Correlated Histochemical and Electron Microscopic Studies of the Esophageal Epithelium in the Salamander, *Hynobius nebulosus*

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Summary. The esophageal epithelium of the adult salamander, *Hynobius nebulosus*, was studied by histochemistry, including periodic acid-Shiff (PAS), alcian blue both at pH 2.5 and pH 1.0, and alcian blue (pH 2.5)-PAS with or without neuraminidase-digestion, and scanning and transmission electron microscopy.

The epithelium was columnar in type, comprising partly pseudostratified and partly two-layered architecture; it consisted mainly of ciliated cells, goblet cells, and basal cells. The ciliated cells consisted of two types, light and dark: both types, especially the latter, frequently contained numerous small mucous granules in their apical portion. Ciliated-mucous cells were also occasionally present. Immature ciliated cells were frequently found. Goblet cells were divided into two types: Type I cells possessed electron lucent mucous granules, which frequently contained dense specific inclusions, and frequently bulged into the lumen; Type II cells had moderately electron-dense mucous granules with no inclusions and a conical apex which did not exceed the level of the lumen. The Type I cells were closely distributed throughout the esophagus, while the Type II were mainly dispersed in the cranial portion, remarkably decreasing in the caudal portion. Correlated histochemical and electron microscopic observations suggested that, in the Type I cell, mucous granules contain acid mucosubstances, while in the Type II, they possess neutral mucosubstances, and that in the Type I cell mucous granules consist of sialic acid-containing glycoproteins and their swollen portions are more highly sulfated than the non-swollen ones.

Although many light microscopic studies on the amphibian esophagus are available (for review PERNKOPF and LEHNER, 1937), the fine structure of this organ has not been previously documented. The esophageal epithelium of amphibians, unlike that of mammals, consists mainly of a ciliated epithelium mingled with goblet cells, and may have a more complex function than mere transport of food from the pharynx to the stomach. Therefore, a thorough study of its fine structure seems both necessary and valuable.

By combining light microscopic histochemistry and scanning and transmission electron microscopy, this study aims at the elucidation of the ultrastructural morphology of the esophageal epithelium in the salamander, *Hynobius nebulosus*. 

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MATERIALS AND METHODS

Adult male salamanders, *Hynobius nebulosus*, ranging from 9 to 12 cm in total length were used for study.

For light microscopy, the digestive tract from the pharynx to the stomach was fixed with 10% neutralized formalin and then embedded in paraffin. Dewaxed serial transverse sections (6 μm) were stained with hematoxylin and eosin, periodic acid-Schiff (PAS) with or without amylase digestion (McMANUS, 1948), alcian blue both at pH 2.5 and pH 1.0 (LEV and SPICER, 1964), or alcian blue (pH 2.5)-PAS (MOWRY and WINKLER, 1956). In addition, some sections were stained with alcian blue (pH 2.5)-PAS following digestion with neuraminidase at 37°C for 6 hr (SPICER and DUVENCI, 1964).

For scanning electron microscopy, the esophagus was opened laterally using small scissors and the internal surface was washed by saline to remove mucous. After being fixed with half-strength Karnovsky's fixative for 3 hr, the tissues were immersed in a mixture of 2% tannic acid and 2.5% glutaraldehyde for 3 hr, and then postfixed in 2% osmium tetroxide for 1 hr. They were dried by the critical point method and sputter-coated with gold-palladium. The epithelial surfaces were observed in a JSM-35C scanning electron microscope at 15 kV.

For transmission electron microscopy, the esophagus with surrounding tissues was cut at the level of the bifurcation of the lung sac into two portions: a cranial portion closely attached to the dorsal surface of the laryngotracheal cavity and a caudal portion liberated in the abdominal cavity. Each portion was briefly fixed in half-strength Karnovsky's fixative and then cut into three pieces: its middle third was fixed in the same fixative for 2 hr. The tissues were rinsed overnight in a buffer containing sucrose and then postfixed with 1% osmium tetroxide in phosphate buffer for 1 hr. Following dehydration in ethanol, the tissues were embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and then examined under a JEM-T8 or 100B electron microscope. For correlated light microscopic studies, thick sections were also cut from Epon blocks and stained either with 1% toluidine blue, or with alcian blue (pH 2.5)-PAS after removing the resin in absolute ethanol saturated with KOH.

RESULTS

The esophagus of *Hynobius nebulosus*, is a roughly straight tube extending from the pharynx to the stomach. Its cranial two thirds are closely attached to the dorsal surface of the laryngotracheal cavity and a caudal portion liberated in the abdominal cavity. Each portion was briefly fixed in half-strength Karnovsky's fixative and then cut into three pieces: its middle third was fixed in the same fixative for 2 hr. The tissues were rinsed overnight in a buffer containing sucrose and then postfixed with 1% osmium tetroxide in phosphate buffer for 1 hr. Following dehydration in ethanol, the tissues were embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and then examined under a JEM-T8 or 100B electron microscope. For correlated light microscopic studies, thick sections were also cut from Epon blocks and stained either with 1% toluidine blue, or with alcian blue (pH 2.5)-PAS after removing the resin in absolute ethanol saturated with KOH.

Light microscopy

In light-microscopic cross sections, the mucous membrane of the esophagus shows eight to twelve folds, which gradually increase in height caudally (Fig. 2). The epithelium is columnar in type, comprising a mixture of pseudostratified and two-layered epithelia along its entire length. The cells composing this epithelium are divided into two main cells, ciliated and nonciliated. The latter cells are mainly subdivided into
goblet cells and basal cells. On the basis of the histochemical staining properties of the cytoplasm filled with mucous granules, we are able to classify the goblet cells into two types, Type I and II. The results are summarized in Table 1. The Type I cells are more numerous than the Type II in any portion of the esophagus. The Type II cells are frequently observed in the cranial portion (Fig. 1), but are remarkably decreased in the caudal portion (Fig. 2).

Both types of goblet cell are columnar or fusiform in profile. The Type I cells often protrude their apically bulging cytoplasm into the lumen, whereas the Type II cells are located rather basally and their conical apex usually does not exceed the level of the luminal surface (Fig. 3). In addition, as shown by electron microscopy (Fig. 6, 8),

|                          | Type I cell | Type II cell |
|--------------------------|-------------|--------------|
|                          | Swollen granules | Non-swollen granules | Swollen granules | Non-swollen granules |
| Toluidine blue in epon section | pale pink | reddish purple | pale blue | blue |
| PAS in paraffin section  | red         | red          | red        | red |
| Alcian blue (pH 2.5) in paraffin section | blue      | blue         | faintly stained | faintly stained |
| Alcian blue (pH 1.0) in paraffin section | blue | pale blue | unstained | unstained |
| Alcian blue (pH 2.5) -PAS in paraffin section | purple | bluish purple | red | red |
| Alcian blue (pH 2.5) -PAS in epon section | blue | pale magenta | magenta | magenta |
| Neuraminidase (AB/PAS) in paraffin section | bluish red | bluish red | red | red |

**Table 1.** Staining properties of secretory granules in two types of goblet cells.

*Fig. 1 and 2.* Part of a transverse section through the cranial (Fig. 1) and caudal (Fig. 2) portions of the esophagus. In the epithelium poorly stained goblet cells (arrows) can be seen among alcianophilic goblet cells. The laryngotracheal cavity surrounded by cartilagines laterales. Paraffin section, alcian blue (pH 2.5) staining. ×100
mucous granules of the Type I cell are often swollen and fused to each other to form a large conglomerate. In contrast, the Type II cell only occasionally displays swollen fused granules, and clear spaces due to partial loss of the limiting membranes are frequently present on the contact surfaces of adjacent granules (Fig. 6, 7). Such swollen granules may differ in staining property from the remaining granules in one and the same cell (Fig. 6, inset). Therefore, the former granules are called “swollen granules”, and the latter, “non-swollen granules” for descriptive purposes.

**Scanning electron microscopy**

In the luminal surface of the esophagus, longitudinal folds parallel to its long axis are present, as seen in light microscopic cross sections. Ciliated cells are prominent in any portion, while nonciliated cells with short microvilli, probably goblet cells, are distributed singly or in small groups among the ciliated cells (Fig. 4). In general, goblet cells seem to be more numerous in the furrows between the folds. The esophageal mucous membrane widens like a funnel near its cranial end and is continuous with the pharyngeal mucous membrane, so that there is no distinct boundary.

On the other hand, at the caudal end of the esophageal mucous membrane, the ciliated cells are completely displaced by goblet cells as soon as they enter the gastric area; the longitudinal mucous folds in the esophagus are additionally transformed into transversely-placed ones in the stomach (Fig. 5). Therefore, a relatively distinct demarcation occurs between the gastric and esophageal mucous membranes.

**Transmission electron microscopy**

In the esophageal epithelium we can identify ciliated cells including immature types, goblet cells at different stages of secretory cycle, as well as basal cells. The characteristics of each of these cells are as follows:

The ciliated cells are subdivided into two types, light and dark cells, the latter appearing very dark due to its electron dense cytoplasm (Fig. 7, 9, 10). They are connected by junctional complexes and desmosomes, either with each other or with adjacent goblet cells. Mitochondria are predominant in an elongated form and accumulate away from basal bodies in the supranuclear portion (Fig. 6). Delicate filamentous bundles are scattered throughout the cytoplasm. Glycogen particles are sparsely distributed in the whole cytoplasm (Fig. 8). The Golgi area is usually located supranuclearly. A few cisternae of the rough endoplasmic reticulum are distributed mainly beside the nucleus. The ciliated cells, especially the dark cells, frequently
contain clusters of small, elongated, electron-lucent bodies in the apical area, from directly beneath the basal bodies to the mitochondria-rich area (Fig. 9). Such bodies vary in size from one cell to another, usually measuring 50–300 nm in short diameter and 300–600 nm in long diameter. These bodies can be easily identified as small-sized mucous granules, because they are very similar in electron density and especially in internal structure to secretory granules characteristic of a certain type of goblet cell,
Ciliated cells of the immature type, especially the dark cells, usually have more numerous and larger mucous granules than the mature cells (Fig. 11): they do not yet have cilia, but clusters of centrioles, procentrioles, or their precursors—dense bodies—which are thought to be the stage prior to procentriole formation (Steinman, 1968; Dirksen, 1971), in the supranuclear region (Fig. 11). In some cases, the mature ciliated cells contain even the same large mucous granules as observed in goblet cells (Fig. 10).

The goblet cells are classified into two types (Fig. 6, 7): one has membrane-bound, electron lucent, round or polygonal secretory granules which frequently contain specific inclusions in a matrix of fine particulate; the other possesses membrane-bound, moderately electron-dense, angular secretory granules, with a fine particulate texture. Mature, non-swollen mucous granules of the former (800–1200 nm in diameter) are smaller than those of the latter (1000–1500 nm). Correlative observation of the electron microscopy of thin sections with their adjacent semithin sections stained with toluidine blue or alcian blue-PAS reveals that the former cells correspond to Type I,

**Fig. 6.** Esophageal epithelium showing two types of goblet cells (I, II) and a light ciliated cell. The arrows show slightly electron-dense, non-swollen mucous granules; the double arrows show fused, swollen mucous granules. B basal cells, M Melker cell. ×3,000. Inset shows a toluidine blue-stained semithin section adjacent to the electron micrograph. In the Type I cell, dark areas (reddish purple) and light areas (pale pink) correspond to non-swollen and swollen granule aggregations in the electron micrograph, respectively. ×700

as shall be mentioned.

Ciliated cells of the immature type, especially the dark cells, usually have more numerous and larger mucous granules than the mature cells (Fig. 11): they do not yet have cilia, but clusters of centrioles, procentrioles, or their precursors—dense bodies—which are thought to be the stage prior to procentriole formation (Steinman, 1968; Dirksen, 1971), in the supranuclear region (Fig. 11). In some cases, the mature ciliated cells contain even the same large mucous granules as observed in goblet cells (Fig. 10).
while the latter cells to Type II (Fig. 6). These two types of mature cell are similar in internal structure to each other, except for the mucous granules: the cytoplasm is electron dense; the nucleus is shifted basally owing to the development of mucous granules; the Golgi complexes are situated supranuclearly and arranged in a U-shape, surrounding a large conglomerate of mucous granules (Fig. 8). Mitochondria, when the cell is filled with mucous granules, are rather sparsely scattered in the periphery; cisternae of the rough endoplasmic reticulum are closely stacked in lamellae around the nucleus; a few filamentous bundles are scattered in the cytoplasm. In addition, profiles of goblet cells which have completely discharged a confluent mass of mucous granules are frequently seen among the ciliated cells. Such cells can also be identified by the remaining atrophic cytoplasm containing swollen mitochondria and dilated cisternae of the rough endoplasmic reticulum (Fig. 8).

The specific inclusions of the Type I cell seem to be morphologically divisible into three main forms: the first form is chromosome-like, dense strings which three-dimensionally form a glomerular structure, and when the strings are fragmented they may appear as many dots (Fig. 7, 13); the second form is arranged in parallel in two to seven dense plates (Fig. 7, 13); the third form appears as small, highly dense rings or concentric circles (Fig. 7, 13). In addition, there seem to be some transitional forms between these three forms.

These three forms usually appear either in combination with each other (Fig. 7,
13) or alone in the same cell. The frequency of mucous granules with specific inclusions varies from cell to cell: in some goblet cells most granules contain inclusions (Fig. 7), but in others granules with inclusions are very rare or completely absent (Fig. 6, 7). In addition, there are occasional non-ciliated, cylindrical epithelial cells whose apex is studded with a few microvilli and often elevated into the lumen (Fig. 12). The apical cytoplasm contains numerous small, round or discoid mucous granules of moderately electron density measuring 100–200 nm in thickness and 200–500 nm in diameter; some of them contain inclusions resembling those mentioned above. Cisternae of the endoplasmic reticulum are particularly well developed. These cells may correspond to the immature goblet cells of Type I, because there are intermediate type cells present which contain large mucous granules besides similar small granules. In contrast, immature Type II cells, which are cuboidal in shape and are filled with numerous lamellar cisternae of the endoplasmic reticulum, are located in the basal side of the epithelium.

Additionally, there are groups of epithelial cells at some sites which show no characteristics of either goblet cells or ciliated cells (Fig. 14). These are cuboidal in shape, lying on the basal cells. The cells have aggregations of small mucous granules in the apical portion, some of which are fused with each other, and numerous short microvilli on their luminal surface, but neither abundant cisternae of the rough endoplasmic reticulum nor procentrioles. The dark cytoplasm is rich in filamentous bundles, and mitochondria are rather sparsely distributed; membrane-bound bodies containing several concentric lamellar structures are frequently observed in the
Fig. 9. Apical cytoplasm of a light ciliated cell and a dark one containing small mucous granules, some of which contain inclusions. ×10,000

Fig. 10. Apical cytoplasm of a dark ciliated cell containing the same-sized mucous granules as seen in the Type I goblet cell, showing exocytosis of mucous granules (arrow). Some of the mucous granules contain inclusions (arrow-heads). ×10,000

Fig. 11. Apical cytoplasm containing small mucous granules of two immature dark ciliated cells. The arrows indicate procentrioles. ×10,000

Fig. 12. Slightly bulged apical cytoplasm having small mucous granules, some of which contain inclusions (arrows), and lamellar arrays of cisternae of the rough endoplasmic reticulum. Such a mucous cell is thought to develop into a Type I goblet cell. ×13,000
The basal cells are flat, triangular, or cuboidal in shape and rest on the basal lamina (Fig. 7). They connect by desmosomes not only with each other but also with other adjacent cell types. In the cytoplasm numerous tonofilaments are scattered in bundles and mitochondria are moderately distributed. Caveolae arranged in a row can be seen along the periphery facing the basal lamina. Some basal cells contain clusters of small mucous granules in the apical cytoplasm (Fig. 8).

Occasionally, round or oval endocrine-like cells with as many as six long cyto-
plasmic processes in favorable sections, are seen surrounded by basal cells. They are rich in filamentous bundles and contain a number of small, round, dense granules with a narrow halo, measuring 50–100 nm in diameter (Fig. 15a). The granules are distributed throughout the cytoplasm, but are especially abundant in the basal cytoplasm. The Golgi area is situated in the supranuclear region; mitochondria are sparsely distributed among the bundles of filaments. This type of cell combines with the surrounding basal cells by small-sized desmosomes. Nerve ending with clear synaptic vesicles frequently attach to or lie close to the surface of this cell (Fig. 15b). These morphological characteristics coincide with the ultrastructure of Merkel cells observed in the amphibian epidermis (Fox and Whitear, 1978).

Another type of endocrine-like cell is occasionally seen in the epithelium (Fig. 16). These are slender cells containing many round, dense granules, about 100 nm in diameter. Most of these granules, except for immature small granules in the Golgi area, are enclosed in a closely fitting limiting membrane. In the cytoplasm, poorly

**Fig. 15.** Merkel cell. a showing small dense secretory granules dispersed mainly in the basal cytoplasm and abundant filamentous bundles. b showing finger-like cytoplasmic processes (arrow-heads) projected into a basal cell. Arrows show nerve endings with clear vesicles lying close to or attached to the cell surface. B basal cell. ×10,000

**Fig. 16.** Part of the supranuclear cytoplasm of another type of endocrine cell, showing small, dense secretory granules, a large lysosomal dense body and a scanty filamentous bundle (arrow). G Golgi apparatus. ×25,000
DISCUSSION

The esophageal epithelium of the adult salamander, *Hynobius nebulosus*, unlike the stratified squamous epithelium of mammals, is columnar, partly pseudostratified and partly two-layered, and consists mainly of ciliated, goblet, and basal cells. Therefore, it rather resembles the epithelium in the respiratory system of this animal species (MATSUMURA and SETOGUTI, 1986). The ciliated cells of the salamander esophagus, however, differ from those in the respiratory passages in that they are divided into dark and light cells and frequently possess mucous granules, and that immature type cells occur more frequently. The difference in electron density of the cytoplasm of ciliated cells has been reported in the tracheal epithelium in man (OSADA, 1964) and the mouse (HANSELL and MORETTI, 1969). Since in the present observation mucous granules occurred more frequently in the dark cells, both cell types may be not artificial but may reflect different cell activities. Ciliated cells containing mucous droplets confirmed by the electron microscope have been described in the tracheal epithelium of the chick (KALNINS and PORTER, 1969) and embryos of *Xenopus laevis* (STEINMAN, 1968). In these animals mucous droplets appeared transitionally prior to ciliogenesis in much smaller amount than in *Hynobius*. The frequent appearance of immature ciliated cells in the esophagus may reflect that, in this organ, the turnover of ciliated cells is quicker than in the respiratory tract, probably because of mechanical pressure from moving food. The present study showed that some ciliated cells contain a number of the same large mucous granules as seen in the goblet cells. Such a cell type combines in one cell the function of ciliated cells with that of mucous cells, and therefore should be termed as a ciliated-mucous cell. It may reflect a low stage of functional differentiation of the cell. In the same animal species we have observed a similar example in the respiratory epithelium, where one cell type plays the roles of both pneumocytes and mucous or goblet cells (MATSUMURA and SETOGUTI, 1984).

In addition, the goblet cells consisted of two types in contrast with those of the respiratory way: the Type I, containing electron lucent mucous granules with occasional inclusions; and the Type II, having moderately electron-dense mucous granules containing no inclusions.

In the present study, light microscopic histochemistry for the demonstration of mucous substances revealed the following characteristics: 1) In alcian blue (pH 2.5)-PAS staining of paraffin sections, the Type I cells were stained in blue or blue-purple, but Type II cells, in red; this implies that the former mainly contain acid mucosubstances, the latter, neutral mucosubstances (MOWRY, 1963; Spicer and DUVENC, 1964). 2) The mucous cells of both types showed diastase-resistant, intensive PAS reactivity in paraffin sections; this is ascribed to the presence of either glycoprotein (Type I cells) or glycoprotein plus neutral mucosubstances (Type II cells). 3) In alcian blue (pH 1.0) staining of paraffin section, the swollen granules of the Type I cells were more strongly positive than the non-swollen ones; this indicates that in the former granules acid mucosubstances are more highly sulfated than in the latter granules (LEV and Spicer, 1964). 4) In alcian blue (pH 2.5)-PAS staining of paraffin sections after neuraminidase digestion, stainability of the Type I cells was changed to bluish red. This partial loss of alcianophilia suggests that the mucous granules of the Type I cells are composed of sialic acid-containing glycoproteins (Spicer and Warren, 1960;
5) Alcian blue-PAS staining of semithin Epon sections showed a somewhat different tincture from that of paraffin sections. This is probably due to the saponification effect of KOH in absolute alcohol used for the removal of resin on the components of mucosubstances.

In some of the Type I cells, mucous granules contained three forms of the specific inclusions: the first form was of chromosome-like strings, the second one of parallel-arranged plates, and the third one of dense circles. In addition, intermediate forms seemed to be present among each form. Such findings strongly suggest that each form of the mucous graule inclusions may be derived from a single form, probably arising with the maturation of mucous granules. In fact, we have found in a certain type of goblet cells of larval *Hynobius* that a small dense body is transformed into these three forms by the unloosing of its dense structure (unpublished data). Goblet cells with similar inclusions have been reported in the oropharyngeal epithelium of larval, postlarval, and adult fire salamanders, *Salamandra salamandra* (L.) (CLEMEN, 1984, 1985a, b): they are named “type III” cells and concentrated in a glandular bud in the epithelium (CLEMEN, 1985a, b). Furthermore, somewhat similar osmiophilic inclusions occur in the mucous granules of mammalian submandibular glands (LUZZATTO et al., 1968; DOREY and BHoola, 1972; TANDLER and MACCALLUM, 1972; YAMASHINA and MIZUHIRA, 1976, RUBY and CANNING, 1978) or of amphibian lingual glands (FAHRMANN 1975; ZYLBERBERG, 1977) and are supposed by some authors (LUZZATTO et al., 1968) to contain enzymes.

As to the biological significance of goblet cells with such inclusions, CLEMEN (1985a) has supposed that secretions from these cells may be associated with the killing of bacteria because of their special location in the front area of the oral cavity.

The Type II goblet cells were characterized by secretory granules containing neutral mucosubstances. This type of cell, with respect to the histochemical properties and fine structure of the secretory granules, seem to resemble CLEMEN’s Type II goblet cells that are widely distributed in the oropharyngeal epithelium of fire salamanders. In the present study, the Type II cells appeared mainly in the cranial portion, especially near the pharyngeal cavity. In addition, we have more frequently found similar, though not identical, cells in the pharyngeal epithelium (unpublished data). Therefore, judging from their location in the esophagus, they might be digestive enzyme-containing cells which migrate downward from the pharyngeal epithelium during the embryonal developmental process and are modified in the esophagus.

In *Hynobius*, swallowed prey is transported to the stomach by the peristaltic action of the esophageal muscles (NOBLE, 1954; REEDER, 1964). In this process, the goblet cells of both types liberate a large amount of mucus and may contribute not only to the smooth passage of the bag through the esophageal lumen but also somewhat to its digestion, since this species does not have any gland—including a mucous gland—in the esophageal wall. To ascertain this hypothesis, however, a comparative observation of the esophageal epithelium of this species with that of other amphibian species possessing glands in the esophageal wall will be needed.

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