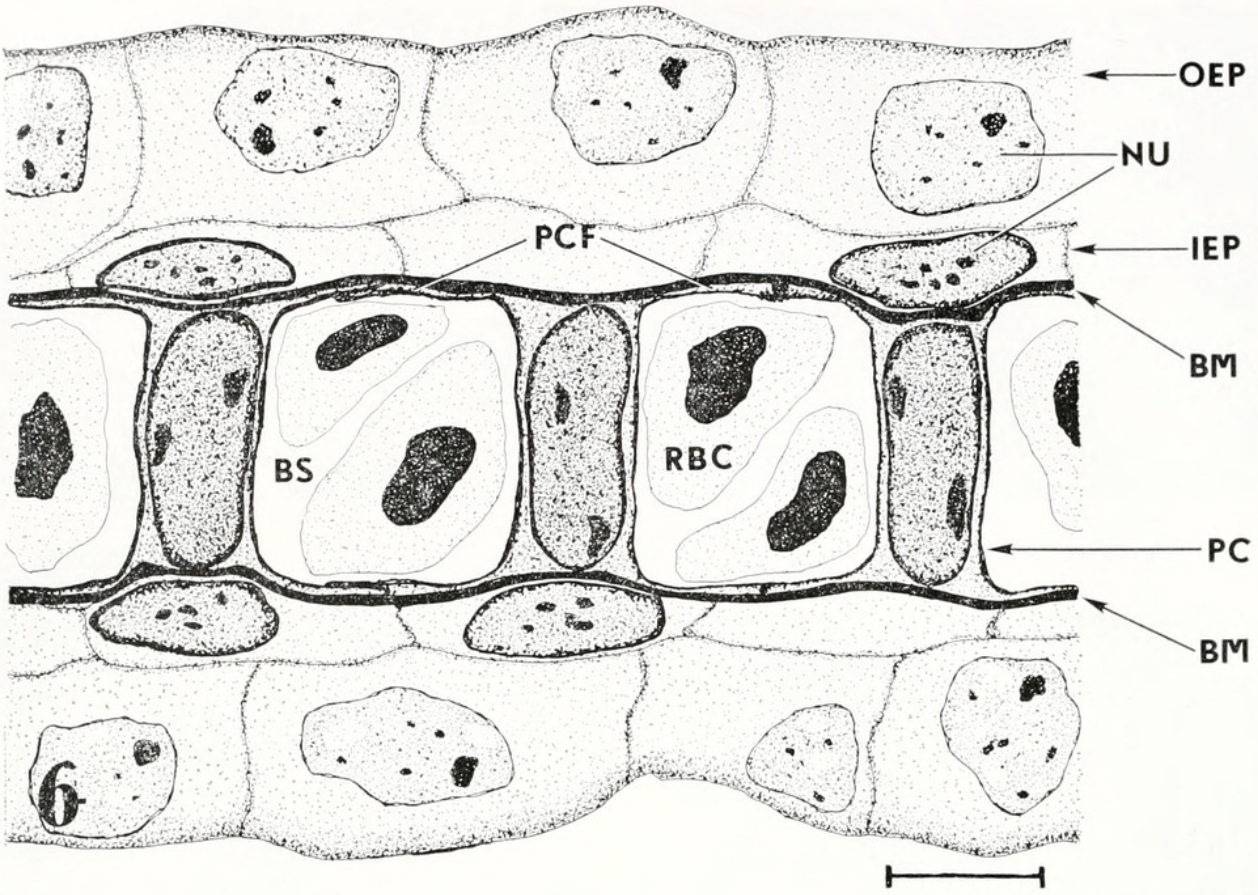

FIGURE 6. Diagram of secondary lamella of an adult eel. Pillar cells (PC) and their cytoplasmic flanges (PCF) delimit blood spaces (BS) within lamella. Two-cell layered epithelium supported by basement membrane (BM) covers secondary lamella. Nuclei (NU) of the inner epithelium (IEP) are more flattened than those of the outer epithelial cells (OEP) and lie over pillar-cell bodies. Scale bar is 5 μm.

RESULTS

General morphology

Gills of the eel consist of four bony gill arches on either side of the pharynx. Gill arches bear two rows of flattened filaments supported for approximately two-thirds of their length by cartilagenous gill rays. Filaments each bear two rows of secondary lamellae.

Cytology and cytochemistry

Gill filaments contain a wide, centrally-located gill ray (Fig. 1) which stains intensely in PAS, Alcian blue pH 0.5, and Alcian blue pH 5.7 with high concentrations of $MgCl_2$ (Table I). Large mucus-secreting cells are found at both base and tip of filaments; cell secretions stain prominently with PAS reactions as well as with Alcian blue pH 2.6. They appear unstained in Alcian blue pH 0.5. A well-defined basement membrane supports the filament epithelium (Fig. 2); this connective tissue encircles afferent lamellar arteries and extends into secondary lamellae as the basement membrane for lamellar epithelia. It is stained intensely by PAS, Alcian blue pH 0.5, and Alcian blue pH 5.7 with concentrations of $MgCl_2$ up to 1.30 M (Table I).

Blood supplied by afferent lamellar arteries flows through blood spaces delimited by pillar cells and their cytoplasmic flanges (Figs. 3, 6). Pillar cell nuclei typically contain two small areas of chromatin material with fine granular material distributed around the margins of the nuclear envelope. Nuclei occupy nearly the entire volume of the cell, with a width of approximately 4 μm and a height of 8 μm.

A two-layered epithelium covers secondary lamellae in adult eels, supported by a basement membrane continuous along pillar-cell flanges. Nuclei of inner epithelial layers frequently lie directly over pillar-cell bodies (Figs. 3, 6), with nuclei flattened against the basement membrane. Several dense areas of chromatin appear near the center of the nucleus, with smaller areas of chromatin adhering to the nuclear envelope. Nuclei of the outer epithelial layer appear more spherical than inner epithelial nuclei; one dark nucleolus is usually visible surrounded by smaller chromatin masses (Fig. 6). Cytoplasm of outer epithelial cells is much less dense than that of inner epithelial cells, with distal portions of cytoplasm staining lightly with Alcian blue pH 2.6. A single-layer epithelium covers secondary lamellae in early juvenile eels (Fig. 4); epithelial nuclei are located over pillar-cell bodies.

Chloride-secreting cells surround afferent lamellar arteries, crowded in the "v" between adjacent secondary lamellae (Fig. 5). Cells are large, with a granular, eosinophilic cytoplasm and a large nucleus with a prominent nucleolus surrounded by small regions of chromatin material (Fig. 5). Crowding of cells in this area of filaments causes them to be elongated, with nuclei typically located at the base of cells. Chloride-cell cytoplasm stains heavily in PAS reactions and exhibits some reaction to Alcian blue pH 2.6, especially at distal portions of cells. In Alcian blue pH 0.5 there is little reaction in chloride cells; further, in Alcian blue pH 5.7, cells reach their extinction point at a critical electrolyte concentration of 0.030 M $MgCl_2$ (Table I).

Discussion

Cartilage supporting gill filaments shows heavy concentrations of sulfated mucopolysaccharides, staining with Alcian blue pH 0.5 (Lev and Spicer, 1964). Polysulfate groups stain selectively as pH is lowered to a point below the pH of carboxyl groups; sulfate groups still dissociated are free to bind the cationic dye. Mowry (1963) reported that, at low pH, hyaluronic acid, heparin, and chondroitin readily stain in Alcian blue; the color reactions are identical but chondroitin comprises only a minor portion of extracellular material. However, the sulfate ester derivatives, chondroitin sulfate A and chondroitin sulfate C, are major structural components of vertebrate cartilage (Lehninger, 1970). In Alcian blue pH 5.7 cartilage reacts intensely when treated with high concentrations of $MgCl_2$ (Table I). Mowry (1970) noted that binding of the cationic dye in 0.30 M or higher concentrations of $MgCl_2$ indicated the presence of sulfated polyanions; the higher the ionic strength of Mg^{2+} the higher the degree of sulfation. Addition of $MgCl_2$ dissolves the stained complexes formed by the polyanion's reaction with Alcian blue (Scott and Dorling, 1965). The critical electrolyte concentration (lowest concentration of Mg^{2+} at which a given polyanion is no longer stainable) is 1.20 M $MgCl_2$ for gill-ray cartilage. The presence of highly sulfated chondroitin

TABLE I

Results of cytochemical tests.

Stain	Mucous cells	Basement membrane	Cartilage	Chloride cells
Hematoxylin and eosin	+	++	+++	+++
PAS	+++++	++++	+++	+++
Feulgen-Picro Amido-black	++	+++	+++	++++
Alcian blue pH 0.5	----	+++++	++++	++
Alcian blue pH 2.6	++++	++	+++	++
Alcian blue pH 5.7 with				
0.00 M $MgCl_2$	+++	+++	+++	++
0.05 M $MgCl_2$	+++	+++	+++	++
0.10 M $MgCl_2$	+++	+++	+++	+
0.20 M $MgCl_2$	---	+++	+++	+
0.30 M $MgCl_2$	---	+++	+++	---
0.40 M $MgCl_2$	---	+++	+++	---
0.50 M $MgCl_2$	---	+++	+++	---
0.60 M $MgCl_2$	---	+++	+++	---
0.70 M $MgCl_2$	---	++++	+++	---
0.80 M $MgCl_2$	---	++++	+++	---
0.90 M $MgCl_2$	---	++++	++	---
1.00 M $MgCl_2$	---	++++	++	---
1.10 M $MgCl_2$	---	++++	++	---
1.20 M $MgCl_2$	---	+++++	++	---
1.30 M $MgCl_2$	---	+++++	---	---

---, No reaction; ++, weak reaction; +++++, strong reaction.

derivatives in cartilage accounts for the intensity of reactions to Alcian blue pH 0.5 and Alcian blue pH 5.7 with high concentrations of $MgCl_2$.

Mucous cells found on filaments at both base and tip have secretions which exhibit properties of acid mucopolysaccharides with vicinal hydroxyl as well as carboxyl groups. Cell secretions stain heavily in Alcian blue pH 2.6, with no staining in Alcian blue pH 0.5. Further, mucous cell secretions reach their extinction point at a critical electrolyte concentration of 0.20 M $MgCl_2$ in Alcian blue pH 5.7 series (Table I). Mowry (1963) stated that basophilia in a 0.30 M or lower concentrations of $MgCl_2$ indicated the presence of polycarboxylates. In addition, Alcian blue is a reliable and sensitive test for carbohydrate polycarboxylates.

Some variation in the exact chemical structure of mucus secretions apparently exists within the *Anguilla* species. Yamada and Yokote (1975) reported the presence of sulfated mucopolysaccharides in mucous cells of the Japanese eel, *Anguilla japonica,* and described staining of cells in a range from 0.10 to 0.60 M $MgCl_2$; those secretions show a higher degree of sulfation than those of *Anguilla rostrata* using similar procedures (Table I). Mucous cells always appear at the base and tip of gill filaments in the eel, as described for trout (Morgan and Tovell, 1973) and many other teleosts (Newstead, 1967; Hughes and Wright, 1970).

Electron microscope studies on epithelial basement membranes in gills from rainbow trout (Morgan and Tovell, 1973) and other teleosts (Hughes and Grim-

stone, 1965; Newstead, 1967) showed a close association of basement membrane and pillar-cell flanges. In the present study, epithelial basement membrane is also in contact with cytoplasmic flanges of pillar cells (Fig. 6); it also surrounds afferent lamellar arteries at bases of secondary lamellae (Fig. 2) and supports gill-filament epithelia. The basement membrane shows heavy concentrations of sulfated mucopolysaccharides, staining intensely in Alcian blue pH 0.5; in Alcian blue pH 5.7 the basement membrane can also be distinguished when treated with concentrations of $MgCl_2$ up to 1.30 M (Table I). Magnesium chloride provides better discrimination of polyanions than other salts (Scott and Dorling, 1965) and the critical electrolyte concentration is a reflection of the type of polyanions present, as well as the concentration. In *Anguilla rostrata,* basement membranes exhibit an even greater degree of sulfation than gill-ray cartilage (Table I).

Blood supplied to secondary lamellae is channeled through blood spaces formed by overlapping pillar cell flanges (Fig. 6). These blood spaces appear to be somewhat similar to capillaries; however, no endothelium could be discerned. Studies on trout (Morgan and Tovell, 1973) and other teleosts (Hughes and Grimstone, 1965; Newstead, 1967; Hughes and Wright, 1970) indicated that blood spaces in secondary lamellae were entirely delimited by extensions of pillar-cell cytoplasm. Morgan (1974) further stated that, developmentally, pillar cells originate directly from mesenchymal cells, and not from cells having affinities to endothelial cells.

Above the pillar cells and basement membrane a two-cell, layered epithelium covers secondary lamellae in adult eels (Fig. 3). Nuclei of inner epithelial cells lie directly over pillar cell bodies (Figs. 3, 6); similar arrangements have been reported to exist in trout (Morgan and Tovell, 1973) and many other teleosts (Newstead, 1967; Hughes and Wright, 1970). Hughes and Grimstone (1965) suggested that location of epithelial nuclei over pillar cell bodies could be adaptive, as little gas exchange would be expected at those points. In the present study, the average water-to-blood distance for adult eels is 5 to 8 μm, but at points where epithelial nuclei are located, the distance is nearly doubled from the free edge to the blood spaces. In early juvenile eels (2–3 g body weight) there is only one epithelial layer in secondary lamellae; nuclei of these cells invariably lie over pillar cell bodies (Fig. 4). Here, the water-to-blood distance is only 3 to 4 μm.

Keys and Willmer (1932) reported only a single layer of epithelium in secondary lamellae of the common eel, *Anguilla vulgaris,* but they gave no information concerning the size of animals used in their study. From the evidence of the two cell layers found in *Anguilla rostrata* and other teleosts, it is apparent that, as the animals mature, a second cell layer appears and the water-to-blood distance increases slightly.

Chloride-secreting cells appear in clusters at bases of secondary lamellae, in close proximity to afferent lamellar arteries (Fig. 5). Cytoplasm of chloride cells exhibits high concentrations of carbohydrate polycarboxylates staining intensely with PAS reactions (Table I) and Alcian blue pH 2.6. In the latter, it is interesting to note that staining is limited to the distal portions of chloride cells; basal portions of cells show no reaction to the stain.

Appearance of chloride cells near afferent lamellar arteries is well known for the Japanese eel, *Anguilla japonica* (Ogawa, 1962; Shirai and Utida, 1970; Utida,

Kamiya, and Shirai, 1971) as well as other teleosts (Vickers, 1961; Munshi, 1964; Newstead, 1967). Getman (1950) suggested that in *Anguilla rostrata,* the location of chloride cells in interlamellar epithelium allows access to a good blood supply and insures exposure to the environment for salt secretion. Shirai and Utida (1970) studied the development and degeneration of chloride cells when eels were adapted to fresh water and to sea water; the secretory mechanism was examined by Utida, *et al.* (1971) to determine the relationship between Na^+-K^+-ATPase and numbers of chloride cells in seawater-adapted animals. In the present study, animals were freshwater adapted, and chloride cells were similar in appearance, location, and stainability to those of freshwater teleosts (Munshi, 1964).

In conclusion, we would like to thank Dr. Joseph A. Vena for his advice and assistance with staining procedures, and Mr. Gerald Nicholls for his suggestions and cooperation on photographic techniques.

Summary

Gills of the American eel were found to be morphologically similar to those of other members of the *Anguilla* species, and to teleosts in general. Gill filaments contain cartilagenous gill rays rich in polysulfates, and stain intensely in PAS and Alcian blue pH 0.5.

Pillar cells delimit blood spaces within secondary lamellae; they were found to be covered by a thin connective tissue supporting a single-layered epithelium in early juvenile animals and a two cell, layered epithelium in adult eels. In the latter, nuclei of the outer layer were much larger and not as densely stained as those of the inner epithelial cells, whose nuclei appeared flattened over pillar-cell bodies.

Basement membranes supporting epithelia of secondary lamellae and gill filaments exhibited heavy concentrations of sulfate groups shown by reactions in Alcian blue pH 0.5 and Alcian blue pH 5.7 with high concentrations of $MgCl_2$.

Chloride cells were found in the interlamellar epithelium, especially surrounding afferent lamellar arteries. They had a granular, eosinophilic cytoplasm with carbohydrate polycarboxylates concentrated in distal portions of cells; nuclei had a prominent, centrally-situated nucleolus surrounded by small chromatin masses.

Results of cytochemical tests for all cell types were reported, and information correlated to previous findings on eel gills in particular, and teleost gills in general.

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