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# A COMPARATIVE MORPHOLOGICAL STUDY OF THE ALIMENTARY CANAL IN THREE COLD WATER PLEURONECTIDS.

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

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## ABSTRACT

The pleuronectidae are known to exhibit considerable species diversity in both habitat and prey preference and have recently become of interest to the aquaculture industry. The Atlantic halibut, the yellowtail flounder and the winter flounder, are three species currently being considered as aquaculture candidates in Eastern Canada. The aim of this investigation was to first determine, whether differences exist in alimentary canal histology between species known to inhabit different environments and to utilize different prey types in the wild and secondly to utilize this information to provide a morphological data base for future studies in digestive physiology and artificial diet development. Samples of each species were captured from wild populations and tissue was removed from five areas along the alimentary canal (esophagus, stomach, pyloric caeca, intestine, and rectum). Following sampling, tissue was processed for both light and electron microscopy. The existence of a distinct surface secretory cell (ESSC) in association with the esophageal mucosa in the winter flounder and the yellowtail flounder coupled with elaborate mucosal folding and a complex mucous histochemistry suggested that this region may have a role in the initiation of chemical digestion in these species. The stomach epithelia in the Atlantic halibut, the winter flounder and the yellowtail flounder, could be divided into three distinct zones based upon ultrastructural differences in the cell types present. Variation in mucous histochemistry across species as well as between regions suggested that different chemo-types of mucus may be important for different digestive functions and that some variations may be species specific. The post-gastric regions of the three pleuronectids could be divided into two main areas based upon the ultrastructural features of the digestion and absorption of specific nutrients: lipid digestion was observed to occur in the intestine and pyloric caeca whereas the intracellular digestion of exogenous protein was observed to occur in the rectum. Numbers of goblet cells within post-gastric regions were not found to be significantly different between species. Numbers were however, significantly different between regions, with a trend indicating an increase in the frequency of goblet cells toward the rectum. Predicted volume fractions for mucus production in the post-gastric alimentary canal were found to be higher in the halibut than the other species.

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To my Parents and wife Sharon

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AB - Alcian Blue
AC - Absorptive cells
BAC - Bacteria
BB - Brush border
BL - Basal lamina
BV - Blood vessel
CAP - Outer cytoplasm of rodlet cell
CHL - Chylomicron particles
CL - Collagen
CM - Circular muscle
EP - Epithelial zones
ESSC - Esophageal surface secreting cell
FI - Fibrous internal substructure
FL - Flocculent material
G - Secretory granules
GC - Goblet cells
Gg - Golgi apparatus
GLa - Gastric glands
GR - Mucous granules
H&E - Hematoxylin and Eosin
LD - Lipid droplets
LI - Lateral membrane interdigitation
LM - Longitudinal muscle
LP - Lamina propria
M - Mucosa
ME - Muscularis externa
MF - Mucosal folds
MG - Mucous granules
MGR - Mucous granule region
MM - Muscularis mucosae
MNC - Mucous neck cells
Mt - Mitochondria
MV - Microvilli
MVB - Multivesicular body
N - Nucleus
OPC - Oxyntico-peptic cells
PAN - Pancreatic tissue
PAS - Periodic acid/Schiff
PSM - Propria-submucosa
RE - Rough endoplasmic reticulum
RL - Rodlets
SE - Stratified epithelial cells
SG - Endocrine granule
SER - Serosa
SM - Submucosa
SMC - Surface mucous cells
VCN - Vesicular cytoplasmic network
VL - Branching villi
ZN1 - Zone 1: surface mucous cells
ZN2 - Zone 2: neck cells
ZN3 - Zone 3: gastric glands

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**3. A comparative histological study of the intestine, pyloric caeca and rectum in three species of pleuronectidae**

# 1. GENERAL INTRODUCTION

Interest in the culture of cold water marine fish species has created an area of increased research over the past number of years. This is a result of the ever increasing pressure on the wild fishery and the reduced availability of wild fish product.

Recent focus is on a range of species, from the more economically important ground fish, like the Atlantic cod, *Gadus morhua* (Kjørsvik et al., 1991) and the Atlantic halibut, *Hippoglossus hippoglossus* (Finn et al., 1991; Kjørsvik and Reiersen, 1992; Murray et al., 1993), to less attractive but technically easier species to culture, such as the Ocean pout, *Macrozoarces americanus*, and the Lumpfish, *Cyclopterus lumpus* (Brown et al., 1992). Unfortunately obstacles to the production of larvae and juveniles, such as consistent good quality eggs and successful first feeding, have proven difficult to overcome.

More recently, investigators have become interested in studying some of the small commercial pleuronectid species for aquaculture. Two such species, presently proposed for study or currently under study include the winter flounder, *Pleuronectes americanus* (Harmin and Crim, 1992; 1993) and the yellowtail flounder, *Pleuronectes ferruginea* (Smigielski, 1979; Walsh, 1992).

For species that are relatively new to the aquaculture industry, like the pleuronectids, a thorough knowledge of the anatomy and physiology of both their somatic and reproductive systems will aid in a better understanding of health, feeding

and growth norms. The better one knows these animals the greater is the possibility for success in their culture.

## 1.1. The Pleuronectidae

The winter flounder, the yellowtail flounder and the Atlantic halibut are members of the order Pleuronectiformes and the family pleuronectidae, the right-eyed flounders.

The Pleuronectiformes in general, as described in Scott and Scott (1988), are fish ranging in size from 0.15 to over 3 meters in length. As adults and juveniles, they are characterized by laterally compressed asymmetrical bodies with both eyes on the same side of the head. One side of the body is unpigmented and rests on the substratum. Dorsal and anal fins extend along much of the body length and a swim bladder is usually absent following the larval stage. They are known to be wide spread, benthic and largely marine.

Flounders are characterized further by the dramatic metamorphic change occurring at the end of the larval stage. This change involves a shift from a pelagic swimming, "normal teleost body form" to the more atypical, asymmetrical, laterally compressed, bottom living form. This developmental modification in the right-eyed flounders involves the left eye migrating to the right side of the body.

## 1.2. Differences Between Species

The three pleuronectid species used in this investigation exhibit distinct differences in their respective natural histories, including water depth frequented, spawning depth, the length of time to first feeding and common prey species.

### 1.2.1. Normal water depth

The Atlantic halibut frequents depths ranging from 165 m to 1000 m (Scott and Scott, 1988). Both the winter flounder and the yellowtail flounder are restricted to comparatively shallow water (Mccracken, 1963; Scott and Scott, 1988). The winter flounder is an inshore shallow water bottom species, found in depths from 1.8 to 36.6 m (Scott and Scott, 1988). The yellowtail flounder inhabits depths of 27 to 364 m, but more generally is found from 37 to 91 metres (Scott and Scott, 1988). The yellowtail flounder is considered an intermediate depth species between the halibut and the winter flounder.

### 1.2.2. Spawning depth

Female halibut are known to spawn at depths between 300 and 700 meters and to produce pelagic eggs of approximately 3.0 mm in diameter (Scott and Scott, 1988; Helvik and Walther, 1992). Haug et al. (1984) noted the occurrence of developing pelagic eggs at 100 to 250 m. Eggs are known to hatch in captivity between 12 and 18 days at 5.0 $^{0}$ C (60 to 90 degree days) (Blaxter et al., 1983; Helvik and Walther, 1992).

Spawning in the winter flounder and the yellowtail flounder occurs on or near the bottom. Egg diameter is very similar between these species, approximating 0.8

mm, which is significantly smaller than that of the halibut egg diameter (Scott and Scott, 1988). Winter flounder eggs have been observed to hatch within 54 degree days and similarly yellowtail flounder have been noted to hatch within 55 degree days (Scott and Scott, 1988).

### 1.2.3. Time to first feeding

In the halibut, time from hatching to successful first feeding was shown in captivity to frequently exceed 210 degree days, although the exact time is still under debate (Blaxter et al., 1983; Pittman et al., 1990; Kjorsvik and Reiersen, 1992). First feeding in winter flounder may occur within 50 degree days after hatching ( H. Murray, unpublished observation). Preliminary observations on the yellowtail flounder suggested first feeding takes place around 3 days post hatch at 6-7 °C (G. Goff, personnal communication) or around 21 degree days (Smigielski, 1979).

### 1.2.4. Differences in feeding strategy and prey type

These species of pleuronectid also exhibit different preferences for both prey type and feeding habits as adults. The Atlantic halibut is a purely piscivorous feeder as an adult, however juveniles primarily feed on invertebrates (De Groot, 1971). Halibut up to lengths of approximately 30 cm feed almost exclusively on annelid worms, crustaceans and decapod shrimp (Scott and Scott, 1988). From 30 to 80 cm both fish and invertebrates are taken. At lengths over 80 cm, the fish feed almost exclusively on other fishes.

Both the winter flounder and the yellowtail flounder prey solely upon invertebrates, however the yellowtail flounder will occasionally take small fish (De Groot, 1971; Libey and Cole, 1979; Collie, 1987; Scott and Scott, 1988).

These differences in feeding habits may reflect the gross anatomy and/or the physiology of the alimentary canal of these animals. Moiseev (1953, cited in De Groot, 1971) showed that the pleuronectids can be divided into three categories based on gross morphology of the gut and feeding characteristics: benthophagus flounders (small mouthed flounders), fish of mixed feeding habits (large mouthed flounders), and predatory flounders (piscivorous flounders). The yellowtail flounder and the winter flounder, being invertebrate feeders as adults, have small soft mouths and jaws and are termed benthophagus according to the above classification whereas the Atlantic halibut exhibits a wide, muscular, many toothed mouth and jaws and is predatory.

De Groot (1971) further suggested possible correlation between the average ratio of the length of the intestine to body length and prey selection in pleuronectids. He pointed out that this ratio decreases from annelid feeding species towards fish feeders.

## 1.3. Structure and Function of the General Vertebrate Alimentary Canal

### 1.3.1. General organization

Welsch and Storch (1976), described a general vertebrate digestive tract as a hollow tube traversing the body in a longitudinal direction. Its lining chiefly consists

of cells specialized for the digestion and absorption of food. Other cells that function in propulsion of the ingested food ( muscle and/or ciliated cells), in lubrication and protection (mucous cells), and in coordination of gut activities (neurons and endocrine cells) are also important for normal digestive processes.

Expanding the above simple model phylogenetically, one finds similarity in function, however, evidence for considerable structural variation occurs on both a macroanatomical and a microanatomical scale. At the macroscopic level a generalized vertebrate alimentary canal is organized into six major areas, consisting of: buccal cavity, pharynx, esophagus, stomach, small intestine and large intestine. The small and large intestine can be further subdivided into smaller regions based upon specialization. For example, the small intestine can be divided into duodenum, jejunum, and ileum, and similarly, the large intestine can be divided into caecum, colon, rectum and anal canal. Generally, on a gross anatomical level the organization of the vertebrate digestive system varies with dietary specialization.

Microanatomically, the whole of the alimentary canal can be described as a tube organized into five concentric layers (Kelly et al., 1984; Stinson and Calhoun, 1993). These layers include a serosa, a muscularis externa (typically an inner circular and an outer longitudinal layer of smooth muscle), a submucosa (connective tissue with vascular and nervous elements), and mucosa (epithelium, lamina propria of loose connective tissue and muscularis mucosa of smooth muscle). The mucosa is frequently thrown into tall folds or villi which aid in increasing the area for absorption of nutrients, water and ions (Welsch and Storch, 1976).

It is the epithelial layer of the mucosa that is important in defining the functionally distinct regions of the gut. The metabolically important epithelial cells of the vertebrate alimentary canal consist of four subpopulations. These include: absorptive cells, mucous cells, specialized secreting cells (i.e. those of the gastric glands), and endocrine cells (Leake, 1975). The relative proportions of these cell types in any given area appear to be a function of the metabolic speciality of that area.

It should also be pointed out that while structure and function of the mucosal layer are generally similar across vertebrate taxa, variations which may be a reflection on the dietary habits or level of phylogenic development. The regions in the vertebrate gut can be divided into three zones based on the major cell type or types associated with the mucosa. These areas include the esophagus, stomach and intestine.

### 1.3.2. Esophagus

The typical vertebrate esophagus is an area of transition from the buccal/pharyngeal area to the stomach (Stinson and Calhoun, 1993). The muscularis externa, however, consists of striated muscle as opposed to the smooth muscle observed in most other areas of the gut. The muscle layers in the mammalian esophageal muscularis are arranged in an oblique or helical fashion as opposed to the circular/longitudinal orientation observed in most other areas of the gut (Leake, 1975).

The nature of the esophageal mucosal layer shows considerable variation across taxa. In the class Amphibia the mucosa is thrown up into deep folds and consists primarily of ciliated cells, whereas in the Reptilia, it is defined by a single, non-folded layer of ciliated cells interspersed with goblet cells (Leake, 1975; Welsh and Storch, 1976).

The avian and mammalian esophageal mucosa is very different from that observed in the above groups. It is highly folded and composed of a stratified squamous epithelium that may or may not be keratinized (Hill, 1971; Welsh and Storch, 1976). The avian esophagus is long and wide to accommodate large pieces of food since chewing is not possible (Leake, 1975). Glands are also present for the secretion of lubricative fluid and the caudal end of the avian esophagus is expanded into a sack-like structure or crop for storage (Leake, 1975).

### 1.3.3. Stomach

Anatomically, the stomach in most vertebrates resembles a large muscular sack for food storage. Morphological variations are usually related to dietary preferences, for example, the avian stomach is a dual structure, consisting of a separate glandular, proventriculus and a muscular, ventriculus or gizzard to grind food (Hill, 1971). In contrast, ruminants possess a stomach with a specialized nonglandular region subdivided into: rumen, reticulum and omasum, and a glandular region, the abomasum, which is similar to the simple stomach in other vertebrates. Structurally, a vertebrate stomach possesses similar tissue layers as observed in other areas of the gut. The muscularis externa is particularly well developed, exhibiting an inner

circular and outer longitudinal layer of smooth muscle. In some species an oblique muscular layer is also present (Stinson and Calhoun, 1993).

The mucosa constitutes the layer that defines the function of the stomach. The lower vertebrates (actinopterygii, amphibia, and reptilia) possess a relatively simple gastric mucosa, divided into only two areas, the cardiac and the pyloric region (Welsh and Storch, 1976; Stinson and Calhoun, 1993). In general, the pyloric region is a small area adjacent to the pyloric sphincter and does not contain typical gastric glands, hence the majority of the gastric mucosa in these lower groups is glandular in nature.

The avian proventriculus consists of a typical glandular gastric mucosa, similar to that of the stomach in other vertebrates. The gizzard or ventriculus possesses a specialized mucosa composed of tubular glands which produce a hard substance similar to keratin called koilin, to aid in the maceration of hard foods in conjunction with stones or other grit picked up while feeding (Leake, 1975).

The mammalian gastric mucosa shows considerable dietary variation. The carnivore stomach is glandular and divided into cardiac, fundic and pyloric zones on the basis of the characteristics of the glands present (Stinson and Calhoun, 1993). In contrast, the ruminant stomach mucosa associated with the fermentation chambers is primarily a nonglandular, stratified squamous epithelium whereas the glandular abomasum contains typical gastric glands.

In general, vertebrate gastric mucosa is known to possess three major epithelial cell types: mucous secreting cells (surface and neck), glandular cells, and

enteroendocrine-like cells. While generally, the mucous cells and the enteroendocrine cells are morphologically similar between higher and lower groups, it is the cells associated with the gastric glands that reveal differences between these groups (Welsch and Storch, 1976).

The gastric glands present in the actinopterygii, amphibians, reptiles and birds have been found to consist of only one type of exocrine secretory cell, the oxyntico-peptic cell or principal cell (Welsch and Storch, 1976). Ultrastructurally, this cell has the characteristics of both an acid secreting cell and an enzyme producing cell.

Mammals possess two distinct cell types associated with the glands of the fundic stomach: the acid secreting parietal (oxyntic) cells, and the enzyme producing chief (peptic) cells. Together these cells perform the same functions as the oxyntico-peptic cell of the lower groups (Welsch and Storch, 1976).

### 1.3.4. Intestine

#### 1.3.4.1 Small intestine

The small intestine in birds and mammals is divided into the duodenum, the jejunum and the ileum, and functions as the primary area for digestion and absorption of nutrients (Stinson and Calhoun, 1993). The amphibian small intestine is divided into a proximal duodenum with thick muscle layers and submucosal glands and a more distal ileum. The reptilian small intestine is similar with additional coiling and a deeply folded mucosa (Leake, 1975). The small intestine contains essentially the same tissue layers as the other areas of the gut. Enzymes and emulsifiers from

the pancreas and the liver are delivered through ducts into the anterior intestine to aid in the digestion and absorption of fats and lipids.

The mucosa is composed of long, finger-like projections called villi, which increase the surface area for the absorption of nutrients (Stinson and Calhoun, 1993). In birds and mammals, the bases of these villi contain specialized glandular areas known as the crypts of Lieberkühn, which may function at least partially in stem cell production (Leake, 1975). Crypts are not observed to be present in other vertebrates. Paneth cells are commonly associated with the intestinal crypts of higher mammals (such as ruminants, the horse, and man) and appear to function as exocrine cells producing bactericidal compounds such as lysozyme (Welsch and Storch, 1976; Wheater et al., 1987).

The cells covering the apical and lateral surfaces of the villi are of two types: the enterocytes which function in absorption and goblet cells which produce mucus.

**1.3.4.2 Large intestine**

The mammalian large intestine is subdivided into the caecum, colon, rectum and anal canal (Stinson and Calhoun, 1993). Non-mammalian vertebrates appear to have much less overall division and organization, in some cases only having a rectum or rectum and anal canal. Large amounts of compact lymphoid tissue are also noted to be associated with this area. Short mucosal folds and a pair of caeca are present in avian large intestine (colon) (Leake, 1975). The rectum has no folding and empties into a cloaca. The large intestine of the amphibians and reptiles shows a

organization similar to that of the avians, with broad, low mucosal folds (Leake, 1975).

The cells lining the mammalian large intestine are similar to those associated with the villi of the small intestine, including both absorptive enterocytes and large numbers of goblet cells (Welsch and Storch, 1976). Ultrastructurally, the enterocytes exhibit features which are known to be correlated with cells active in triglyceride synthesis and in water absorption (Welsch and Storch, 1976).

The cells lining the avian large intestine are typical enterocytes interspersed with numerous goblet cells (Hill, 1971). The aves, reptiles and the amphibians all possess a cloaca as the terminal portion of the large intestine. This region acts as a common chamber into which the digestive and urogenital tracts converge (Hill, 1971; Leake, 1975). It is commonly lined with a short columnar epithelium that changes to a stratified squamous epithelium near the vent or outer opening.

## 1.4. Structure and Function of the Fish Alimentary Canal

### 1.4.1. General organization

Early in its life history the larval fish possesses a digestive system characterized by a straight, undifferentiated tube, which during development becomes compartmentalized into distinct regions described as foregut, midgut and hindgut (Govoni et al., 1986). Later this structure gives way to the complex, segmented and multifunctional alimentary canal of the adult fish.

Considerable variation exists between species with regard to the general structure of the adult teleost digestive system. Typically, a generalized fish alimentary canal consists of buccal cavity, pharynx, esophagus, stomach, pyloric caeca, intestine and rectum. The latter four areas are known to be the most important in digestion and absorption. Detailed reviews of the morphology of the adult fish digestive system have been completed by Barrington (1957), Kapoor et al. (1975), and Fänge and Grove (1979). Govoni et al. (1986) have reviewed larval fish gut histology and physiology.

### 1.4.2. The esophagus

The esophagus appears as an extension connecting the bucco-pharyngeal area with the stomach, thus acting as an area functioning primarily in transport of food. The esophagus possesses a number of functions in different fishes (Kapoor et al., 1975). These range from initial pregastric digestion due to the presence of gastric-type glands to absorptive functions suggested by the presence of extensive numbers of blood vessels in the lamina propria.

Histologically, the esophagus consists of numerous mucosal folds. Epithelial stratification has been observed in some species (Al-Hussaini, 1946; 1947), however, the general theme is a simple columnar epithelium intermixed with large numbers of goblet cells (Dawes, 1929; Martin and Blaber, 1984; Morrison, 1987 ). The large numbers of goblet cells suggest for esophagus, a function in the lubrication of food for smooth passage and protection of delicate lining of the epithelium.

The muscularis externa of the esophagus shows significant variation with regard to type and organization of muscle layers. In the Atlantic cod, two layers of striated muscle, an inner longitudinal layer and a thicker outer circular layer are present (Bishop and Odense, 1966; Morrison, 1987). In the red mullet, *Mulloides auriflamma* there is only one layer of circular, striated muscle present in the esophagus (Al-Hussaini, 1946). This contrasts with the Northern pike, *Esox lucius*, where Bucke (1971) described smooth muscle layers in the muscularis of the esophagus.

### 1.4.3. Stomach

Developmentally, the stomach is the last section of the fish alimentary canal to become functional (Govoni et al., 1986). Before metamorphosis and prior to the formation of the stomach, it is the hindgut that is involved in the digestion of protein, a function usually reserved for the stomach (Govoni et al., 1986). Protein digestion is known to take place intracellularly in the hindgut mucosal cells following the pinocytotic uptake of particulate proteinaceous material (Watanabe, 1981; 1984).

During the time between larval and adult forms, most species of teleosts will develop a functional stomach, however, a few species are known to continue to maintain a larval-like stomachless condition (Al-Hussaini, 1947; Govoni et al., 1986). Those fishes not possessing a functional stomach were thought to have neither gastric glands and nor pyloric sphincter (Fänge and Grove, 1979), however, it should be pointed out that this may not be accurate for all "stomachless fishes". Other

reviewers have reported the presence of gastric gland-like structures in some species of stomachless fish (Vegas-Velez, 1972; Fänge and Grove, 1979).

In those teleosts possessing a typical stomach, the structure historically has been described as distinctive in both function and form.

The stomach of the red mullet is divided into two distinct areas, based on the characteristics of the mucosa and the muscularis externa. These are compared to that in mammals and termed the cardiac and pyloric regions (Al-Hussaini, 1946). While this observation appears as a general theme in most species of teleost some descriptive variation between species does exist. The stomach of the European eel, *Anguilla anguilla*, for example, is divided into cardiac, fundic and pyloric areas (Clarke and Witcomb, 1980). No regional differentiation is observed in the tilapia, *Tilapia nilotica*. In an attempt to resolve this problem for observational and descriptive purposes the stomach of tilapia has been arbitrarily divided into initial, middle and terminal regions (Osman and Caceci, 1991).

The mucosa of the stomach in most fishes has been shown to be unique compared to that found in the other areas of the alimentary canal. It is organized into deep invaginations or folds which lead to gastric pits into which the glands open (Dawes, 1929; Bucke, 1971; Clarke and Witcomb, 1980; Buddington and Doroshov, 1986; Morrison, 1987).

The epithelium lining the gastric folds is similar histologically and histochemically in most groups of fish studied to date. Early studies describe the cells as tall columnar that exhibit staining patterns which indicate mucus production

(Dawes, 1929; Al-Hussaini, 1946). Later investigations show the cells have a strong Alcian blue (AB) and periodic acid/Schiff (PAS) reaction, verifying the function of the cells as producers of mucus (Bishop and Odense, 1966; Bucke, 1971; Sis et al. 1979; Morrison, 1987; Osman and Caceci, 1991).

More recently, it has been shown that the mucosal epithelium is composed of a second type of mucous cell. This cell, termed a mucous neck cell exhibits a foamy eosinophilic cytoplasm and gives a positive AB/PAS reaction (Noaillac-Depeyre and Gas, 1978; Osman and Caeci, 1991).

The gastric epithelium observed in a non-teleost fish like the white sturgeon, *Acipenser transmontanus*, has been shown to be different from that of the teleost, consisting predominantly of goblet cells interspersed with ciliated cells (Buddington and Doroshov, 1986). The surface epithelial cells give way to glands that, depending on the species, are simple, compound, or occasionally branched tubular. These glands are most frequently found in the cardiac region of the stomach, and in most instances are not observed in the pyloric region (Bishop and Odense, 1966; Clarke and Witcomb, 1980; Martin and Blaber, 1984; Buddington and Doroshov, 1986). The only available exception to this rule appears in the tilapia where gastric glands are present in the initial, middle and terminal regions (Osman and Caceci, 1991).

Historically, only one type of cell has been identified with these glands (Dawes, 1929; Al-Hussaini, 1946; Bishop and Odense, 1966; Bucke, 1971; Reifel and Travill, 1978; Rebolledo and Vial, 1979; Clarke and Witcomb, 1980; Ezeasor, 1981; Martin and Blaber, 1984; Anderson, 1986; Osman and Caceci, 1991; Grau et al.,

1992). Noaillac-Depeyre and Gas (1978) coined the term "oxyntopeptic cells" for them, further suggesting that they might perform a function reminiscent of the roles of the parietal and chief cells found separately in the stomach of mammals.

Endocrine-like cells have been shown to be associated with the gastric mucosa of some fish species (Noaillac-Depeyre and Gas, 1978; Ezeasor, 1981). Three types of endocrine-like cells have been distinguished in the gastric epithelium of the rainbow trout, *Oncorhynchus mykiss* according to shape, size and density of cytoplasmic granules.

### 1.4.4. The Intestine, pyloric caeca and rectum

In most fish species, it is the pyloric caeca, intestine and rectum that provide the important areas for absorption and digestion (Kapoor et al., 1975; Fänge and Grove, 1979). However, variations in the structure and function of these regions were noted (Fänge and Grove, 1979).

Developmentally, the intestine and rectum form from the larval midgut and hindgut areas respectively whereas the pyloric caeca appear late in development as extensions from the foregut, corresponding with the formation of a functional stomach (Govoni et al., 1986; Pedersen and Falk-Petersen, 1992). Functionally, the role of these areas change during the period of development. The pyloric caeca which initially function in lipid digestion and absorption, continue in this role by significantly increasing the absorptive area of the proximal intestine and providing a surface area for the action of enzymes (Buddington and Diamond, 1986). In contrast, the hindgut, which gives rise to the posterior intestine and rectum, aids in the

digestion of protein in larval and stomach-less fish and ceases to function in this role following the formation of a functional stomach (Govoni et al., 1986; Watanabe, 1984).

Histologically, the pyloric caeca, intestine and rectum of the adult or juvenile fish are organized in the same fashion as previously discussed with reference to the esophagus and the stomach. While the mucous producing cells are morphologically similar in the three areas, the enterocytes show some regional ultrastructural differences across species suggesting functional dissimilarities (Buddington and Doroshov, 1986). Based on this point and taking into consideration morphological variation among species, these areas can be divided into at least two zones : the pyloric caeca/intestine and the rectum.

#### 1.4.4.1 Pyloric caeca/intestine

In general, the mucosa of the pyloric caeca and the intestine appear very similar suggesting comparable functions. These areas are lined with a simple columnar epithelium interspersed with goblet cells (Dawes, 1929; Al-Hussaini, 1946, 1947; Bishop and Odense, 1966). Ultrastructural evidence outlining the presence of chylomicra within elements of the smooth ER and in the intercellular spaces, suggests that the main role of the intestine is the absorption of lipid following lumenal emulsification and digestion (Ezeasor and Stokoe, 1981; Morrison, 1987). The pyloric caeca when present, significantly increase the surface area of the anterior intestine for the absorption of lipids (Bishop and Odense, 1966; Ezeasor and Stokoe, 1981). The caeca are also important in the absorption of carbohydrates in the goldfish,

*Carassius auratus* (Elbal and Agulleiro, 1986). The caeca of the Luderick, *Girella tricuspidata* carry out both absorptive and secretory functions as characterized by the different cells present (Anderson, 1986). Equivalent functions have been suggested for the caeca of the Mediterranean amberjack, *Seriola dumerili* (Grau et al., 1992).

### 1.4.4.2 Rectum

The mucosa of the rectum and anal canal is composed, almost totally, of goblet cells occasionally interspersed with short columnar cells (Bucke, 1971; Martin and Blaber, 1984; Elbal and Agulleiro, 1986). The number of goblet cells appear to vary with area and species, increasing towards the posterior intestine in the Northern pike (Bucke, 1971) but showing no significant difference in number between anterior and posterior intestine in the goldfish (Elbal and Agulleiro, 1986).

Generally, the mucosa of the rectum is highly folded and associated with a well developed and richly vascularized submucosa. The muscularis externa has also been noted to be well developed and in some cases at least twice as thick as that which is present in the middle intestine. Sis et al. (1979) observed this to be true in the channel catfish. Similarily, Martin and Blaber (1984) and Grau et al. (1992) pointed out comparable features in the glassy perchlet and the amberjack, respectively. Epithelial cells are typically columnar in shape, and hence similar to those found in other areas (Grau et al., 1992).

Functionally, the rectum or hindgut has been theorized to be an important region with regard to the supplemental intracellular digestion of protein in many species of fish. This feature was previously thought to only occur in the hindgut of

larval teleosts and mature stomachless fish species (Govoni et al., 1986). However, ultrastructural evidence suggests an active uptake of proteinaceous material even in mature animals with functional stomachs (Ezeasor and Stokoe, 1981; Govoni et al., 1986).

## 1.5. Gastrointestinal Mucus

### 1.5.1. Function of mucus in the vertebrate alimentary canal

Vertebrate gastrointestinal mucus serves important functions for the alimentary canal epithelium. These include: protecting the epithelium from pathological and/or physical damage and protection of epithelium from chemical damage, such as autodigestion (Allen et al., 1982; Mantle and Allen, 1989).

Considering that the gut mucosa lines a lumen through which material passes, material that may be abrasive to the epithelium, a protective or lubricating physical barrier to damage is clearly essential. Entero-invasive pathogens in the gut lumen must penetrate the mucous barrier to gain access to the mucosa, thus the mucus acts as a barrier to invasion by possible pathogens (Mantle and Allen, 1989). Similarly, indigenous bacteria colonizing the gastrointestinal tract are embedded in the mucous layer and thereby physically separated from the mucosa, limiting their potential to cause tissue damage.

Another function of alimentary mucus is to provide protection from chemical damage or autodigestion. This feature is especially important in the stomach and anterior duodenum where the mucosa can be exposed to fluids of low pH.

Epithelial cells of the gut produce bicarbonate, which in turn acts in neutralizing acid solutions. Florey (1955), and Heatley (1959) pointed out that acid can penetrate the mucous layer covering the epithelium. They suggested that the hydrogen ions permeating down through the mucous layer would react with bicarbonate secreted by the epithelium and thus be neutralized.

The total production of bicarbonate by intestinal epithelial cells is sufficient to counter solutions at pH of about 4 such as found in the anterior intestine, but it is not sufficient to neutralize solutions of pH 1.5 and below frequently found in the stomach (Allen et al., 1982; Mantle and Allen, 1989). Since hydrogen ions are slowed in traversing the mucous layer, it was suggested that the mucus may act as a mixing barrier rather then a physical barrier to ion movement in the stomach. By acting as an unstirred layer the mucus could prevent the relatively small amounts of bicarbonate from being exposed to the large volume of acid solution associated with the lumen and thus act as a more efficient neutralizer (Allen et al., 1982; Mantle and Allen, 1989).

Protein digesting enzymes like pepsin are associated with the gastric epithelium, raising the threat of autodigestion. Pepsin will not diffuse through the mucous layer over the epithelial cells (Allen et al., 1982). However, a continuous proteolysis of the lumenal surface of the mucous layer does occur, resulting in a continuous degradation of the layer (Allen et al., 1982; Mantle and Allen, 1989). Since mucus is produced as a continuous secretion, it can maintain itself as a barrier even during digestion by pepsin. The cells producing acid and pepsinogen are not

protected by a mucous coat, thus they may be resistant to autodigestion (Mantle and Allen, 1989).

### 1.5.2. Biochemistry of mucus

Purified, undegraded, vertebrate gastrointestinal mucous glycoproteins consist of 60-80% carbohydrate, 15-25% protein and approximately 5% ester sulphate (Mantle and Allen, 1989). These macromolecules range in molecular weight from 1 X $10^6$ to 44 X $10^6$.

The general structure of a mucous glycoprotein molecule consists of a single protein backbone chain which is partly glycosylated (Silberberg and Meyer, 1982; Mantle and Allen, 1989). The amino acid composition is highly characteristic, consisting of either threonine or serine in the glycosylated region and aspartate and large numbers of cysteine residues associated with the non-glycosylated region. Sugar groups are linked to the protein through O-glycosidic bonds to the threonine and serine. The cysteine residues form intra and/or inter-molecular disulphide bonds (Silberberg and Meyer, 1982).

Evidence suggests that the attached sugar groups and the associated presence or absence of acid radicals determine the characteristics of any specific mucin. It is known that mucins may exist as neutral, acid sulphated, acid carboxylated or acid sulphated sialomucins (Bancroft and Cook, 1984). Differences in the sugar composition of the glycoproteins from pig gastric and colonic mucus relate to the proportion of sialic acid residues (Allen et al., 1982). Greater numbers of sialic acid residues are present on the colonic glycoprotein. Significant differences were also

noted between the sugar compositions of glycoproteins in the small intestine and the gastric and colonic mucus.

These observations suggest differences in function, related to the respective areas. The actual nature of these functional differences is not known for most vertebrate groups.

### 1.5.3. Histochemical detection of mucus

The identification and localization of different chemical types of mucus in tissue may be achieved through histochemical stains. The most common procedure for the general detection of carbohydrate containing compounds in fixed tissue, including mucins, is the PAS reaction (Bancroft and Cook, 1984; Pearse, 1985). This reaction will typically stain carbohydrate containing compounds a deep magenta. Technically, substances containing 1,2-glycol groups or their amino or alkylamino derivatives are oxidized by periodic acid to form dialdehydes, which combine with Schiff's reagent to from an insoluble magenta coloured compound.

To further define the chemical nature of mucins, stains like Alcian blue (AB) may be utilized with considerable accuracy. This dye contains positively charged groups capable of salt linkage with certain polyanions. The most common polyanions involved consist of the sulphate and carboxyl radicals of the acid mucins. AB staining solutions of pH values 0.2, 1.0, and 2.5 will stain specifically for highly sulphated acid mucins, both weakly and strongly sulphated acid mucins, and general acid mucins respectively (Bancroft and Cook, 1984). A staining procedure utilizing both AB and

PAS in succession will allow an accurate determination of a full range of chemically variable mucins.

### 1.6. Research Objectives

Morphological adaptations to specialized functions are known to be characteristic of the digestive systems of many vertebrate species and many of these variations are reflective of the mode of feeding or food preference (Stinson and Calhoun, 1993). De Groot (1971) suggested a correlation between gross morphology of the alimentary canal and prey preference in pleuronectid teleost fishes. This apparent correlation raises the possibility that structural differences also appear at the cellular level with relation to feeding in these species. Distinct histological differences have been observed between a bottom feeding fish, the red mullet and a planktonic feeding fish, the *Atherina forskali (Rupp)* (Al-Hussaini, 1946; 1947). These observations suggest the same is true for other marine species as well, thus giving merit to further work of this kind to increase our general understanding of these species.

The recent increased interest in the aquaculture of non-traditionally cultured species has resulted in a demand for more information regarding the anatomy and physiology of these species. Indeed, histological studies of the gut of fish are necessary to assess disease problems, nutritional stress, and environmental toxicity, as well as physiological adaptation to salinity changes (Grau et al., 1992).

In order to facilitate the culture of new species of pleuronectids and to determine whether correlations exist between alimentary canal histology, diet preference and natural environment, the present study compares the gut histology of three species, the winter flounder, the yellowtail flounder and the Atlantic halibut. All have different prey preferences, inhabit different enviromental niches and are candidates of interest to aquaculture.

The objectives of this research are :

1. To compare the morphology of the esophageal and gastric mucosa in the three species of pleuronectid teleost utilizing both light and electron microscopy.

2. To compare the morphology of the pyloric caecal, intestinal, and rectal mucosa in these species utilizing both light and electron microscopy.

3. To compare and identify the types and distribution of mucous cells in the esophagus, stomach, pyloric caeca, intestine and rectum of these fish utilizing specific histochemical techniques and quantitative analysis at the light microscope level.

4. To improve our understanding of the digestive systems of three flatfish species and thereby assist in their evaluation as candidates for marine aquaculture in Canada.

# 2. A COMPARATIVE HISTOLOGICAL STUDY OF THE ESOPHAGUS AND STOMACH IN THREE SPECIES OF PLEURONECTIDAE

## 2.1. Introduction

Several studies have reported the morphology of the alimentary canal of cold water marine teleosts (Mackay, 1929; Bishop and Odense, 1966; Tyler, 1972; Mattisson and Holstein, 1980; Meister et al., 1983; Morrison, 1987; Kjørsvik et al., 1991; Olafsen and Hansen, 1992). However, only a limited number of reports concern pleuronectid species. These include investigations of both adult gut (Dawes, 1929; Trier and Colony-Moxey, 1980; Mcleese and Moon, 1989; Jenkins et al., 1992) and larval or juvenile gut (Macdonald, 1987; Kjørsvik and Reiersen, 1992; Murray et al., 1993). Few studies have compared the histology of the alimentary canal across species of pleuronectids. Studies like these are becoming more valuable, as interest in the culture of these species expands and workers require more information with regard to feeding and nutrition.

The pleuronectidae as a group utilize a wide range of prey species (De Groot, 1971; Braber and De Groot, 1973). Similarly, they also inhabit a wide range of environments extending from shallow, brackish water areas to zones of extreme depth (Scott and Scott, 1988).

The percentage of gut length occupied by the esophagus and stomach in pleuronectids is thought to be important in the determination of prey preference and feeding behavior in the wild (De Groot, 1971; Macdonald, 1987). Piscivorous fish like

the halibut, will grasp relatively large prey intact and digest it almost totally in the stomach, thus they require a large esophagus and stomach for transport, storage and extended digestion time (Davenport et al., 1990). In contrast, invertebrate feeders, like the winter flounder, the yellowtail flounder or the lemon sole take small prey, in combination with indigestible matter, and at a higher frequency, thus they do not have the storage capacity and elasticity that the fish eaters need (Davenport et al., 1990). These variations suggest that histological differences also exist, which may act in accommodating these divergences.

Mucous secretions are an important component of the esophageal and gastric mucosa in fish as well as other vertebrates. Studies have shown that gastric mucus acts as an adherent mucous gel providing a protective buffer zone between the epithelia and the lumenal environment (Allen et al., 1982; Mantle and Allen, 1989). This is especially important in the stomach where low pH and digestive enzymes like pepsin present a constant threat of damage to the underlying epithelial cells. Similarily, the mucus secreted by goblet cells of the esophagus, has been shown to exhibit a complex carbohydrate histochemistry in many species of fish and thus may indicate function other than a barrier to abrasion (Reifel and Travill, 1977).

It is hypothesized that the type of food material and length of stay in the stomach influences the lumenal environment. Where this is true the chemical nature of the protective mucous barrier varies among species to accommodate differences in diet and the rate of gastric emptying, thus correcting for different extremes of the lumenal environment.

The purpose of this study is to compare the histology and mucous histochemistry of the esophageal and gastric regions of pleuronectids known to utilize different prey species in the wild (winter flounder, yellowtail flounder versus Atlantic halibut) and species thought to feed on similar prey (winter flounder and yellowtail flounder). The information gained from this study will aid in determining if differences exist that may be important to consider during the future development of ongrowing diets for these fish in culture.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Fish sampling protocol

#### 2.2.1.1 Atlantic halibut

Halibut were captured from an area approximately 20 km south of Sandyville, on the south coast of Newfoundland, Canada. Fish were taken using standard commercial long line trawls, baited with freshly chopped herring.

Six fish were taken in total, four of which were deemed suitable for tissue sampling. Suitability, was determined by the speed and the ease of handling specimens on board ship. Fish forklength ranged from 79 cm to 169 cm. Fish between 79 and 100 cm were used for tissue sampling. All fish were noted as immature females. Gut tissue was removed immediately following capture.

#### 2.2.1.2 Winter flounder

Winter flounder were captured from an area approximately 5 km northwest of North Rustico, Prince Edward Island, Canada. Fish were taken by gill netting.

Eight fish were sampled in total with an average size of 25 ± 3 cm forklength. Five fish were males and three females. Fish were either processed immediately upon capture or held alive in aerated ambient seawater on board ship until processing was completed.

#### 2.2.1.3 Yellowtail flounder

Yellowtail flounder were captured by divers at an inshore location near Witless Bay, Newfoundland, Canada. These fish were maintained in aerated cold seawater for transport to the Ocean Sciences Centre, Logy Bay, Newfoundland. Following transport, fish were transferred to tanks maintained with flowing ambient seawater around 2 $^{0}$C . Fish were held in this system for a period of 15 days prior to processing. Animals were offered frozen capelin and shrimp on a daily schedule and were kept under normal light conditions.

Six yellowtail flounder were sampled with average forklengths of 28 ± 2 cm. Three fish were males and three were females. All fish were processed systematically following measurements.

### 2.2.2. Fish processing protocol

#### 2.2.2.1 Measurements and observations

Forklengths of fish were taken immediately prior to removing tissue. Lengths of stomach, intestine, and rectum were also obtained for individual fish. Indications of active feeding were based upon presence or absence of food material in stomach or intestine.

#### 2.2.2.2 Sampling of tissue

Fish were euthanized by a blow to the head followed by cervical severance. The body cavity of the winter flounder and the yellowtail flounder, was immediately opened via an incision beginning at the vent and running dorsally to the lateral line and then cranially until a flap of tissue could be folded away exposing the viscera. Esophagus, cardiac stomach, and pyloric stomach were identified by gross anatomical appearance either *in situ* or after subsequent removal. The Atlantic halibut were eviscerated by a fisherman, eliminating the opportunity to obtain samples of esophagus from this species.

Bands of tissue from 0.25-0.5 cm in length were removed from the above areas according to the following criteria:

1. Samples of esophagus were removed from an area immediately adjacent and cranial to the gastro-esophageal junction. 2. Samples of cardiac and pyloric stomach were removed from areas immediately adjacent to and approximately 0.5 cm caudal to the gastro-esophageal junction, and approximately 0.5 cm anterior to the gastro-intestinal junction, respectively.

Following removal, tissue was immediately immersed in approximately 10 ml of cold fixative. Tissues were fixed for 24 to 72 hours at 4 $^{0}$C in either Bouin's fluid, 10% neutral buffered formalin, modified Karnovsky's fixative, or glutaraldehyde (Appendix A).

### 2.2.3. Tissue processing

#### 2.2.3.1 Electron microscopy

Tissue for electron microscopy was trimmed and washed three times in either cold 0.06M or 0.1M sodium cacodylate buffer at pH 7.2 or 7.3 respectively. The tissue was post-fixed for two hours in 1% osmium tetroxide in the same buffer at 4 $^0$C. Tissue was next dehydrated in an ascending series of ethanols (50%, 70%, 95%, 100%) cleared in propylene oxide and infiltrated with Epon 812/Araldite 502 resin (Marivac Ltd., Halifax, N.S.). Following infiltration overnight in a desiccator, individual pieces of tissue (~ 2 mm$^2$) were embedded in fresh resin. Polymerization of resin into blocks was carried out in a 60 $^0$C oven for approximately eight hours. Six blocks per region were produced for each of eight winter flounder, four halibut, and six yellowtail flounder.

Three sections (0.5 $\mu$m thick) were cut from each of the six blocks on a Reichart-Jung Ultracut E ultramicrotome using glass knives, mounted on glass slides and stained with 1% toluidine blue in 1% borax. After viewing all sections using a compound microscope for optimal tissue orientation and quality, the three best blocks were chosen for thin sections. Three prethin (0.5 $\mu$m thick) sections and nine ultrathin sections (70-90 nm thick) were cut from each block with glass knives. Three of the ultrathin sections were picked up on each of three 200 mesh, uncoated, copper grids. Sections were contrasted with uranyl acetate and Sato lead stain (Sato, 1968). Grids were examined using a Hitachi H-600 electron microscope operated at 75 kV. Micrographs were taken on Kodak Electron Microscope Polyester sheet film, and

developed in Kodak D-19. Final prints were produced on Kodak Polycontrast Resin Coated paper with an automatic print processor.

Dimensions of specific structures from electron micrographs eg. granules and membrane projections, were taken by a metric ruler, measuring length or diameter of twenty representative examples of the object of interest in at least five micrographs. The mean and standard error of each set of measurements was recorded.

#### 2.2.3.2 Light microscopy

Tissue for light microscopy was washed three times in 70% ethanol for 1 hour each, dehydrated through ascending ethanol series, (70%, 95%, 100%) cleared in two washes of xylene, and finally infiltrated with and subsequently embedded in paraffin wax.

Paraffin sections (6 - 8 $\mu$m thick) were cut on a A.O. Spencer 820 rotary microtome, floated on warm distilled water and picked up on glass slides. Slides were dried overnight on a slide warmer (~ 55 °C) and then stored in slide boxes at room temperature, prior to staining. Sections were stained with either hematoxylin and eosin (H & E), AB pH 1.0 or 2.5, or AB/PAS pH 1.0 or 2.5 (Bancroft and Cook 1984). Sections representing similar regions from all the species were stained in the same lot, and at the same time to reduce variability in stain intensities due to different staining runs. Stains and their representative reactions are noted in Appendix B. Positive controls consisted of tissue sections from rainbow trout

intestine, rat intestine and lamprey cartilage. These were know to give positive reactions for AB/PAS.

Following staining, sections were examined for descriptive histology or histochemical reactions. Representative areas were photographed using an Olympus BH2 compound microscope and a Zeiss Photomicroscope III, respectively.

**2.2.3.3 Criteria for mucous histochemistry staining reactions**

Positive reactions for mucous cell histochemistry are described as follows:

(A). AB pH 2.5 : reactions ranging from light to dark blue were considered positive for all acid mucins.

(B). AB pH 1.0 : reactions ranging from light to dark blue were considered positive for sulphated acid mucins.

(C). AB pH 2.5 or 1.0 followed by periodic acid-Schiff reagent : i) A reddish purple reaction at AB/PAS pH 2.5 was considered positive for a combination of neutral and acid mucins. A parallel reaction at pH 1.0 giving the same deep red to reddish purple result was considered positive for a combination of sulphated acid mucin and neutral mucin or nonsulphated acid mucin.

ii) A reddish purple reaction at AB/PAS pH 2.5 with a parallel reaction at pH 1.0 giving a magenta result, was considered positive for a combination of non-sulphated acid mucin and neutral mucin.

iii) A magenta reaction at AB/PAS pH 2.5 was considered as an indicator of the presence of neutral mucins.

## 2.3. RESULTS

### 2.3.1. Light microscopy

#### 2.3.1.1 Esophagus

The general structure of the posterior esophagus was similar between the winter and the yellowtail flounders. It consisted of a series of distinct tissue layers, the mucosa, the propria-submucosa, the muscularis externa, and the serosa. An obvious muscularis mucosae which subdivides lamina propria from submucosa was not observed in the esophagus of either species. In subsequent situations where a muscularis mucosae is not present, the connective tissue adjacent to the epithelia will be referred to as a propria-submucosa.

The mucosa was organized into a series of large, complex, branching folds consisting of an epithelial layer and an adjacent propria-submucosa (Figure 2.1). The epithelium was composed of two cell types, goblet cells and cuboidal epithelial cells (Figure 2.2).

In the zone between the mucosal folds the epithelia was composed of a stratified layer of cuboidal epithelial cells interspersed with goblet cells (Figure 2.2). Towards the distal end of the mucosal folds, the extent of the stratification of the cuboidal epithelium appeared to increase from two layers at the lowest point between the folds to several layers at the tips of the folds. This feature may be a function of the plane of section. The apparent density of goblet cells in many cases, also decreased as one moved from between the folds to the tips.

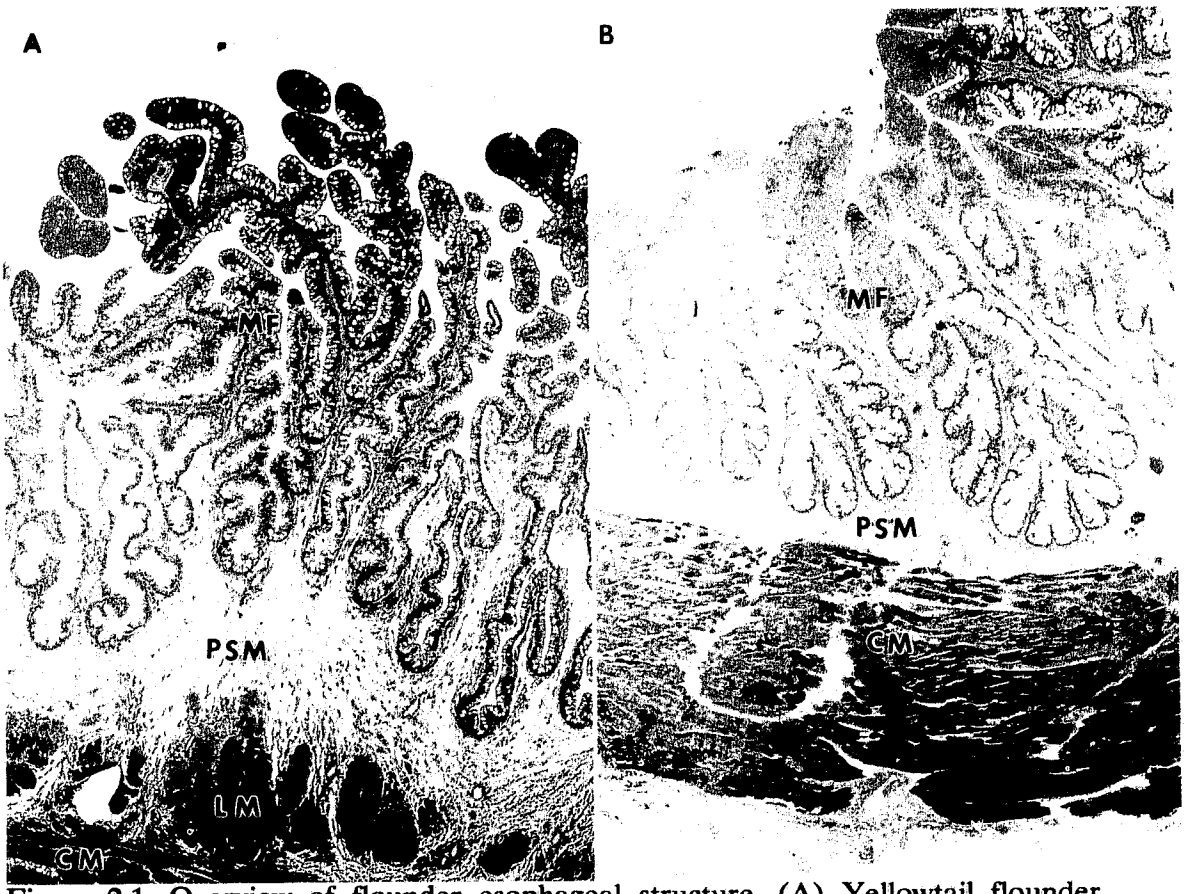


Figure 2.1. Overview of flounder esophageal structure. (A) Yellowtail flounder esophagus (B) Winter flounder esophagus. MF, mucosal folds, PSM, propria-submucosa, LM, longitudinal bundles of muscle, CM, circular muscle. (X40).

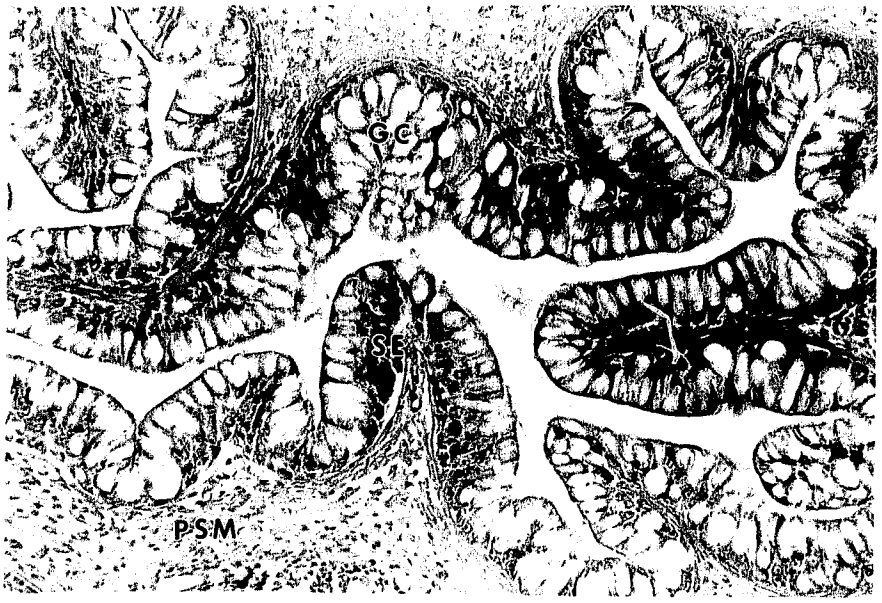



Figure 2.2. High magnification of an esophageal mucosal fold from the yellowtail flounder, demonstrating abundant goblet cells (GC) and stratified epithelial cells (SE). PSM, propria-submucosa. (X250).

The propria-submucosa immediately adjacent to the epithelium appeared slightly denser then that more distal. Both zones had a moderate degree of vascularization. The majority of the propria-submucosa was composed of a loose connective tissue in the yellowtail flounder, and an irregular dense connective tissue in the winter flounder (Figure 2.1).

Bundles of striated, longitudinally arranged muscle were also present in the propria-submucosa of both flounders, but not in association with the externa (Figure 2.1). The muscularis externa of the esophagus in both flounders was composed of one thick layer of circular striated muscle.

A relatively thin adventitia was observed adjacent to the muscularis externa in both flounders. This layer was composed of eosinophilic collagen fibers and was frequently found to be associated with large nerve bundles in the yellowtail flounder.

The transition from esophagus to cardiac stomach was abrupt in both species. Minor folds were observed with one side exhibiting an esophageal-type mucosa and the other a distinctly gastric mucosa.

**2.3.1.2 Stomach**

The stomachs of the Atlantic halibut, winter flounder and the yellowtail flounder were organized in a similar fashion based upon the general layered theme of the typical gut plan. Like the esophagus, the layers consisted of the mucosa, submucosa, muscularis externa, and the serosa.

In general, the gastric mucosa in all three species was found to be organized into folds composed of a compact connective tissue lamina propria and an

epithelium. In the winter flounder, these folds had a scalloped or serrated lumenal surface and appeared more elongated than those found in the other species (Figure 2.3). In contrast, the gastric folds of the halibut and the yellowtail flounder appeared shorter and exhibited a relatively smooth lumenal surface.

Based upon their histology, the stomach of the yellowtail flounder and the winter flounder could be divided into two distinct regions, a cranial glandular or cardiac stomach and a caudal aglandular or pyloric stomach. The cardiac stomach appeared to encompass approximately 3/4 of the stomach area in these fish. In comparison, the halibut stomach did not appear to possess a corresponding aglandular zone.

The gastric folds of the cardiac region in all three species could be divided into three distinct zones (Figure 2.4). Zone 1 consisted of surface mucous cells exhibiting a columnar shape and basal nuclei with an eosinophilic apical cytoplasm (Figure 2.5). Deeper into the clefts and between folds, in Zone 2, the surface cells give rise to columnar mucous neck cells with basal nuclei but exhibiting a very translucent apical cytoplasm (Figure 2.6). Zone 3 was characterized by the presence of simple, branched tubular glands (Figure 2.7). The glands were composed of only one type of cell which was pyramidal in shape with a basal nucleus and a very granular, eosinophilic apical cytoplasm.

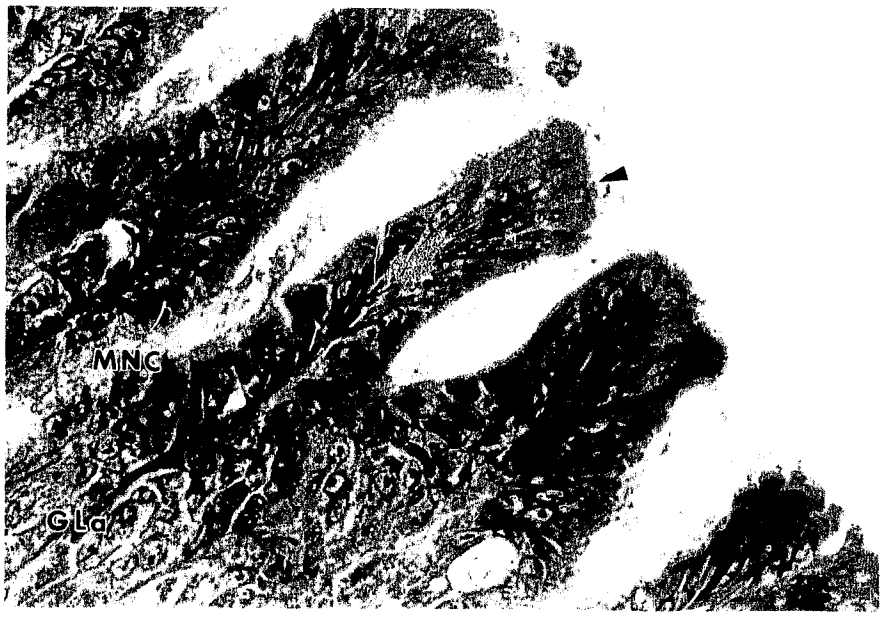



Figure 2.3. Micrograph of winter flounder gastric mucosal folds from the cardiac region, demonstrating a serrated surface (arrowhead). GLa, gastric glands, MNC, neck cells (X660).

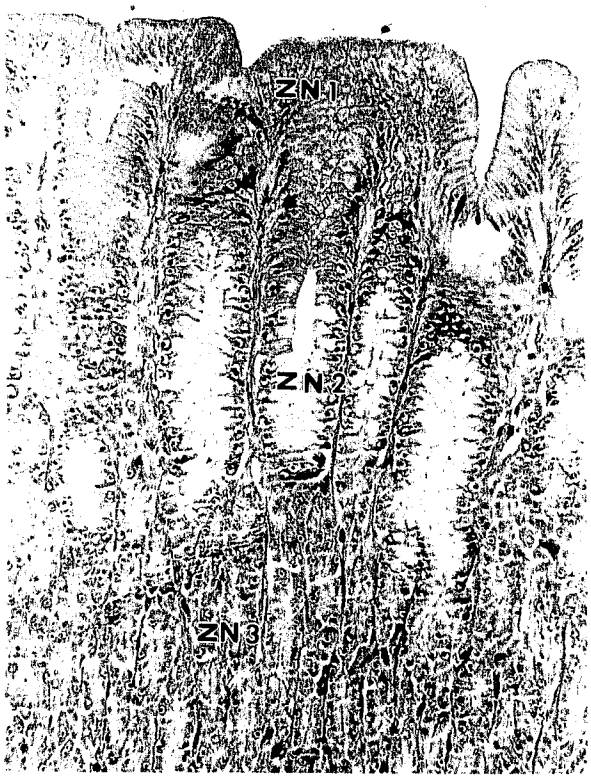



Figure 2.4. Overview of the cardiac stomach in the yellowtail flounder demonstrating the three cellular zones associated with the cardiac region. ZN1, zone 1: surface mucous cells, ZN2, zone 2: mucous neck cells, ZN3, zone 3: gastric glands (X250).

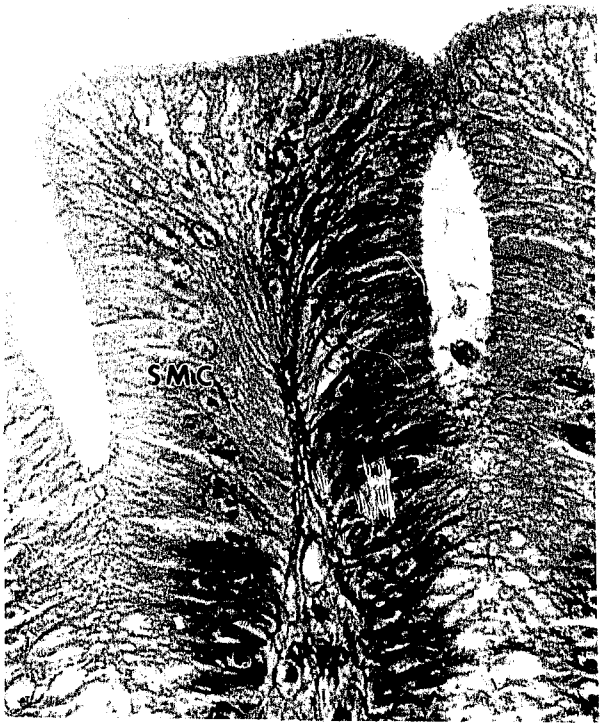



Figure 2.5. Detail of a mucosal fold from Zone 1 in yellowtail flounder cardiac stomach, showing the characteristic surface mucous cells (SMC) typically found in this Zone (X660).

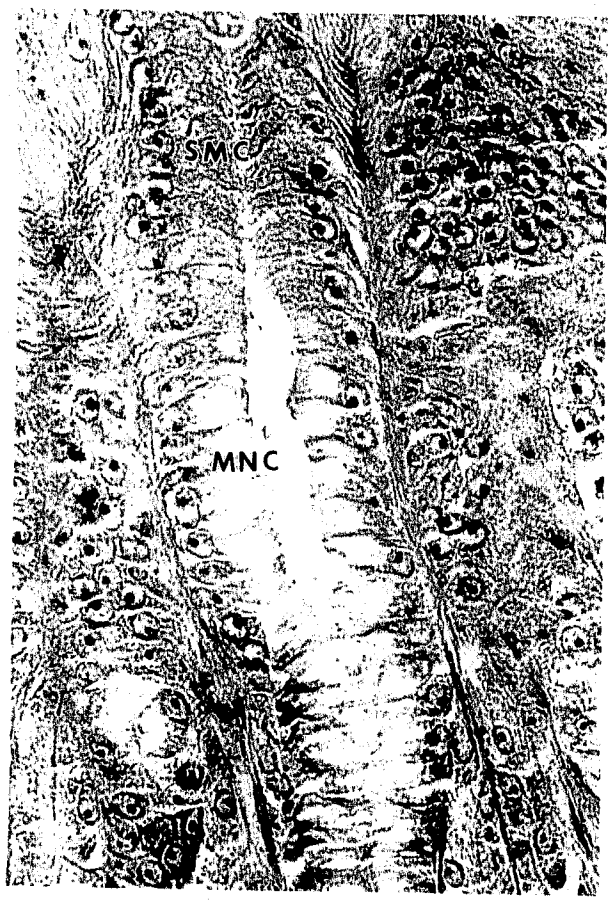



Figure 2.6. Detail of Zone 2 from yellowtail flounder cardiac stomach, demonstrating typical mucous neck cells (MNC), characterized by a clear apical cytoplasm and basal nucleus. SMC, surface mucous cells (X660).

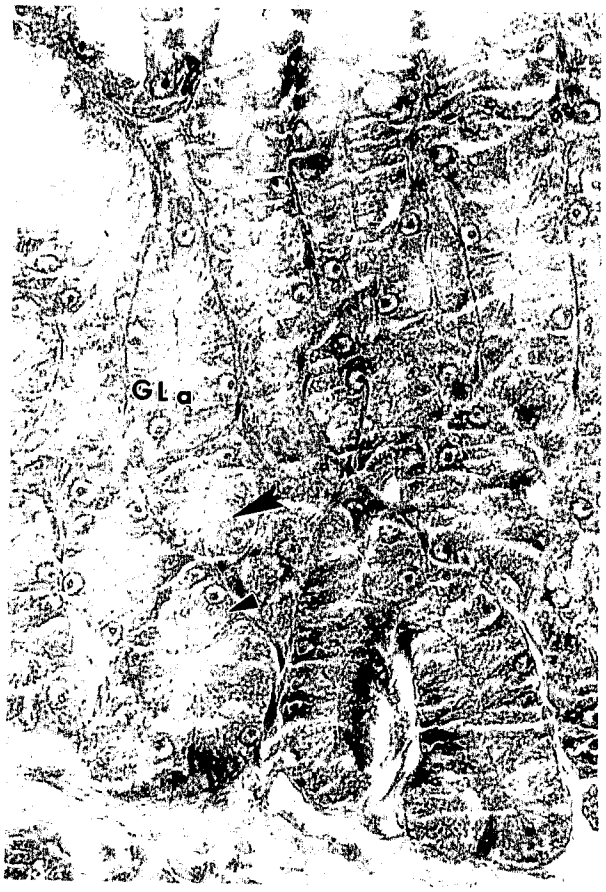



Figure 2.7. Detail of Zone 3 from yellowtail flounder cardiac stomach, demonstrating the tubular gastric gland (GLa) configuration and gland cells (arrowheads)(X660).

The structure of the submucosal layer found immediately beneath the Zone 3 glandular layer showed some variation between species. In the yellowtail flounder, a distinct muscularis mucosae of smooth muscle was observed adjacent to the glandular region (Figure 2.8). The submucosa was composed of a vascularized, loose connective tissue. No distinct demarcation, such as a muscularis mucosae, was observed between the lamina propria and submucosa in the winter flounder and the halibut stomachs (Figure 2.9). In the winter flounder, the propria-submucosa near the glandular zone was a dense irregular connective tissue but more distally it was composed of a loose connective tissue (Figure 2.10). This feature appeared consistent between the cardiac and pyloric areas. The propria-submucosa in the Atlantic halibut was very different from that of the other two species. It was composed of a dense irregular connective tissue, infiltrated with strands of smooth muscle with little vascularization and innervation (Figure 2.9).

The muscularis externa appeared similar in organization between the three species. In general, it consisted of an inner circular and an outer longitudinal layer of smooth muscle. In the cardiac region the circular layer of smooth muscle was occasionally infiltrated by striated muscle extending from the muscularis externa of the esophagus. In the pyloric stomach the circular layer appeared thicker in comparison to that of the cardiac region due to the appearance of the pyloric sphincter (Figure 2.11).

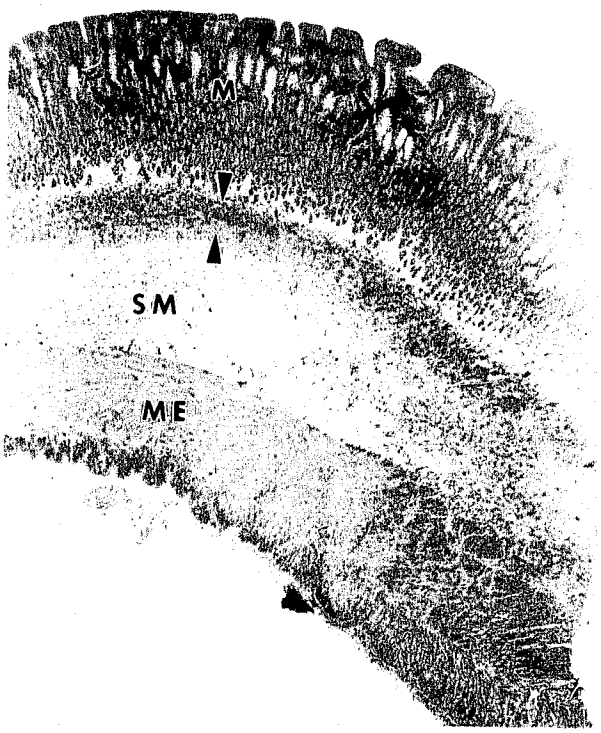



Figure 2.8. Overview of the tissue layers associated with the cardiac stomach in the yellowtail flounder demonstrating the mucosa (M), submucosa (SM), a distinct muscularis mucosae (between arrowheads) and a muscularis externa (ME) (X40).

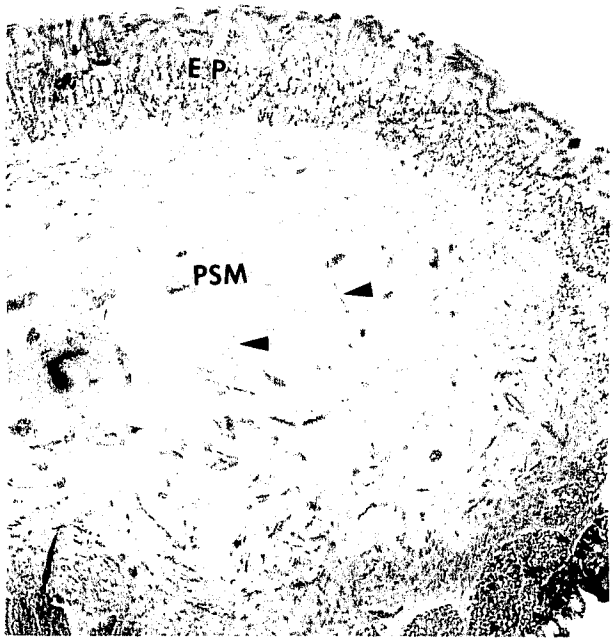



Figure 2.9. Overview of the connective tissue propria-submucosa (PSM) associated with the stomach of the Atlantic halibut illustrating the dense nature of the tissue adjacent to the Epithelial Zones (EP). Note infiltration by smooth muscle fibers (arrowheads) (X40).

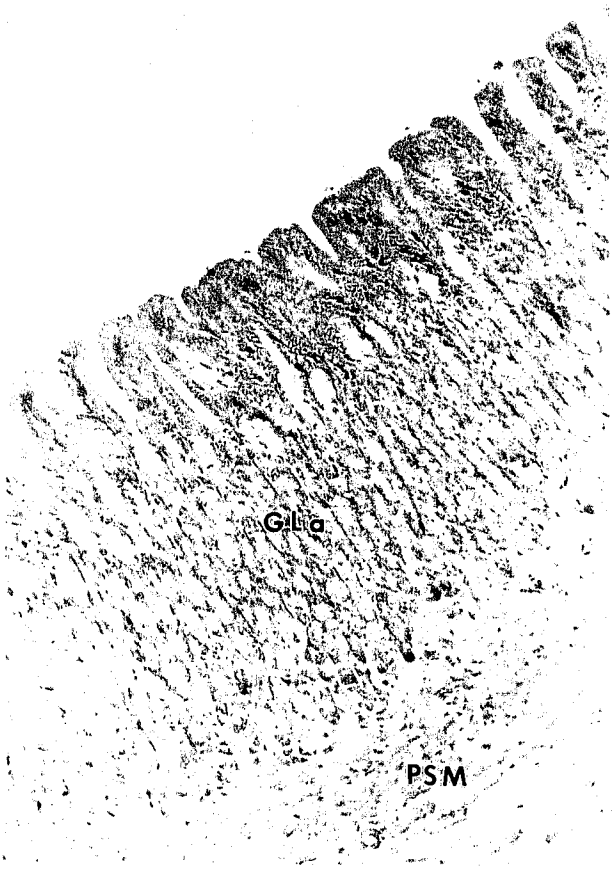



Figure 2.10. Detail of propria-submucosal (PSM) connective tissue next to Zone 3 in the winter flounder cardiac stomach showing the compact nature of the connective tissue adjacent to the gastric glands (GLa) (X250).

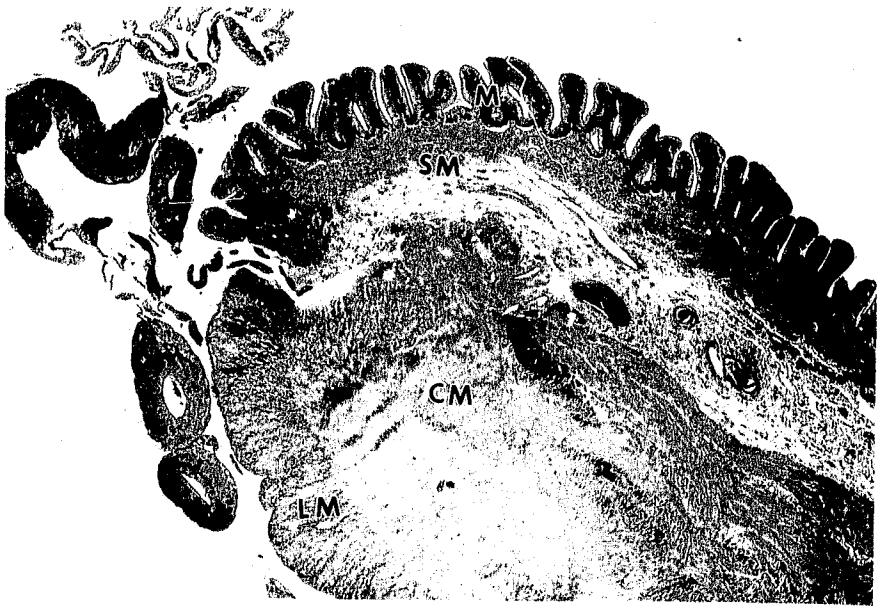



Figure 2.11. Overview of the tissue layers associated with the pylorus of the yellowtail flounder showing the typical thickened inner layer of circular muscle (CM) identified with the development of the pyloric sphincter. LM, longitudinal muscle, SM, submucosa, M, mucosa (X40).

### 2.3.2. Mucous Histochemistry

#### 2.3.2.1 Esophagus

The cells associated with the posterior esophageal mucosa of the winter and yellowtail flounders gave comparatively different histochemical results for mucus when stained at the same time with the same mucous histochemistry protocol (Table 2.1; Figure 2.12-2.14). Both the esophageal epithelial and goblet cells showed some positive staining reactions.

Staining results revealed that both the goblet cells and the surface epithelium of the winter flounder stained positive only for sulphated acid mucins (Figure 2.12 and 2.14). In the yellowtail flounder, the goblet cells were observed to stain positive for both acid nonsulphated and sulphated acid mucins (Figure 2.13). The surface epithelial cells, however, were positive for only acid nonsulphated mucins.

The colour of the AB staining reaction was also observed to vary between species, being blue/green in the winter flounder (Figure 2.12) versus a deep blue in the yellowtail flounder (Figure 2.13). Surface epithelial cells stained similar to that of the goblet cells in the winter flounder and the yellowtail flounder (Figure 2.14 A and B).

#### 2.3.2.2 Stomach

The mucous cells associated with the stomach of the Atlantic halibut, winter flounder and yellowtail flounder exhibited different histochemical reactions for mucus, suggesting that the chemical nature of the gastric mucus present varied between species.

TABLE 2.1. MUCOUS HISTOCHEMISTRY : ESOPHAGUS

| | Winter flounder | | Yellowtail flounder | |
|---|---|---|---|---|
| | Goblet cells | Surface epithelia | Goblet cells | Surface epithelia |
| Neutral mucins | - | - | - | - |
| Nonsulphated acid mucins | - | - | + | + |
| Sulphated acid mucins | + | + | + | – |
| Cellular combinations | – | – | – | – |


Figure 2.12. Esophageal mucosal folds from the winter flounder exhibiting differential staining of goblet cell mucus. Tissue stained with AB/PAS. Note the regular light blue/green staining of the goblet cells (arrowheads) at both (A) pH 2.5 (X250) and (B) pH 1.0 (X100).

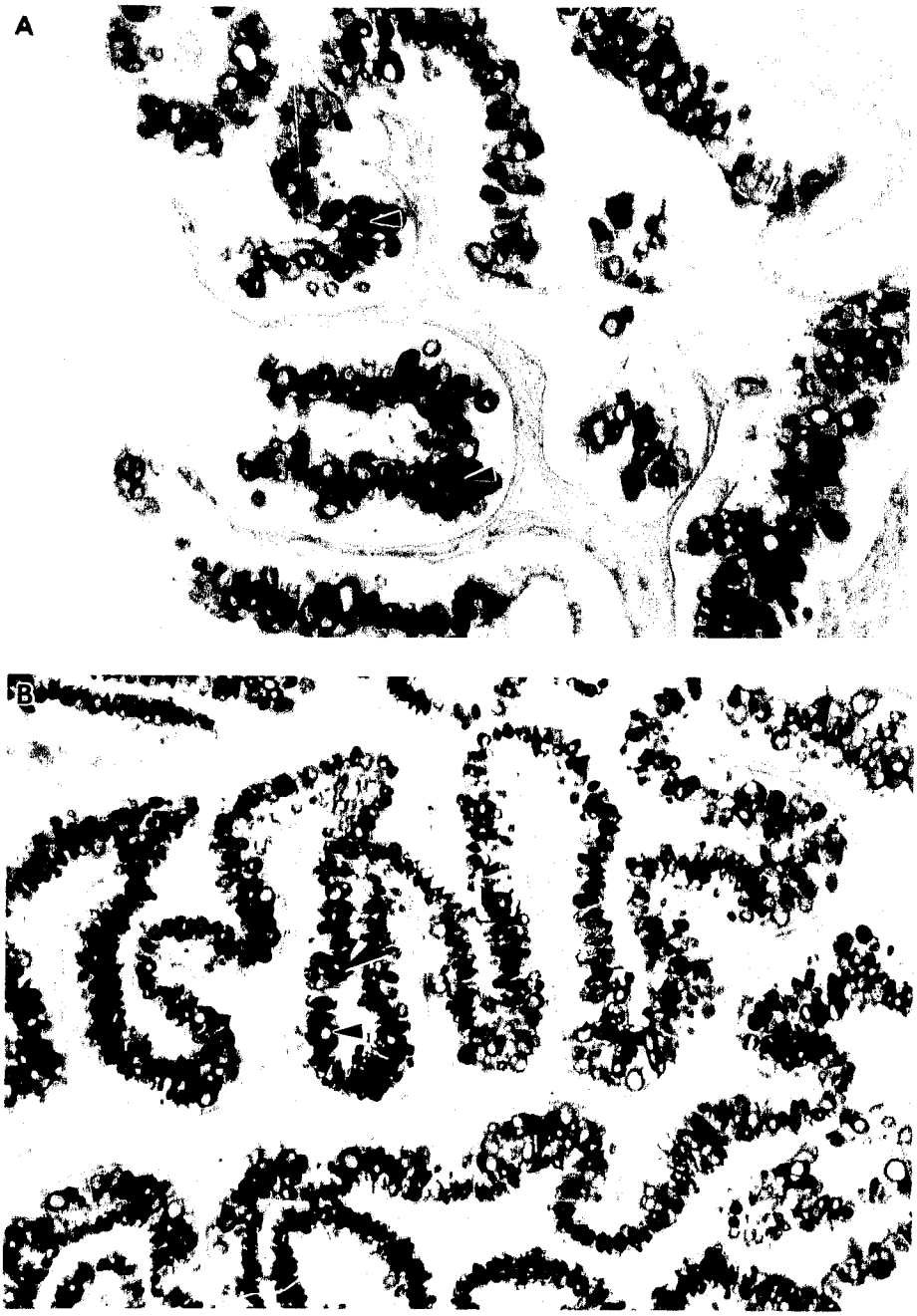



Figure 2.13. Esophageal mucosal folds from the yellowtail flounder exhibiting differential staining of goblet cell mucus. (A) Tissue stained with AB/PAS, pH 2.5. Note the regular deep blue to purple staining of the goblet cells (arrowheads). (X250). (B) Tissue stained with AB/PAS, pH 1.0. Note the presence of both magenta and blue/purple staining goblet cells within the same epithelium (arrowheads) (X165).


Figure 2.14. High magnification of the tips of esophageal mucosal folds showing histochemical staining of esophageal surface secreting cells (arrowheads). (A) yellowtail flounder fold stained with AB/PAS, pH 1.0 (X660) (B) winter flounder fold stained with AB/PAS, pH 1.0 (X660).

#### 2.3.2.2.1 Atlantic halibut

The supranuclear region of the surface mucous cells associated with the halibut gastric mucosa stained moderately for within cell combinations of neutral and nonsulphated acid mucins. The mild positive reaction with AB at pH 2.5 and no reaction with AB at pH 1.0, indicated that nonsulphated acid mucins appear to make up only a small proportion of the total mucus in the cell (Table 2.2; Figures 2.15 and 2.16).

Mucous neck cells exhibited a similar reaction to that of the surface mucous cells, staining positive for cellular combinations of neutral and nonsulphated acid mucins (Figure 2.15). No reaction with AB at pH 2.5 or 1.0 indicated a dominance of neutral mucins (Figure 2.16).

The apical portion of the cells associated with the gastric glands of this fish exhibited a slightly positive reaction with PAS, suggesting the presence of neutral staining mucins.

#### 2.3.2.2.2 Winter flounder

Staining reactions for the surface mucous cells associated with the gastric mucosa of the cardiac and pyloric regions of the winter flounder stomach, indicated the presence of neutral mucins only. The reaction in this zone appeared isolated to the most apical region of the gastric cells (Table 2.2; Figures 2.17, 2.18, and 2.19).

The mucous neck cells of the winter flounder stained similar to the surface cells, exhibiting a positive reaction for neutral mucins (Figure 2.19). Like that

TABLE 2.2. MUCOUS HISTOCHEMISTRY : STOMACH

| | Atlantic halibut | | | Winter flounder | | | Yellowtail flounder | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sur | Nec | Gla | Sur | Nec | Gla | Sur | Nec | Gla |
| Neutral mucins | - | + | + | + | + | + | - | - | + |
| Nonsulphated acid mucins | – | – | – | – | – | – | – | – | – |
| Sulphated acid mucins | – | – | – | – | – | – | – | – | – |
| Cellular combinations | + | – | – | – | – | – | + | + | – |

Table codes : Sur = Surface mucous cells

Nec = Neck mucous cells

Gla = Gland cells

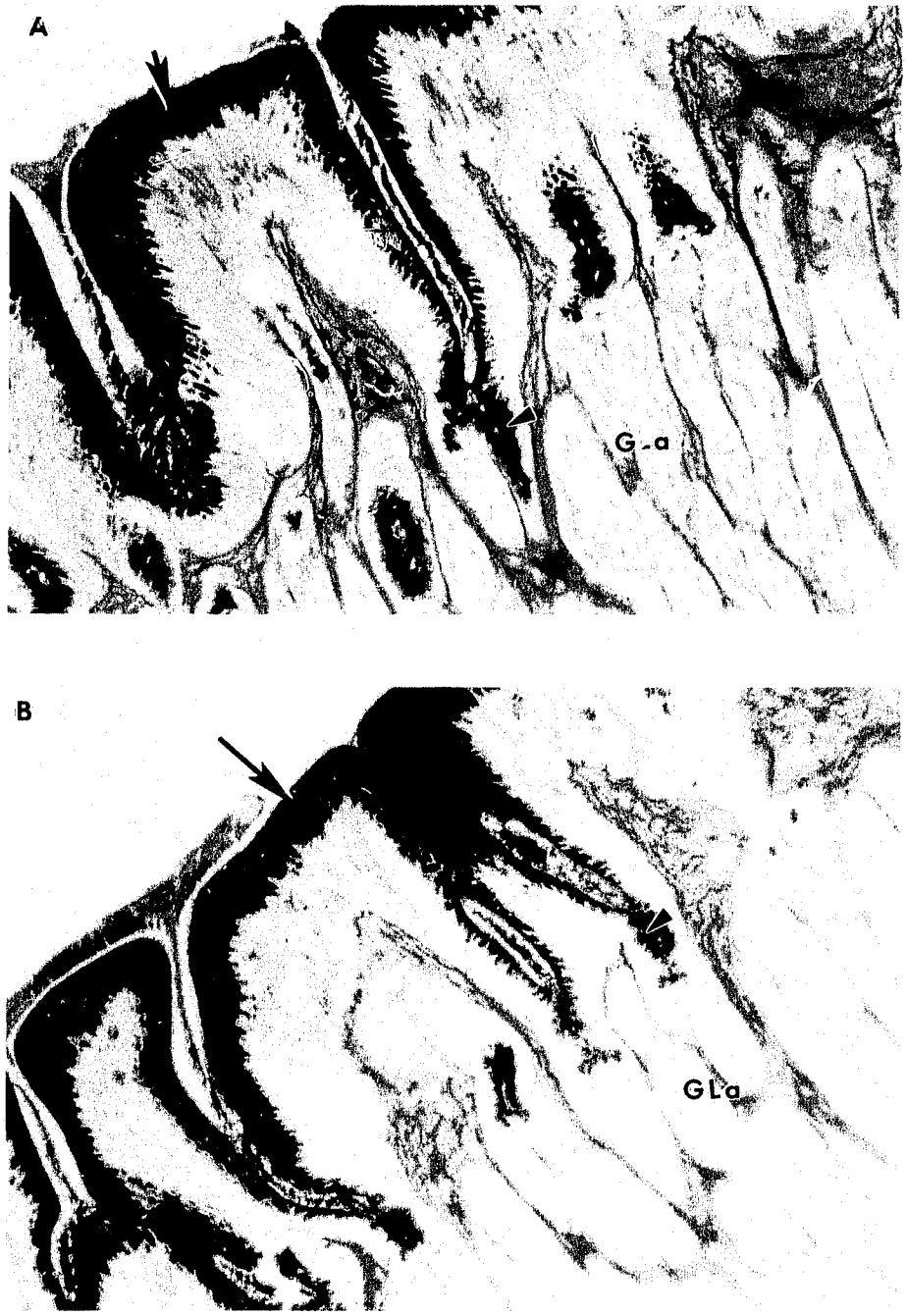



Figure 2.15. Alcian blue/PAS differential staining of gastric surface cell (arrows) and neck cell mucus (arrowheads) from the Atlantic halibut. GLa, gastric glands. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

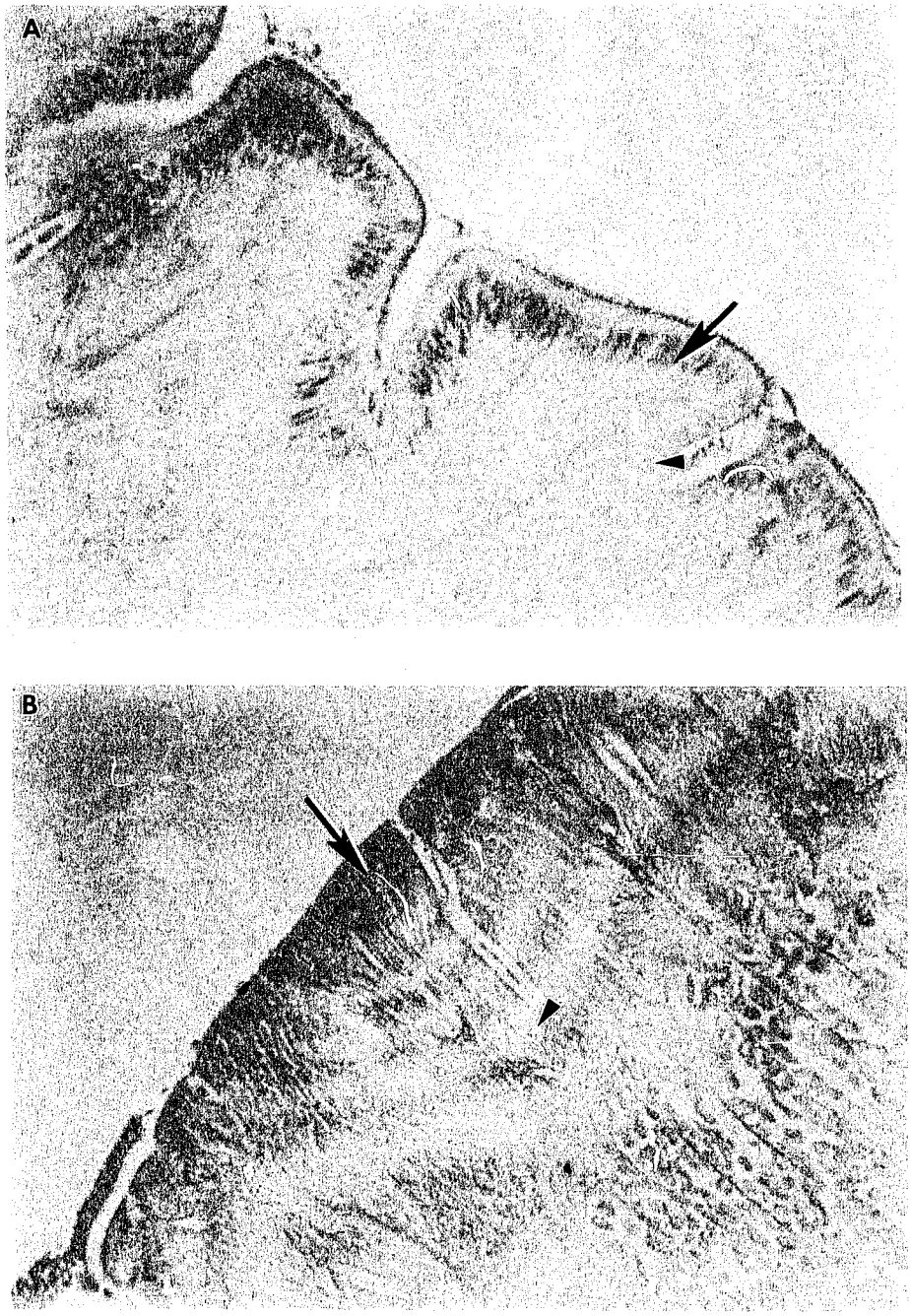



Figure 2.16. Alcian Blue differential staining of gastric surface cell (arrows) and neck cell mucus (arrowheads) from the halibut. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

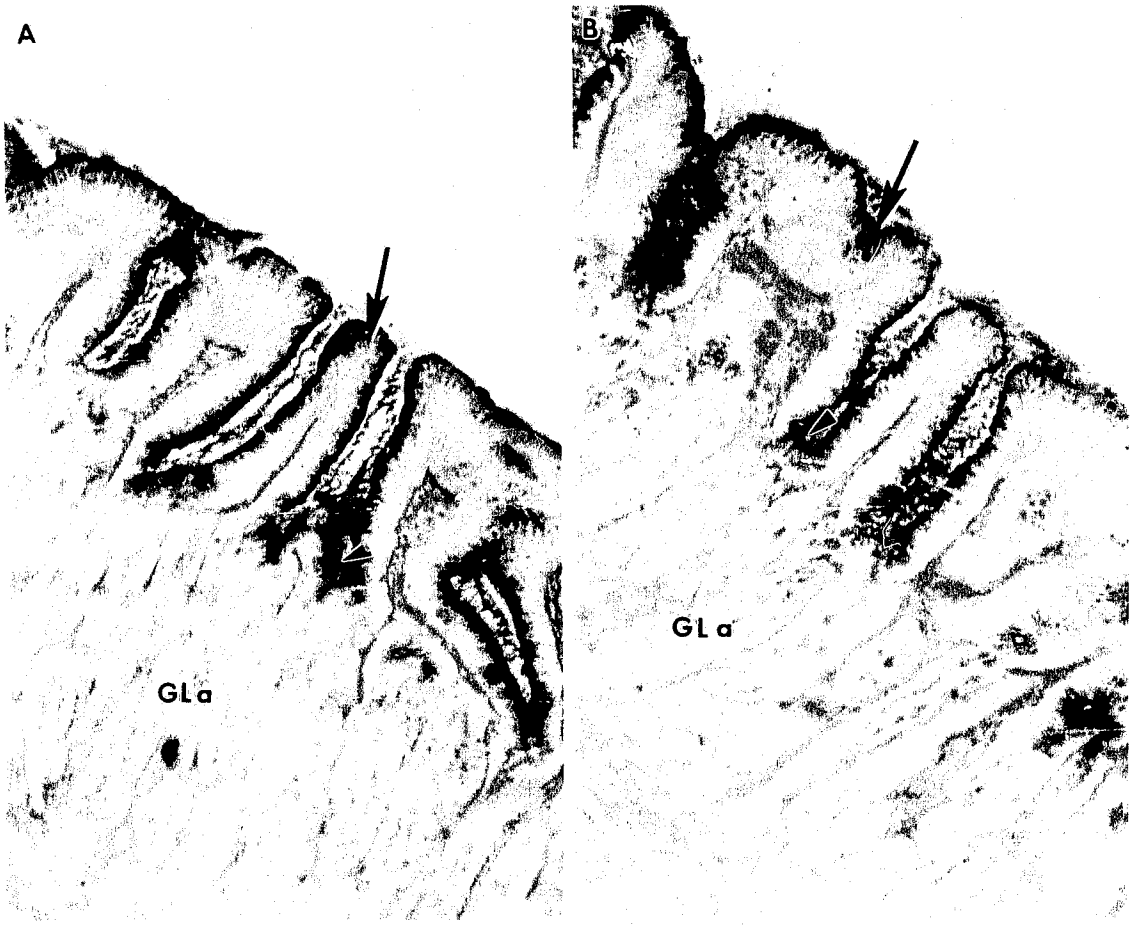



Figure 2.17. Alcian Blue/PAS differential staining of gastric surface cell (arrows) and neck cell mucus (arrowheads) from the winter flounder. GLa, gastric glands. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

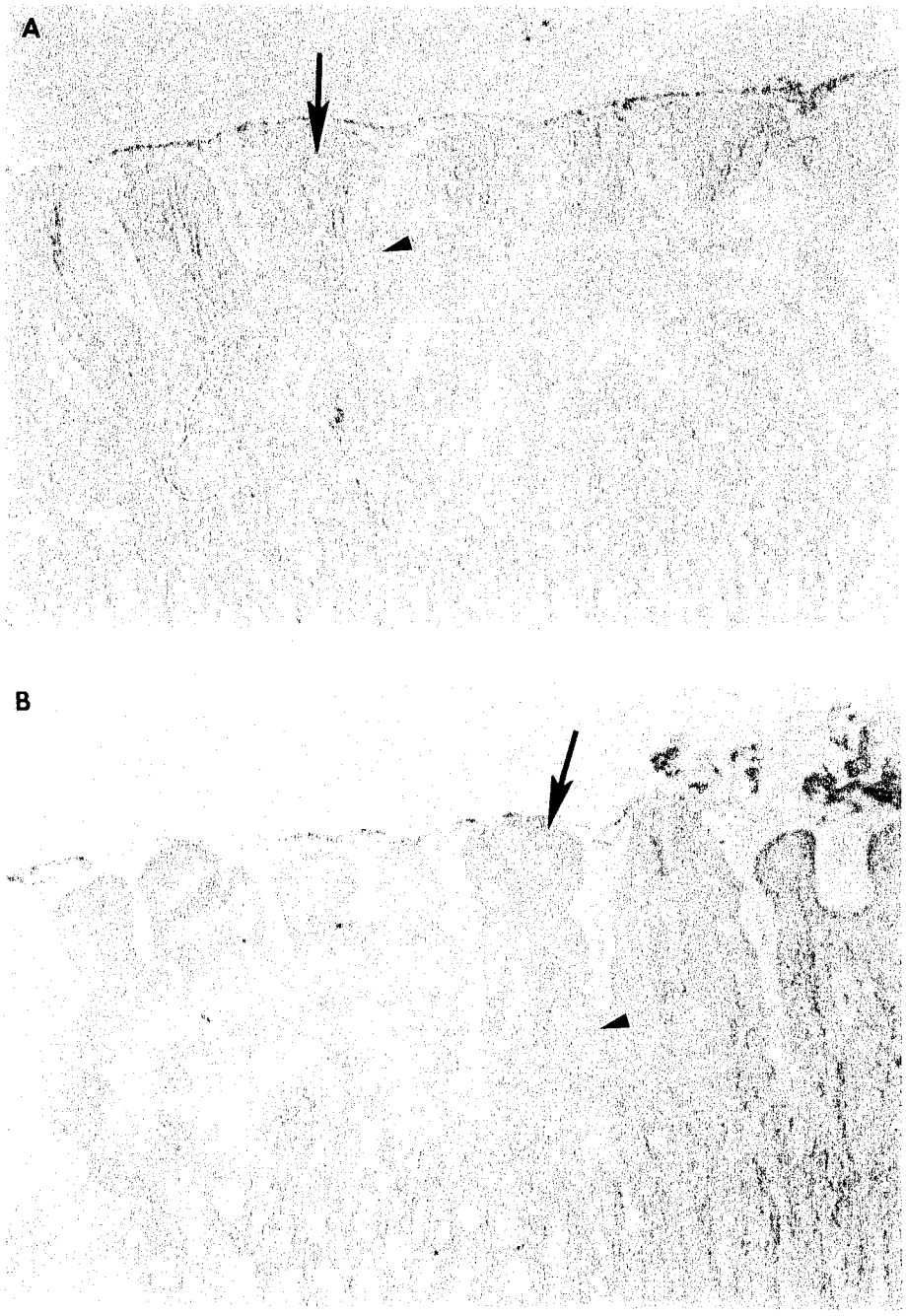
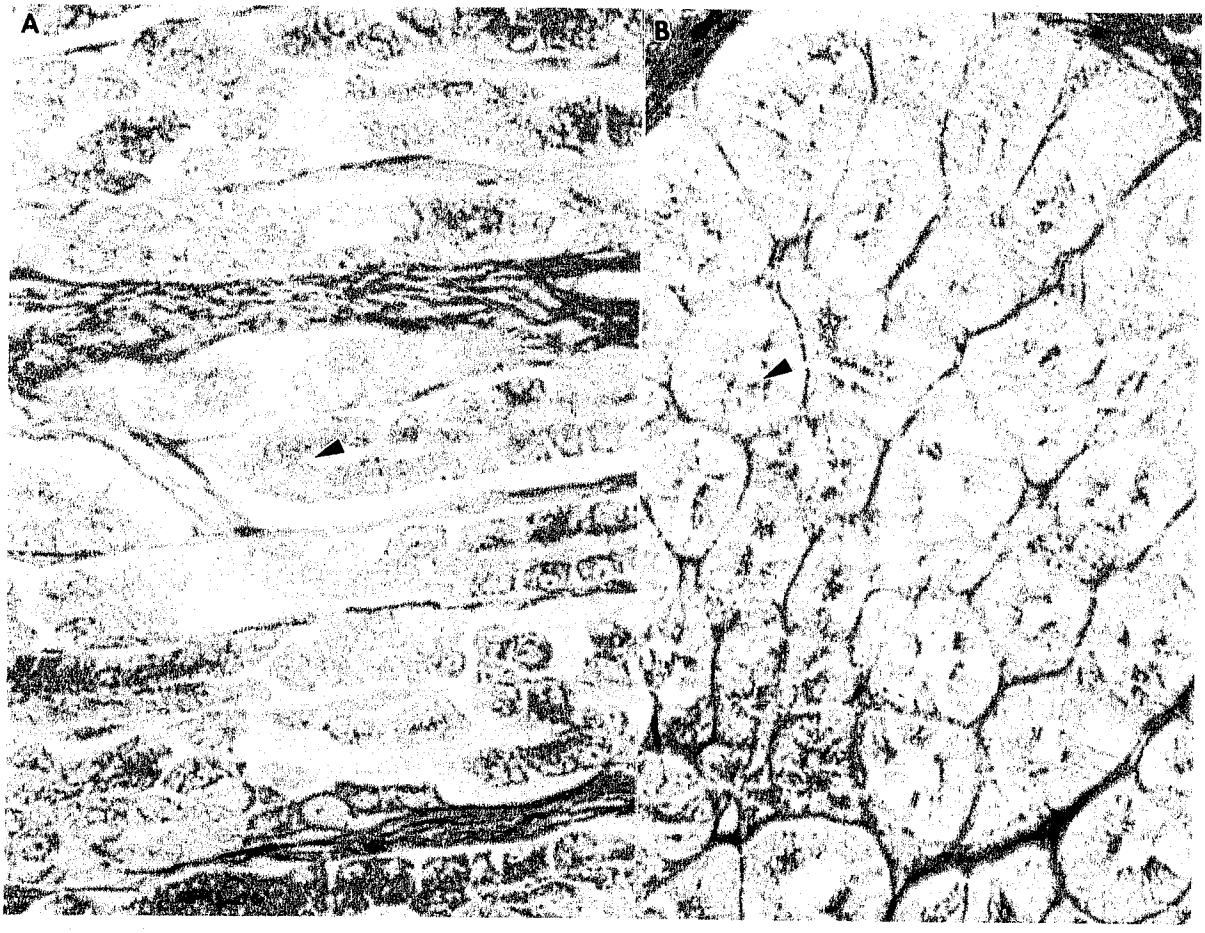



Figure 2.18. Alcian Blue differential staining of gastric surface cell (arrows) and neck cell mucus (arrowheads) from the winter flounder. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

Figure 2.19. Alcian Blue/PAS pH 1.0 differential staining of gastric surface cell from the winter flounder pyloric stomach (arrows) (X250).

Figure 2.20 A-C: Alcian blue/PAS staining of gastric gland cells from (A) the halibut, (longitudinal section) (B) the winter flounder (transverse section) (C) yellowtail flounder (longitudinal section). Note magenta staining at the apex of the glandular cells (arrowheads) (X660).


A
B


C

observed in the halibut neck cells, the reaction product in the winter flounder was dispersed through the cytoplasm as apposed to being isolated in one distinct area.

The apical portions of the gastric gland cells were also stained weakly positive for neutral mucins (Table 2.2; Figure 2.20).

#### 2.3.2.2.3 Yellow tail flounder

The entire supranuclear zone of the surface mucous cells in both the cardiac and pyloric stomachs stained intensely positive for combinations of neutral and nonsulphated acid mucins (Table 2.2; Figures 2.21-2.24). The intensity of the AB reaction suggests a larger proportion of acid mucins then that observed in the other two species. Weak staining for AB at pH 1.0 also suggested the presence of some sulphated acid mucin. The distribution of the staining reaction was observed to be similar to that in the halibut surface cells.

The mucous neck cells of the cardiac stomach stained strongly for neutral mucins. When stained with AB at pH 2.5 or 1.0 without PAS, similar cells showed weak staining for both nonsulphated and sulphated acid mucins. As was noted in the previous species the staining product was found to be dispersed throughout the cytoplasm (compare Figures 2.16, 2.19, 2.21). The apical regions of the glandular cells stained weakly positive for neutral mucins.

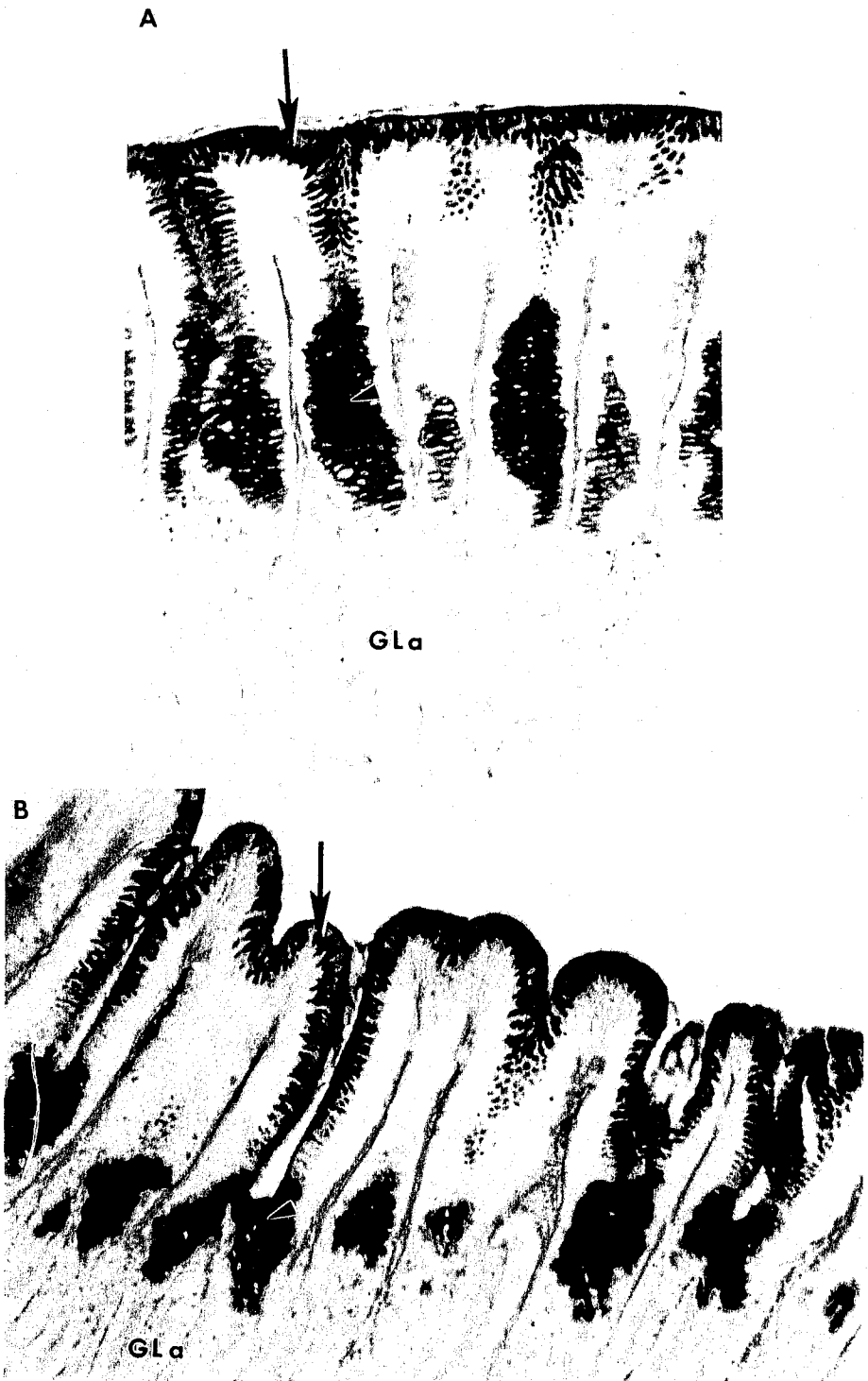



Figure 2.21. Alcian Blue/PAS differential staining of gastric surface cell (arrows) and neck cell mucus from the yellowtail flounder (arrowheads). GLa, gastric glands (A) pH 2.5 (X250) (B) pH 1.0 (X250).

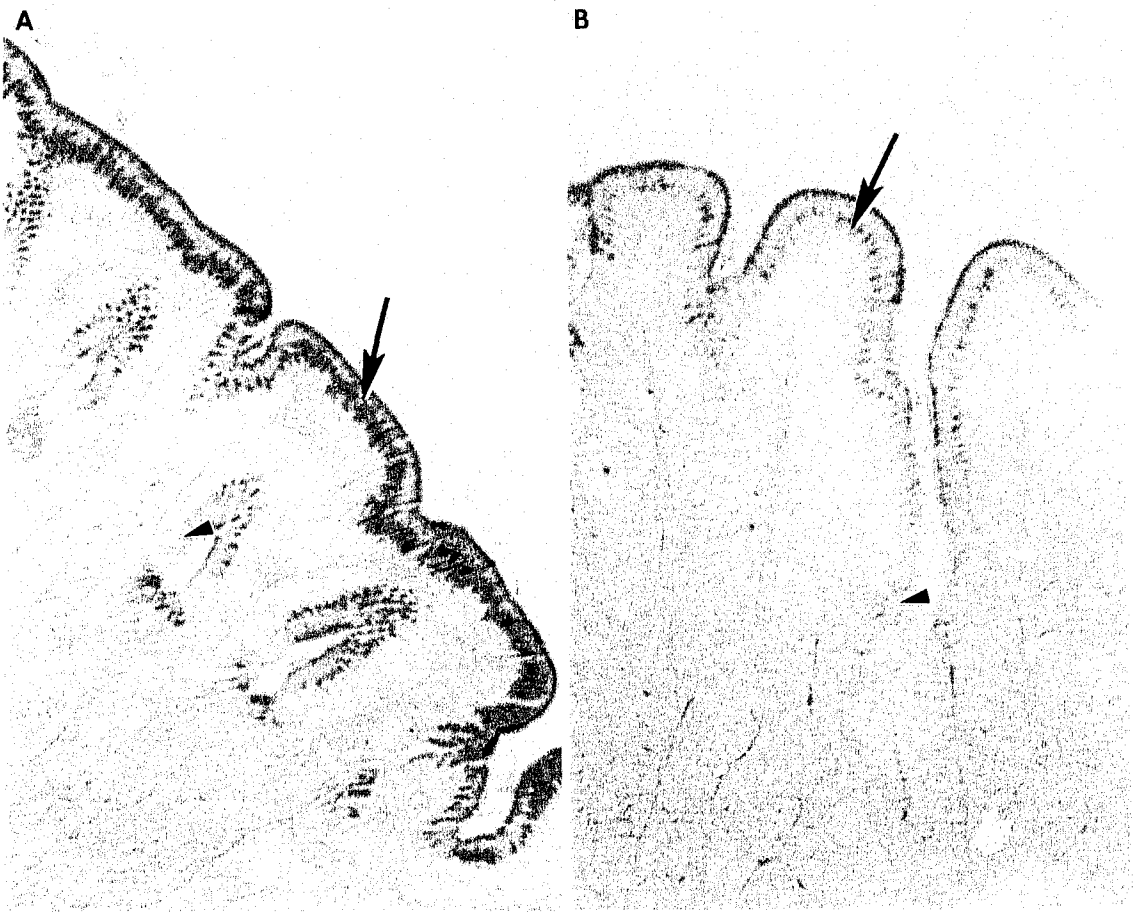



Figure 2.22. Alcian Blue differential staining of gastric surface cell (arrows) and neck cell mucus from the yellowtail flounder (arrowheads). (A) pH 2.5 (X250) (B) pH 1.0 (X250).


Figure 2.23. Alcian Blue/PAS differential staining of gastric surface cell mucus from the pyloric region of the yellowtail flounder stomach (arrows). (A) pH 2.5 (X250) (B) pH 1.0 (X250).


Figure 2.24. Alcian Blue differential staining of gastric surface cell mucus from the yellowtail flounder pyloric stomach (arrows). (A) pH 2.5 (B) pH 1.0 (X250).

### 2.3.3. Electron Microscopy

#### 2.3.3.1 Esophagus

The posterior esophageal mucosa in the winter flounder and the yellowtail flounder was composed of two main cell types: goblet cells and stratified esophageal surface secreting cells (ESSC's) (Figure 2.25 A; B).

In the winter flounder, the cuboidal shaped ESSC's bordering the esophageal lumen, exhibited an apical membrane organized into evenly spaced, spike-like projections (microridges) approximately 561 ± 10 nm long (Figure 2.26A). Adjacent cells were joined via junctional complexes and lateral membranes exhibited distinct interdigitations.

The peripheral cytoplasm displayed numerous filaments. These appeared especially prominent immediately beneath the apical plasma membrane forming part of the terminal web. Filaments were also noted to extend into the microvilli and along the lateral membrane where they were closely affiliated with spot desmosomes.

The cytoplasm of these cells was further characterized by numerous Golgi associated granules (average diameter = 250 ± 19 nm) and rough endoplasmic reticulum. The contents of the granules were electron lucent and frequently contained the remnants of a flocculent material exhibiting a variable electron density (Figure 2.26A). Commonly, two or more granules were fused and many exhibited ruptured membranes.

Figure 2.25. Lumenal surface of the posterior esophageal epithelium showing the two main types of cells present in (A) winter flounder (X6720) and (B) yellowtail flounder (X8750). GC, goblet cell, ESSC, esophageal surface secreting cell, N, nucleus of undifferentiated surface cell.

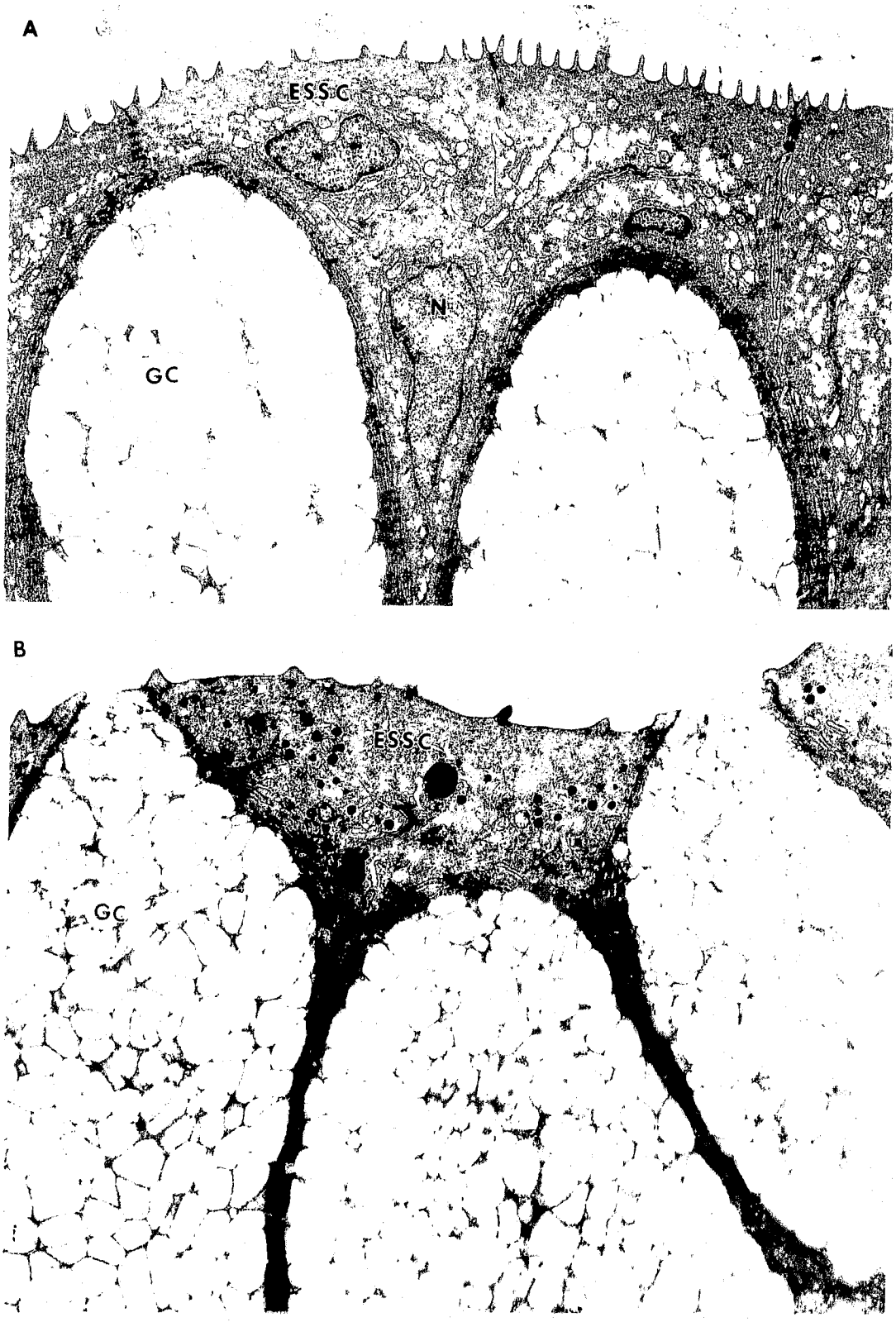
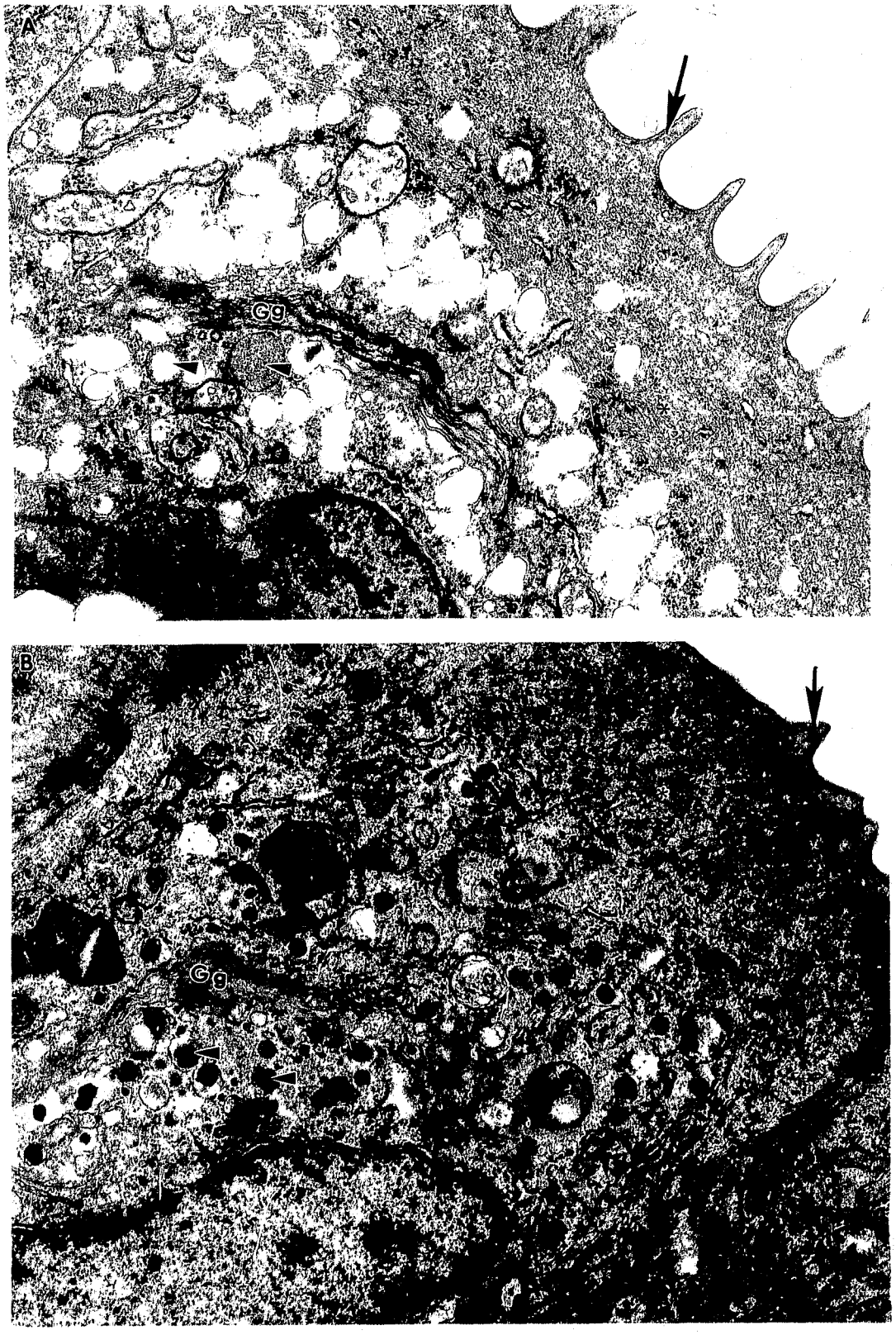

A
ESSC
N
GC
B
ESSC
GC

Figure 2.26. Esophageal surface secreting cells showing Golgi (Gg) associated granules (arrowheads), filaments (asterisk) and apical membrane microridges (arrows). Note species differences in granule morphology. (A) winter flounder (X25500) (B) yellowtail flounder (X21360).


A
Gg
B
Gg

The lumenal surface of the yellowtail flounder esophageal epithelial cells was smooth except for occasional short, irregularly distributed, membrane projections (Figure 2.26B). The cells were joined to their neighbours via apical junctional complexes. Occasional spot desmosomes were noted to be affiliated with interdigitations of the lateral and baso-lateral membranes.

The cytoplasm contained an extensive network of filaments, which appeared to be most abundant in the apical and lateral regions. A prominent Golgi apparatus with numerous associated dense, spherical membrane-bound granules (average diameter = 180 ± 10 nm) was also noted to be present in the cytoplasm (Figure 2.26B). The granules exhibited a range of electron densities, suggesting the possibility of different product types or stages of granule maturity. Granules were usually observed as separate entities.

Goblet cells associated with the winter flounder and the yellowtail flounder esophageal mucosa were similar between species. The apical plasma membrane of the cells showed no surface specializations and appeared to present a much smaller surface area than that of the surface epithelial cells (Figure 2.25B). The supranuclear cytoplasm of the cell was dominated by numerous tightly packed, mucigen granules which exhibited a coalesence of the granule membranes. The basal portion of the cell exhibited complex zones of RER and a Golgi apparatus. The Golgi was identified with the mucous granules. The nucleus was distinctly euchromatic and displayed a prominent nucleolus (Figure 2.27).

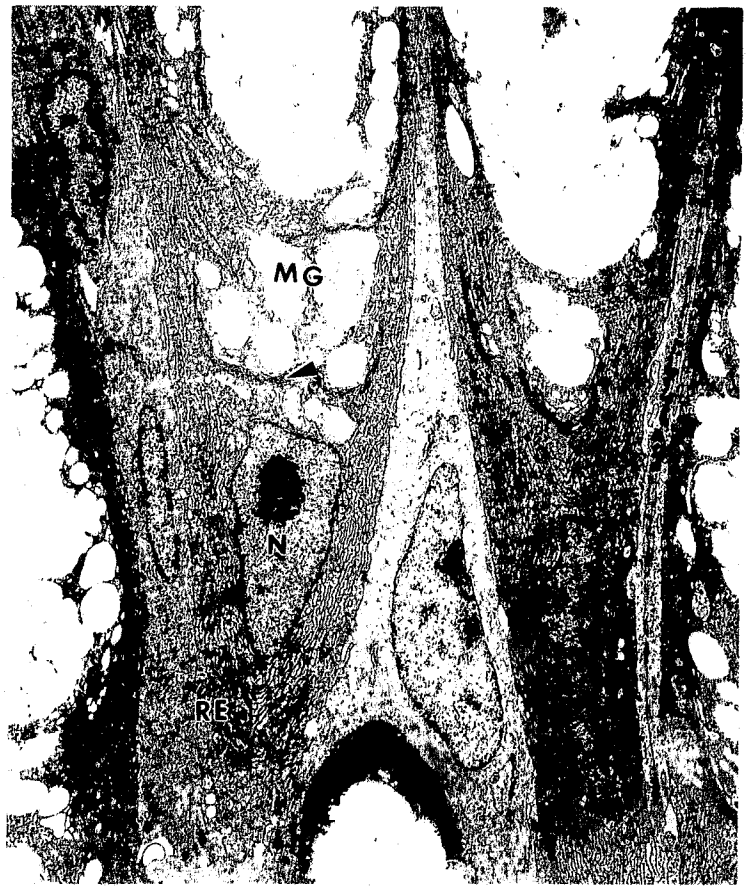



Figure 2.27. Basal portion of esophageal goblet cells from winter flounder s'.owing the close association of mucous granules (MG) with Golgi (arrowhead), abundant rough endoplasmic reticulum (RE) and nucleus (N) with prominant nucleolus (X5250).

#### 2.3.3.2 Stomach

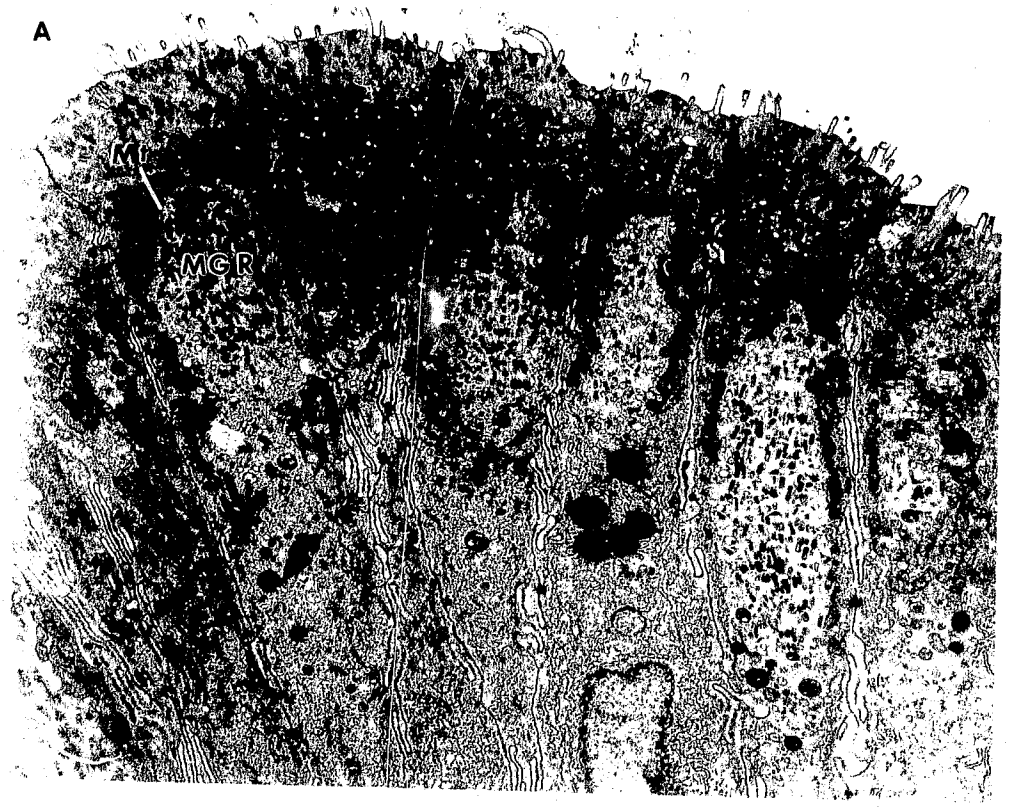
The epithelium of the gastric mucosa associated with the winter flounder, Atlantic halibut, and the yellowtail flounder was observed to be composed essentially of four cell types, A) surface mucous cells B) mucous neck cells, C) glandular cells and, D) enteroendocrine-like cells. Each of these cells appeared similar between species having distinct ultrastructural characteristics, which provided a basis for their identification as well as a means for functional interpretation.

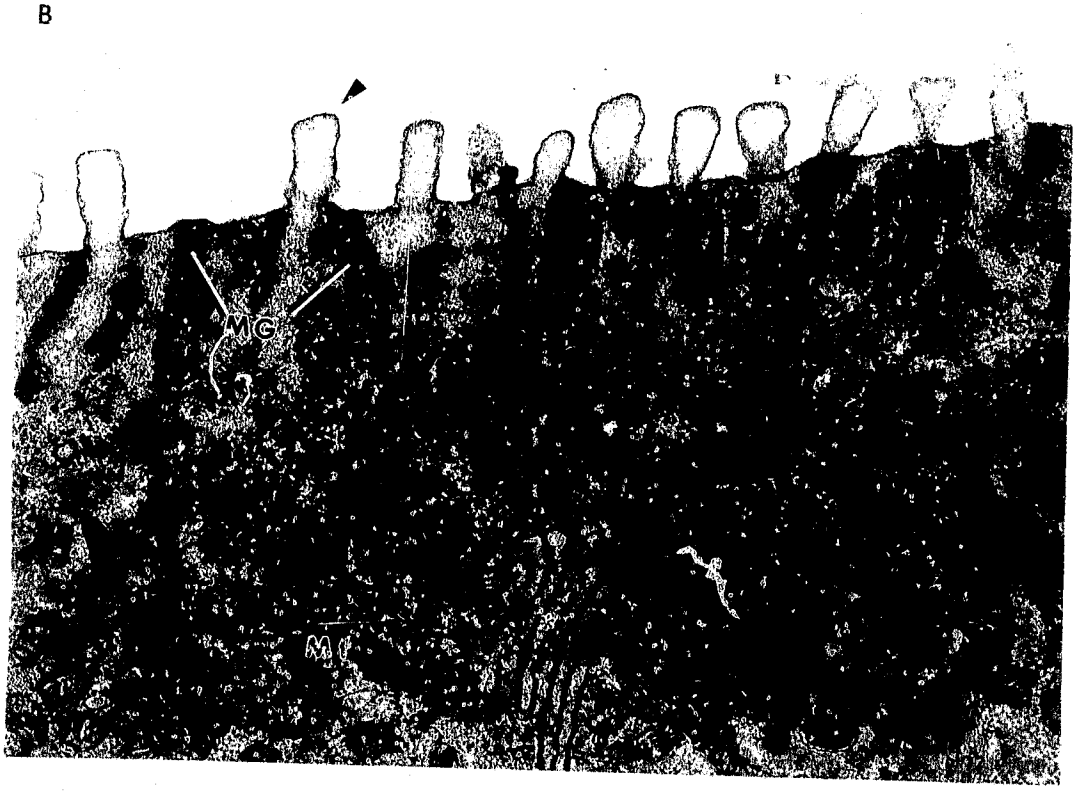
##### 2.3.3.2.1 Surface mucous cells

The columnar surface mucous cells were characterized by the presence of dense, elongate, membrane bound granules. The majority of granules appeared localized in an area of the apical cytoplasm just below the terminal web. The mucous granules were not compartmentalized or fused together like those observed in goblet cells, instead the granules existed as individual entities. Large numbers of mitochondria were observed to surround the mucus containing zone of the cytoplasm (Figures 2.28A and B). A Golgi apparatus, extensive RER, numerous free ribosomes and a complex network of filaments were also noted to be associated with the zone of mucous granules.

The apical membrane of the mucous cells was folded into irregularly distributed, short microvilli decorated with a fine filamentous glycocalyx (Figure 2.28B). Frequently, individual mucous granules were lined up directly beneath the apical membrane, oriented such that their long axes were aligned perpendicular to the surface, apparently in readiness for exocytosis.

Figure 2.28. Pleuronectid gastric surface mucous cells. (A) Overview of surface mucous cells from the winter flounder stomach showing cytoplasmic mucous granule region (MGR) with associated mitochondria (Mt) and lateral membrane interdigitations (X6230). (B) Lumenal surface of Atlantic halibut surface mucous cells showing short microvilli with associated glycocalyx (arrowheads), prominent membrane interdigitations (asterisk) and electron dense mucous granules (MG) in apparent preparation for exocytosis. Note junctional complex (arrow) (X26700).


A
M
MGR


B
MG
M

The lateral and basolateral membranes showed extensive interdigitation. Apical junctional complexes and spot desmosomes were found between adjacent cells.

**2.3.3.2.2 Mucous neck cells**

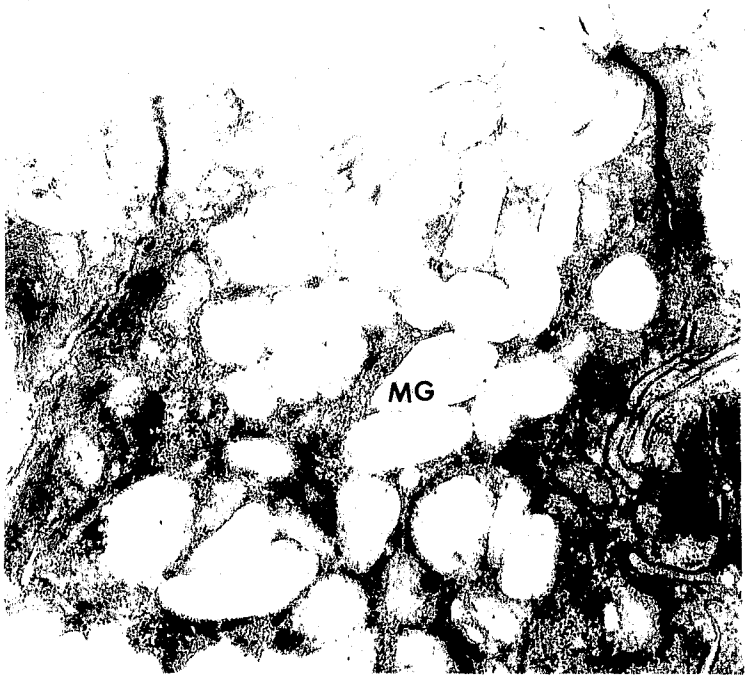
The mucous neck cells appeared distinct from the surface mucous cells; the supranuclear cytoplasm was filled with large, ovoid, electron lucent granules (Figure 2.29A). Adjacent cells were joined through junctional complexes. The apical plasma membrane was folded into scattered short microvilli and the basolateral membranes showed extensive interdigitation. A network of filaments, and cisternae of RER were interspersed amongst the granules. In many cases the granules were fused and exhibited a filamentous sub-structure (Figure 2.29B). Occasionally, dense cores were observed within the granules, giving them a "bullseye" appearance (Figure 2.29A).

The basal cytoplasm contained an irregularly shaped, euchromatic nucleus, and exhibited numerous free ribosomes, mitochondria, and many cisternae of RER.

In the yellowtail flounder, the morphology of the granules within the neck cells varied with the position of the cells. Neck cells deep within Zone 2, adjacent to the glands of Zone 3, contained granules with distinct corrugated membranes and one or two dense cores (Figure 2.30). These appeared more frequently in cells closer to the glands. These positional differences in morphology were not seen to occur in mucous neck cells of the Atlantic halibut or winter flounder.

Figure 2.29. Pleuronectid gastric mucous neck cells. (A) Overview of mucous neck cells from the halibut showing electron lucent cytoplasmic mucous granules (MG) and extensive lateral membrane interdigitation (LI) (X7500). (B) Apical cytoplasm of halibut neck cells detailing the fibrous ultrastructure of the mucous granule contents (MG) and the abundance of cytoplasmic filaments (asterix). Note the occasional fusion of granules (arrowheads) (X22500).


A
MG
LI


B
MG

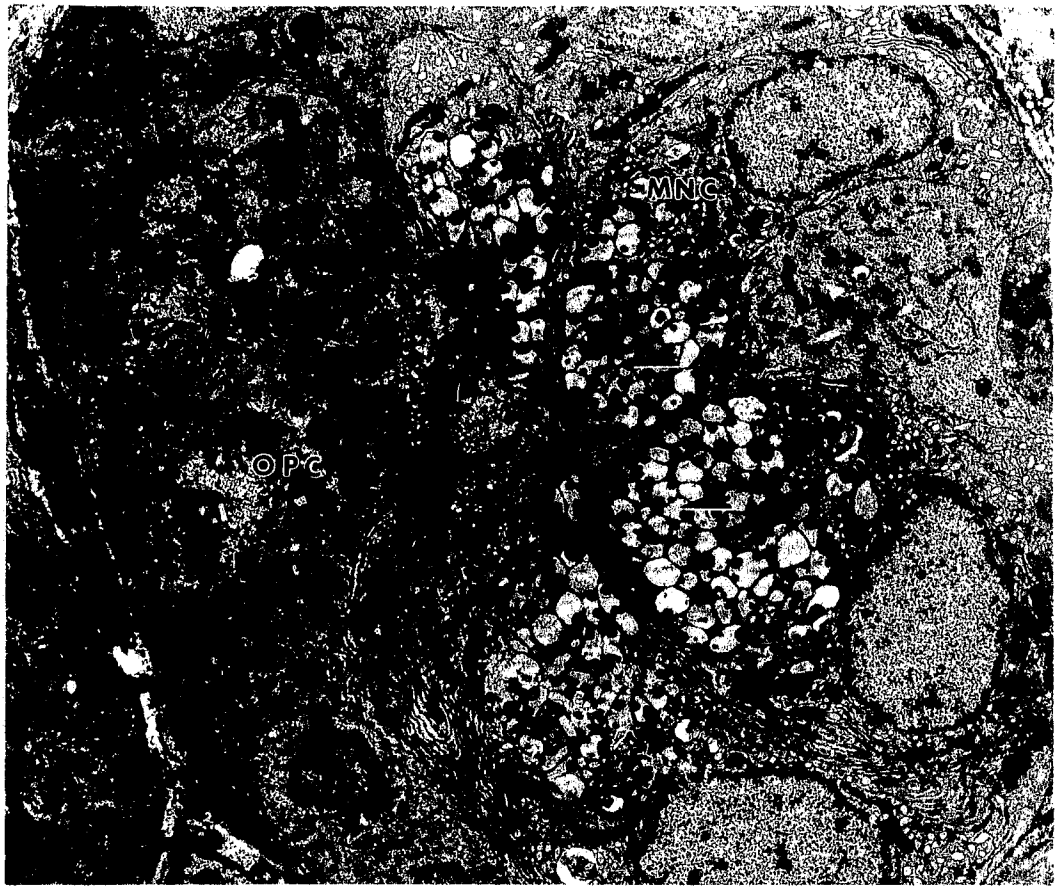



Figure 2.30. Overview of the transitional region from mucous neck cells (MNC) to oxyntico-peptic cells (OPC) in the yellowtail flounder stomach. Note the abundance of dense cores or "bullseyes" (arrowsheads) in association with the neck cell mucous granules (X5400).

#### 2.3.3.2.3 Gastric gland cells

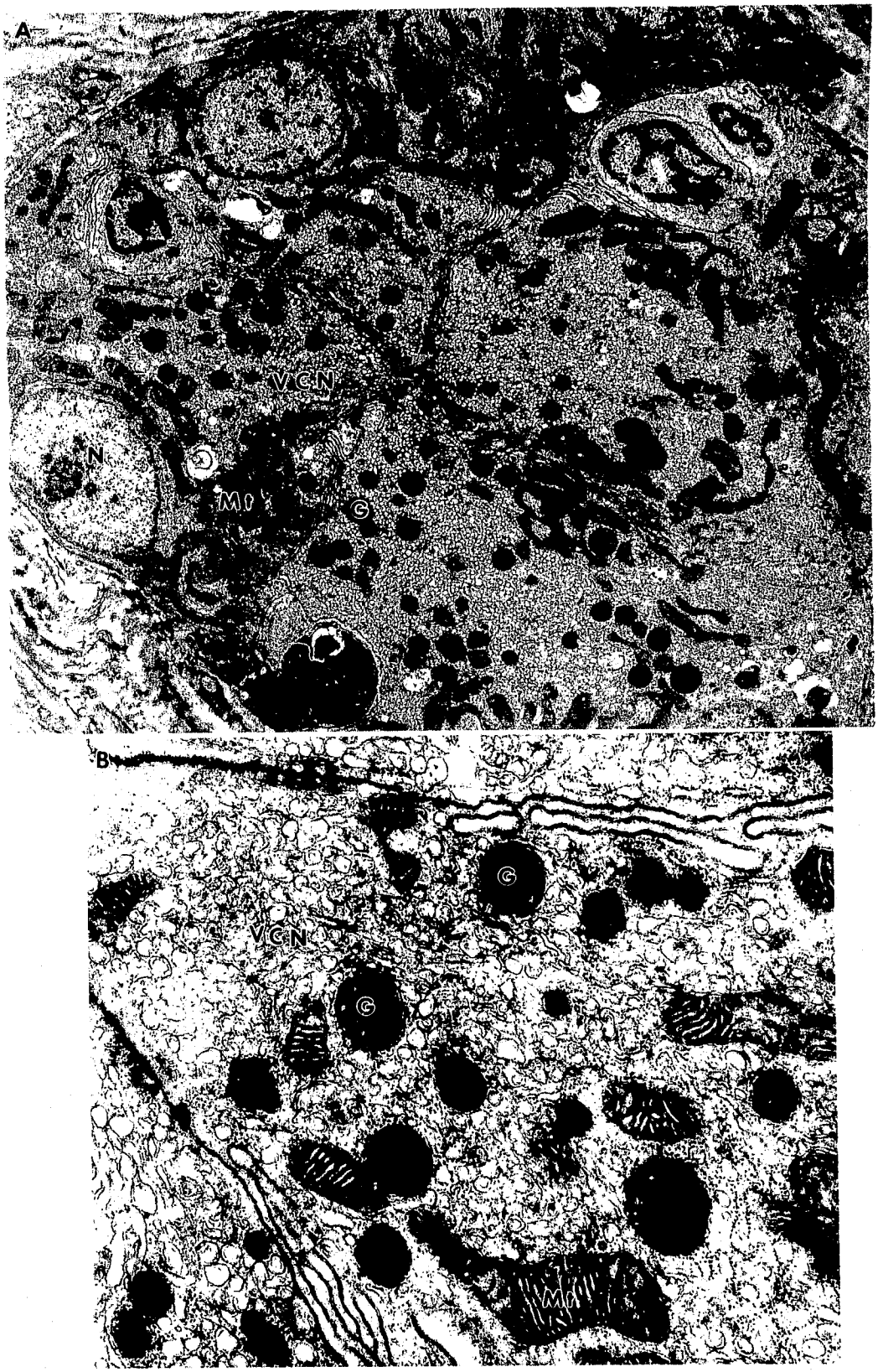
The gastric glands of the winter flounder, yellowtail flounder and the Atlantic halibut were composed of one cell type (Figure 2.31A). The cells were pyramidal with lumenal microvilli and junctional complexes, along with interdigitations of the lateral plasma membrane. The cytoplasm contained spherical, membrane bound, granules with variable electron density. The granules had an average diameter (N=25) of 965 ± 36 nm in the halibut, 792 ± 29 nm in the winter flounder and 575 ± 65 nm in the yellowtail flounder. The mean granule diameters of the winter flounder and the halibut were not significantly different ($p>0.05$). The yellowtail flounder granule diameter was, however, significantly different from that found in the above ($p<0.05$).

The apical cytoplasm was characterized by the presence of a complex tubulo-vesicular network of smooth vesicles (Figure 2.31B). The basal cytoplasm contained large numbers of free ribosomes as well as many cisternae of RER. The most striking feature of this region of the cell was the numerous mitochondria, exhibiting unusually prominent, parallel cristae (Figure 2.31A; B).

#### 2.3.3.2.4 Enteroendocrine-like cells

Two types of enteroendocrine-like cells were identified with the epithelium of the gastric mucosa. All were found within Zone 1, distributed between the bases of the surface mucous cells and their cytoplasm did not seem to extend to the lumenal surface.

Figure 2.31. Pleuronectid oxyntico-peptic cells. (A) Overview of gastric gland demonstrating oxyntico-peptic cells with basal nuclei (N), numerous mitochondria (Mt), secretory granules (G) and vesicular cytoplasmic network (VCN) (X5100). (B) Higher magnification of the apical region of oxyntico-peptic cell showing detail of the vesicular cytoplasmic network, secretory granules and mitochondria with prominent cristae (X25330).


A
VCN
N
Mi
G
B
G
VCN
G
Mi

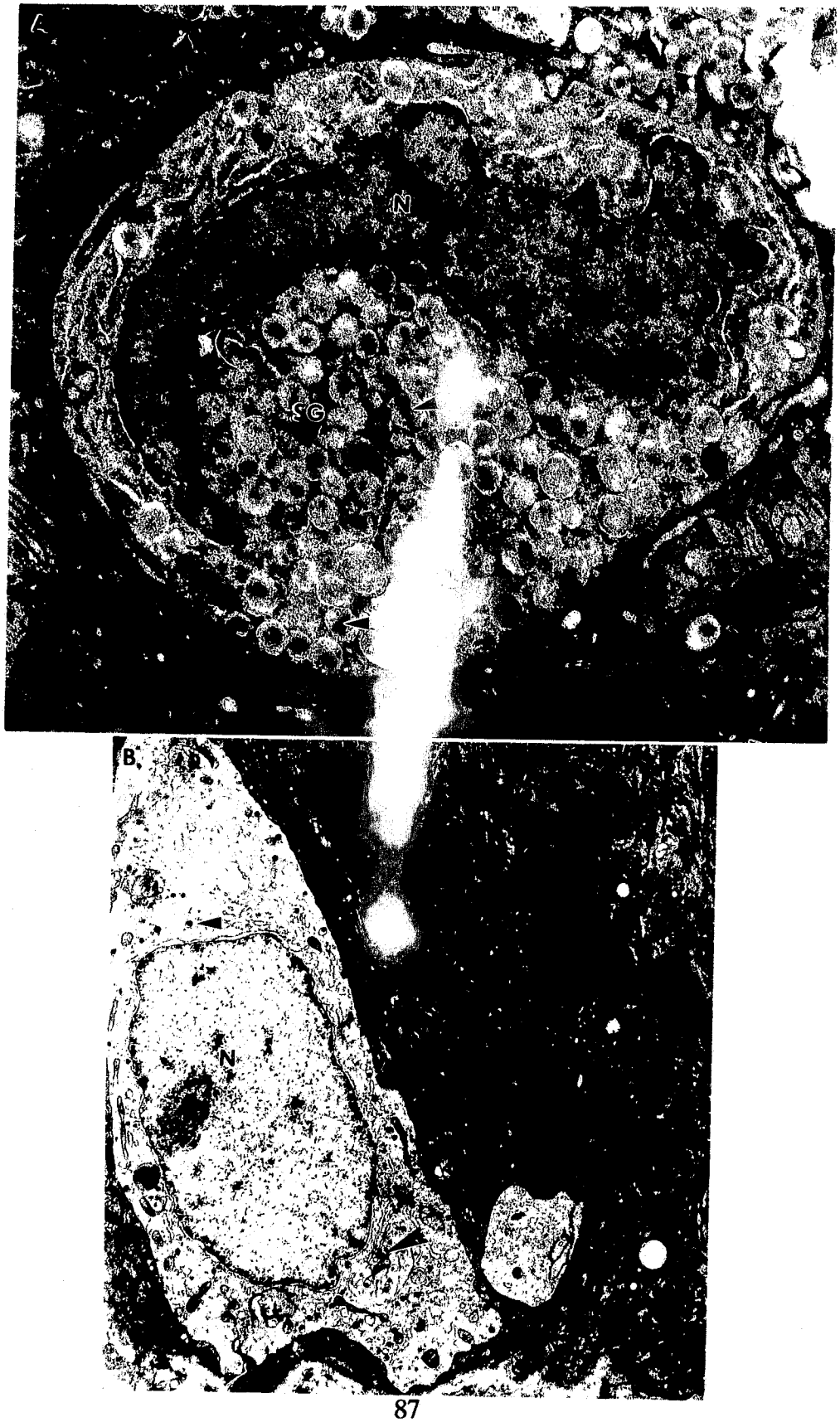
The Type I cells were present in the winter flounder and the halibut only. They were normally oval in shape with elongate irregular nuclei. The cytoplasm was filled with many spheroid, membrane bound granules, with variable electron density (Figure 2.32A). Some granules occasionally exhibited a dense filamentous cylindrical core. Granule diameter differed between the two species, ($p<0.05$), with a mean ($N=25$) of 354 ± 7.8 nm in the halibut and 303 ± 9 nm in the winter flounder.

The Type II cells were found to be present in all three species. In general, they were pyramidal or triangular in shape possessing a large, centrally located, euchromatic, oval nucleus (Figure 2.32B). The cytoplasm contained very dense, membrane bound, granules with distinct haloes. The granule diameters ($N=25$) were similar between species averaging at 115 ± 4 nm ($p>0.05$). The cytoplasm of these cells appeared consistently less electron-dense than that of the mucous cells that surrounded them.

## 2.4 Discussion

The histology of the esophagus in the winter flounder and the yellowtail flounder and the stomach in all three pleuronectids under investigation was similar in basic organization to that of teleosts studied previously. Each region consisted of the layers commonly found associated with the alimentary canal of most vertebrates including the mucosa, submucosa, the muscularis externa and the serosa (Barrington, 1957; Kapoor et al., 1975; Fänge and Grove, 1979). The lamina propria and the submucosa were indistinguishable as separate entities in the esophagus and stomach

Figure 2.32. Pleuronectid gastric enteroendocrine-like cells. (A) Type I enteroendocrine-like cell, exhibiting irregular elongate nucleus (N) and characteristic granules (SG), some of which have a denser filamentous cylindrical core (arrowheads) (X16000). (B) Type II enteroendocrine cell showing triangular shape with large central nucleus (N) and small dense secretory granules with distinct haloes (arrowheads) (X9000).


A
N
SG
B
N

of the winter flounder and the halibut. This feature has been previously noted in the stomach of the fresh water perch (Noaillac-Depeyre and Gas, 1978), in the esophagus of ten other freshwater teleosts (Reifel and Travill, 1978), and in the esophagus of the sea bream, *Sparus auratus,* a marine species (Elbal and Aguilleiro, 1986). Only in the yellowtail flounder stomach was there a distinct muscularis mucosae between the lamina propria and the submucosa.

### 2.4.1. Esophagus

The lack of a distinction between the lamina propria and the submucosa observed in the esophagus of the yellowtail flounder and the winter flounder is not unusual for teleosts and has been associated with the absence of a muscularis mucosae (Reifel and Travill, 1978). The differences in relative compactness of the connective tissue of the submucosa in the winter flounder and the yellowtail flounder suggest different support and protection needs for the esophageal mucosa in these fish. The winter flounder appears to demonstrate a more robust structure.

The observation of an esophageal muscularis externa composed of one thick circular layer of striated muscle in the yellowtail flounder and the winter flounder was found to be inconsistent with that in the perch (Hirji, 1983), the cod (Morrison, 1987) and the striped bass (Groman, 1982). Longitudinal striated muscle appeared to occur as isolated bundles in the submucosa. This organization of longitudinal muscle proved similar to that described in the esophagus of the plaice, *Pleuronectes platessa* and the stiped bass (Dawes, 1929; Groman, 1982).

The existence of only one defined layer of circular striated muscle in the esophagus of fish is considered to be unusual in teleosts (Anderson, 1986). A similar state of organization has been observed in the bottom feeding mullet, *Mulloides auriflamma* (Al-Hussaini, 1946). This characteristic has been suggested to imply that the esophagus may have a mixing or triturating effect on the food. In both the winter flounder and the yellowtail flounder, the absence of a well defined longitudinal muscle layer, may reflect their bottom feeding life styles. Both species are known to ingest inorganic matter with their chosen prey types (Libey and Cole 1979; Scott and Scott, 1988).

The highly folded nature of the mucosa in the esophagus of the winter flounder and the yellowtail flounder was comparable to that in other species. Similar mucosal folds have been observed in association with the esophagus of the channel catfish (Sis et al. 1979), the striped bass (Groman 1982), the Atlantic cod (Morrison, 1987), and the amberjack (Grau et al., 1992). Sis et al., (1979) suggested that these folds may allow for stretch during swallowing in the channel catfish. The fact that the flounder species from this study are characterized by relatively small mouths (Libey and Cole, 1979; Scott and Scott, 1988), would suggest that a large amount of stretching is not required, and certainly would not account for the elaborate foldings of the esophageal mucosa in these species. The extensiveness of the mucosal folding suggests a functional purpose in increasing surface area. Reifel and Travill (1977) reported that the initiation of digestion may be an important function of the

esophagus in teleosts. The increase in surface area resulting from foldings of the esophageal mucosa in the present species may reflect this function.

The caudal esophageal epithelium of the winter flounder and the yellowtail flounder consisted of two main cell types, stratified cuboidal epithelial cells and goblet cells. The extent of the stratification of the epithelial cells was shown to increase near the tips of the mucosal folds. The existence of a prominent rough endoplamic reticulum and Golgi associated membrane-bound granules as well as an extensive filamentous cytoskeleton indicates a dual function (digestive enzyme secretion and structural support) for the stratified surface epithelium in both species of pleuronectid.

The prominent apical cytoskeleton with associated junctional complexes and extensive interdigitations of the lateral membrane indicates a role in mucosal support. Hirji (1983) stated that in the perch, the stratified epithelial cells of the esophagus may provide support for the goblet cells. Structural evidence from the present study supports this proposal for the pleuronectids.

In the winter flounder, the cuboidal epithelial cells bordering the lumenal surface possessed an apical membrane characterized by numerous evenly spaced projections, supported by filamentous extensions of the apical cytoskeleton. These projections are likely profiles of microridges associated with the apical membranes of these cells. Numerous authors have previously described complex microridge formations in association with the epithelial cells of the teleost esophagus (Sperry and Wassersug, 1976; Sis et al., 1979; Ezeasor and Stokoe, 1980; Meister et al., 1983;

Morrison, 1987). It has been proposed that the ridges may have dual function: protection from mechanical trauma and channelling mucous secretions (Ezeasor and Stokoe, 1980; Morrison, 1987).

The lack of a similarly organized apical membrane in association with the surface epithelial cells from the yellowtail flounder esophagus suggests, based upon the proposed functions of the microridges, that the esophageal mucosa in this species may be less adapted to withstand mechanical trauma than that of the winter flounder.

Stomach analysis data (Libey and Cole, 1979; Scott and Scott, 1988) provides evidence for these species feeding at different times of the day, (winter flounder are daytime sight feeders whereas the yellowtail flounder are nighttime feeders), although they feed on similar prey species and are both essentially bottom feeders. The microanatomical differences observed in the esophagus of these species during the present study, however, suggests that the winter flounder may be more versatile in prey choice and thus is probably a more successful opportunistic feeder then the yellowtail flounder.

The existence of electron-dense granules or lucent mucous granules in association with the Golgi apparatus in the esophageal surface epithelial cells of these species indicates also a secretory function for the surface epithelial cells. Similar dense granules have been observed in the surface epithelial cells of the esophagus of the perch (Hirji, 1983) and the dab, *Limanda limanda* (Meister et al., 1983), however, no functional purpose was proposed for them. Linss and Geyer (1968) described cells from the esophagus of the pike that exhibited granules indicative of secretory

activity and suggested a possible antimicrobial or digestive function for the secretions. Likewise, Cataldi et al. (1987) described cells in the esophagus of the seabream which were similar in shape to mucous secreting cells but contained eosinophilic granules, and they thought that these cells may produce digestive secretions. In the yellowtail flounder and the winter flounder, the close association of the granules with a Golgi apparatus again suggests that these cells are functioning in the storage and transport of a specific secretory product. Differences in the morphology of these granules between flounder species imply different secretory products. The lucent and coalescing nature of the granules in the winter flounder surface epithelial cells is indicative of a mucous producing cell, whereas the smaller more electron dense haloed granules in the cells of the yellowtail flounder are more reminiscent of an enzyme producing cell (Ghadially, 1988).

The occurrence of positive histochemical reactions for nonsulphated and sulphated acid mucins in association with the yellowtail flounder and the winter flounder esophageal surface epithelial cells, respectively, suggests that the secretion product is glycoprotein based and supports the idea of a mucous-like product for the winter flounder and a possible enzyme product for the yellowtail flounder. The actual functional nature of either of these secretions is not yet known based on their position and granule morphology, but one might hypothesize a digestive function, similar to the salivary alpha-amylase found in mammalian saliva (Jamieson, 1988). The observations in the present study appear to be a first observation for these species, and indicates a role in addition to support.

Numerous authors have suggested that the chemically variable mucus produced by esophageal goblet cells may have a role in digestion (Reifel and Travill, 1977; Hirji, 1983). It seems plausible to suggest that a secondary mucin or enzyme of surface epithelial origin may combine with goblet cell mucus to provide a digestive function for the esophagus. The excessive numbers of goblet cells present in the esophagus of the pleuronectids support a requirement for increased protection and lubrication for the mucosa in this area of the alimentary canal for both species. This observation may be a reflection of the bottom feeding lifestyle exhibited by both the winter flounder and the yellowtail flounder due to the large amount of inorganic matter. Ultrastructurally, the goblet cells in both of these species were typical of those found in the esophagus of other species of fish as well as higher vertebrates (Hirji, 1983; Neutra and Padykula, 1984; Elbal and Agulleiro, 1986; Morrison, 1987).

The occurrence of different histochemical reactions for mucus in the esophageal goblet cells of the winter flounder compared to that of the yellowtail flounder suggests the possibility of functional differences for the mucus in the different species. These functional variations may be related to the proposed differences in surface epithelial cell secretions. Generally, teleost esophageal mucous cells have been noted to contain at least two combinations of nonsulphated acid mucus, sulphated acid mucus, or neutral mucus ( Bucke, 1971; Reifel and Travill, 1978; Sis et al., 1979; Groman, 1982). Mucous histochemical differences observed in different teleosts indicate additional functions beyond lubrication including digestion (Reifel and Travill, 1978).

The combination of elaborate foldings of the mucosa, specialized mucous or enzyme secreting epithelial cells and variations in goblet cell mucus histochemistry all allude to a pregastric initiation of digestion in these species of pleuronectid.

### 2.4.2. Stomach

The stomach of the pleuronectids from this study were found to possess the same tissue layers observed in other teleosts ( Sis et al., 1979; Groman, 1982; Elbal and Agulleiro, 1986; Cataldi et al., 1987; Morrison, 1987; Grau et al., 1992). In the flounders, the transition from esophageal to gastric mucosa was abrupt. A similar abrupt transition has also been observed in the amberjack and the Atlantic cod (Morrison, 1987; Grau et al., 1992).

The histological separation of the stomachs of the yellowtail flounder and the winter flounder into cardiac (glandular) and pyloric (aglandular) regions was consistent with observations in other teleosts (Reifel and Travill, 1978; Ezeasor, 1981; Morrison, 1987). The absence of an aglandular region in the stomach of the Atlantic halibut was unusual and comparable with that observed in the stomach of the tilapia (Osman and Caceci, 1991), the rainbow trout (Weimreb and Bilstad, 1955) and the catfish, *Clarias lazera* (Stroband and Kroon, 1981).

The division of the glandular region mucosa into three vertical zones based upon the histological and histochemical appearance of the cells present is not a consistent occurrence for teleosts, however the division of the stomach in higher vertebrates into as many as four zones is not uncommon (Neutra and Padykula, 1984;

Davison, 1989). While most authors have acknowledged the presence of a morphologically distinct surface mucous cell in the teleost stomach (Zone 1) (Noaillac-Depeyre and Gas, 1978; Ezeasor, 1981; Morrison, 1987) , only a few investigators have reported a zone characterized by ultrastructurally and histochemically unique mucous cells associated with the neck region of teleost stomach (Zone 2) (Reifel and Travill, 1978; Noaillac-Depeyre and Gas, 1978). No mention of mucous neck cells has been made in association with the gastric mucosa of the rainbow trout (Ezeasor, 1981) or the striped bass (Groman, 1982). Similarily, cells found in the neck region of the gastric folds in the Atlantic cod were not morphologically different from the surface mucous cells (Morrison, 1987).

Within the pleuronectids of this study, the major distinquishing feature of the surface mucous cells and the mucous neck cells, with the exception of variations in position, was the appearance and storage pattern of the cytoplasmic granules. Surface cells were defined by the presence of dense, elongate, membrane bound granules. These granules were confined to the apical cytoplasm and were closely surrounded by filaments and large numbers of mitochondria. Similar microfilaments in the surface mucous cells of the perch have been proposed to be important in the movement of individual granules to the apical membrane for exocytosis (Noaillac-Depeyre and Gas, 1978). In the present species, they may be also involved in orientation of the granule along the apical plasma membrane.

The mucous neck cells in these pleuronectids were defined by large, ovoid, electron-lucent granules. Cytoplasmic filaments were noted in close association with

the granules and in many cases the granules themselves appeared to exhibit variations in their substructure. The observed difference in shape and granular appearance between neck cells in close proximity to the glands and those nearer the surface in the winter flounder and the yellowtail flounder suggests a maturation effect. The consistent occurrence of dense cores associated with those neck cell granules most adjacent to the glands in the yellowtail flounder is reminisent of similar structures observed in the perch (Noaillac-Depeyre and Gas, 1978). These cores were not as common in the neck cells of the halibut or the winter flounder. Noaillac-Depeyre and Gas (1978) proposed that these cores may be protein based, however, the authors could not offer a function. The apparent disappearance of the cores in neck cells further along the mucosa folds in the yellowtail flounder suggests a role in the maturation of the neck cell mucus translated into morphological differences in substructure of mucous granules. The observation of distinct mucous neck cells in all three fish species from this study but the absence of similar cells in other species, supports Ezeasor (1981) in his contention that teleost gastric glands can be of two types, those with and without neck cells. The ultrastructural differences between mucous granules from cells of the different regions suggest that there are different roles for these cells in the gastric mucosa of fish. Similar structural and functional differences have been noted with regard to the mucous neck cells of mammals (Neutra, 1988).

The general variations in gastric mucous histochemistry between the winter flounder, yellowtail flounder and the halibut imply possible differences in the

chemistry of the microenvironment of the gastric lumen, therefore, requiring different chemo-types of mucus to efficiently protect under different gastric conditions. These differences may encompass variations in pH and/or the chemical nature of enzyme secretions (pepsinogen/pepsin), and may be a function of diet. Osman and Caceci (1991) observed that the neck cell in the stomach of the tilapia secretes both acid and neutral mucins. They stated that these mucins, besides protecting the mucosa, also regulated the pH of gastric fluid and may explain the variations in gastric fluid pH from different species of tilapia feeding on different diets.

Reifel and Travill (1978) observed considerable variation in the gastric mucous histochemistry from a variety of freshwater teleosts and proposed that no particular type of mucus could be correlated with a freshwater piscivorous diet. They did observe in general, however, that the amount of sulfomucin in the gastric pit cells increased as the size of food particles decreased.

Observed variations in mucous histochemistry between zones in the same species and ultrastructural differences between mucous cells suggest that at least two types of chemically different mucus can be produced in the same general region. This was observed to be most distinct in the yellowtail flounder where neutral mucins were dominant in association with the neck cells and nonsulphated acid to sulphated acid mucins were observed to be prominent in the surface mucous cells. The differences between zones in the halibut were not as obvious as in the yellowtail flounder, however, nonsulphated acid mucins were common in the surface mucous cells whereas neutral mucins were prominent in the neck. The presence of only

neutral mucins in the winter flounder stomach may imply that this species is quite divergent in gastric mucous chemistry from the other pleuronectids. Different gastric conditions, reflected in variable mucous histochemistry, are important for different diets across species of tilapia (Osman and Caceci, 1991). A similar conclusion could be arrived at when one observes the differences between the halibut and the flounders, a fisheater versus invertebrate eaters. The variations observed between the winter flounder and the yellowtail flounder present a more challenging problem, since stomach analysis data indicate that these species feed on similar prey and in fact overlap territories (Libey and Cole, 1979; Scott and Scott, 1988). If the contention of Osman and Caceci (1991) also applies to these pleuronectids then the histochemical data here would indicate differences in prey preference. Clearly, more work is required to further define the reasons for differences in gastric mucous chemistry in these species.

The region of mucosa histologically defined as Zone 3 in the winter flounder, the halibut and the yellowtail flounder, corresponds with the gastric gland region in other species (Barrington, 1957; Kapoor et al., 1975; Fänge and Grove, 1979; Groman, 1982). This area was similar between species and exhibited simple branched tubular glands, which opened at the base of the folds. Historically, these glands were noted to consist of only one cell type, and were thus comparable to oxyntico-peptic cells of the amphibians, reptiles and birds, which are known to produce both acid and pepsinogen (Hill, 1971; Welsch and Storch, 1976).

Ultrastructurally, the oxyntico-peptic cells (GOPC) were similar among the three species in this study, and were comparable to the cells comprising the gastric glands of other teleosts (Noaillac-Depeyre and Gas, 1978; Ezeasor, 1981; Elbal and Agulleiro, 1986; Morrison, 1987). These cells are distinguished by an apical cytoplasm consisting of complex networks of smooth membrane saccules and frequent electron-dense spherical granules. These ultrastructural features have been correlated with the production of both HCl and pepsinogen, respectively (Noaillac-Depeyre, 1978; Ezeasor, 1981; Elbal and Agulleiro, 1986). The presence of a basal cytoplasm, exhibiting ample RER and numerous mitochondria with prominent cristae provide further support for the oxyntico-peptic cell being a secretory cell, characterized by high rates of oxidative metabolism (Anderson, 1986).

The present study also shows enteroendocrine-like cells associated with Zones 1 of the mucosa. These cells could be divided into two different types based upon the morphology of secretory granules. Type I was common in the winter flounder and the halibut, and appeared to be similar to the Type III cell described in association with the stomach mucosa of the rainbow trout (Ezeasor, 1981).

The pleuronectid Type II cell was present in all three species and was shown to be similar to the Type II cell described in the rainbow trout and the cell in the perch (Noaillac-Depeyre and Gas, 1978; Ezeasor, 1981).

The enteroendocrine-like cells observed in the stomach of these pleuronectids were never seen to reach the lumen, suggesting that they may be of the "closed type". This appears generally different from other fish, since most investigators have

recorded the dominant presence of the more primitive "open type" enteroendocrine cell in association with both the stomach and the intestine (Rombout, 1977; Noaillac-Depeyre and Gas, 1978; Ezeasor, 1981; Holmgren et al., 1986). The observation of the "closed type" cells in the stomach of the pleuronectids from this study is reminiscent of the enteroendocrine cells in the stomach of mammalian species (Rombout, 1977). This observation may be a reflection on the advanced phylogeny of the pleuronectid with reference to other teleosts.

The different types of enteroendocrine-like cells described in this study probably secrete various peptides, since biochemically several gastrointestinal peptides have been localized in the gut mucosa of teleosts (Holmgren et al., 1986; Bjenning and Holmgren, 1988). Unfortunately, few studies are available which examine the nature of these hormones in pleuronectids. Bjenning and Holmgren (1988) were able to summarize known neuropeptide secretions (e.g., bombesin and substance P) in the stomach of the pleuronectids, *Platichthys flesus* and *Pleuronectes platessa*. Since many of these substances are also associated with enteroendocrine cells (Holmgren et al., 1986), it may be logical to suggest that similar compounds may originate from the cells described in the present study. Since the pleuronectids from this study have oxyntico-peptic cells which secrete both HCl and pepsin, it seems possible that there may be control of these secretions by gastro-intestinal peptides. Pepsin secretion is stimulated in the cod by members of the tachykinin family i.e. substance P (Holstein and Cederberg, 1985, cited in Holmgren et al., 1986). Somatostatin inhibits gastric acid secretion in the cod (Holmgren et al., 1986).

The deep gastric connective tissue layers of the species in this study showed some variation. The propria-submucosa of the winter flounder and the submucosa of the yellowtail flounder were observed to be composed of a loose connective tissue, exhibiting considerable vascularization and nervous infiltration. A muscularis mucosae defined by a layer of smooth muscle adjacent to Zone 3 was observed only in the yellowtail flounder. This layer was also prominent in the pyloric aglandular region of this species. A submucosa defined by a similar connective tissue layer was noted to be associated with the gastric submucosa of the common eel and the tilapia (Clarke and Witcomb, 1980; Osman and Caceci, 1991). No distinct stratum compactum was observed in either the winter flounder or the yellowtail flounder.

The propria-submucosal region observed immediately below Zone 3 in the Atlantic halibut stomach was distinctly different from that of the other two species. The presence of a very compact eosinophilic connective tissue infiltrated with scattered smooth muscle fibers suggests great elasticity and tensile strength of the organ. This single layer may be comparable to the stratum compactum in other species (Burnstock, 1959; Bucke, 1971; Grau et al., 1992). In the halibut, a voracious piscivorous feeder, the significant thickness and associated strength of this layer may be a feature to provide support to a stomach adapted for holding and subsequently digesting large teleost prey. The absence of a similar construction in the other species of pleuronectid reflects a diet of less "vigorous" prey. The muscularis externa associated with the stomach of the winter flounder, Atlantic halibut and the yellowtail flounder are comparable to that observed in other species, exhibiting an inner circular

layer of smooth muscle and a thinner outer longitudinal layer in close association with the thin serosa (Clarke and Witcomb, 1980; Morrison, 1987; Grau et al., 1992).

As observed in the common eel, the muscularis externa from the yellowtail flounder and the winter flounder exhibited a thickened inner circular layer of smooth muscle in the pyloric region, probably in association with the formation of the pyloric sphincter (Clarke and Witcomb, 1980). Similarily, as seen in relation to the eel, striated muscle extensions in the cardiac region of the stomach, extending from the muscularis of the esophagus, suggests some voluntary control over the anterior stomach in these species. Martin and Blaber (1984) noted a similar configuration of striated muscle in the anterior cardiac stomach of species of glassy perchlet. In the flounders, the strands of striated muscle quickly give way to the typical alimentary canal muscularis externa.

In conclusion, observations on the histology of the esophagus in the winter flounder and the yellowtail flounder suggest that the winter flounder is more robust in esophageal structure and thus may be a more successful opportunistic feeder then the yellowtail flounder.

Ultrastructural and histochemical examination of the esophageal mucosa in the winter flounder and the yellowtail flounder indicates that two different types of secretory cells are present, the goblet cell and the ESSC. The ultrastructural and carbohydrate histochemical differences in the granules from the ESSC imply that the chemistry of the secretory product may differ between species. The similarities in basic cell structure and position indicate, however, that the basic functions of the cell

may be comparable between the species. It is proposed that the secretions from these cells may be important as enzyme initiators of pregastric digestion. The combination of a large surface area due to elaborate folding of the esophageal mucosa, a mixing effect initiated by contractions of the prominant circular muscularis, and possible digestive secretions from the ESSC, suggest that the esophagus of the winter flounder and the yellowtail flounder may function in the pregastric digestion of prey. Further work is required (e.g., enzyme cytochemistry) to elucidate the composition and function of the ESSC granules.

In conclusion, the structure of the stomach in the pleuronectids from this study was similar among the species and indicated that the mucosa could be divided into three distinct zones based upon cell type present. These species possessed gastric glands with distinct mucous neck cells (Zone 2), an observation not consistent with all species of teleost, supporting the theory that teleost gastric glands may exist with or without distinct mucous neck cells.

The marked differences in the gastric mucous histochemistry observed between species suggest that the chemical nature of lumenal environments during digestion may be different and may reflect natural diet. Clearly, the obvious differences observed here require further investigation.

# 3. A COMPARATIVE HISTOLOGICAL STUDY OF THE INTESTINE, PYLORIC CAECA, AND RECTUM IN THREE SPECIES OF PLEURONECTIDAE

## 3.1. Introduction

The intestine, pyloric caeca, and rectum have been studied extensively in many species of fish (Barrington, 1957; Kapoor et al., 1975; Fänge and Grove, 1979; Morrison, 1987) and are considered of primary importance in the digestion and absorption of nutrients. Each region has mucosal specializati ıs that maximize the efficiency of the secretion, absorption, and digestive functions (Buddington and Diamond, 1986; Stinson and Calhoun, 1993). Many studies have related gross morphology to diet (Tyler, 1972; Kapoor et al., 1975; De Groot, 1979). A few have also examined possible correlations between mucosal histology and diet (Al-Hussaini, 1946; 1947; Martin and Blaber, 1984; Anderson, 1986).

The pleuronectids as a group, are known to exploit a wide realm of habitats and prey types. Many species utilize prey assortments ranging from fish to marine invertebrates (Scott and Scott, 1988).

The Atlantic halibut, winter flounder, and the yellowtail flounder are three species of pleuronectid presently being studied for possible exploitation through aquaculture. These species are known to feed on different prey types in the wild. The Atlantic halibut is a purely piscivorous feeder when over 80 cm in length,

whereas the winter flounder and the yellowtail flounder are invertebrate feeders and mixed feeders respectively (Scott and Scott, 1988).

Some authors have suggested that different diets or prey types may be reflected in the number and kinds of specific cell types in the gut (Anderson, 1986).

In order to determine if comparative histological differences exist in the alimentary canal of shallow water, invertebrativorous and deep water, piscivorous pleuronectids, this study will:

1. Examine and compare the histology of the epithelium in the intestine, pyloric caeca and rectum from the Atlantic halibut, winter flounder, and the yellowtail flounder with reference to diet.

2. Examine and compare the mucous histochemistry, number per unit area, regional distribution and respective dimensions of goblet cells across these three absorptive regions and species, and comment theoretically on mucous production per area of gut.

Information gathered from studies such as this will aid in expanding present knowlege of the structure and function of the digestive system in pleuronectids and provide information to further advancement into the culture of these species.

### 3.2. Materials and Methods

(Please see Chapter 2, sections 2.2 to 2.2.3.3 for detailed fish and tissue processing protocol).

#### 3.2.1. Sampling of tissue

Bands of tissue 0.25-0.5 cm long were removed from the intestine, pyloric caeca, and rectum according to the following criteria:

1. Intestinal samples were removed from an area midway between the gastro-intestinal junction and the intestinal-rectal junction.

2. Samples of pyloric caeca were removed from an area midway along the structure.

3. Rectal samples were taken from an area midway between the intestinal-rectal junction and the rectal-anal junction. Junctions were distinguished grossly as areas of constriction separating distinct regions of gut.

#### 3.2.2. Quantitative analysis

##### 3.2.2.1 Distribution of goblet cells

The numerical distribution of goblet cells through the post-gastric alimentary canal was compared in the three species. Quantification involved measuring randomly defined areas of specific regions and subsequently counting the goblet cells that were present within these areas. Area determination and counting were carried out on a "Bioquant" computerized quantification system (BQ system IV; R & M Biometrics Inc.). Microscope slides containing sections of gut stained with AB/PAS, were placed on a compound microscope with an affixed video display camera. The image of the section was projected onto a computer monitor screen.

Randomization was achieved by using a random number generator to produce a specific value corresponding to a numbered block on a grid affixed to the computer monitor screen. Determination of the random area was carried out at 25X magnification and the measurements followed at 400X magnification.

Four fish were utilized from each species and measurements continued until approximately 30 cells were counted per region for each fish. Statistical analysis was carried out on SAS (version 6.04). Analysis involved an analysis of variance through Split-plot design model (Steel and Torrie, 1980), with species as the main plot and regions within fish as the subplot. The error term for calculation of the F-value was that which encompassed all samples. The resulting F-value was large enough ($p=0.0001$) to guarantee significance in the absence of the consideration of the error term for specific subsamples (SS subsamples (SP x Fish x Region)).

**3.2.2.2 Cross species comparison of goblet cell dimensions**

Using the same system as above, 30 random goblet cells were measured for length and width in each region of gut and across species. Statistical analysis was carried out on the MINI-TAB (version 7.1; VAX/VMS VERSION 1989) statistics package and involved a two-way analysis of variance and a one-way analysis of variance to compare the dimensional area of the goblet cells as well as the individual cell lengths and widths between regions and between species. Statistics were based on the model for analysis of variance presented in Huntsberger and Billingsley (1981)

#### 3.2.2.3 Prediction criteria for calculation of mucous volume

Predicted limits for mean total mucous volume were calculated for each species over the same regions by utilizing the above data and making the following assumptions:

a) The shape of the mucus containing region of the goblet cell in each species can be modeled by an ellipsoid and hence the volume could be calculated as that for an ellipsoid.

b) The stereology term $\bar{D}$, can be predicted to be greater than the minor and less than the major diameter of a goblet cell modeled as above. $\bar{D}$ is defined as the average of the diameters of a theoretical number of objects at different orientations (Weibel, 1979).

## 3.3. Results

### 3.3.1. Light microscopy

#### 3.3.1.1 Intestine and pyloric caeca

The general organization of the intestine and pyloric caeca was similar to that of the general vertebrate plan, with essentially all the same layers present in the fish. Exocrine pancreatic tissue was observed in association with the intestinal serosa of both the flounders, and with the serosa of the pyloric caeca in all species. The muscularis externa in all three species consisted of two layers of smooth muscle, an outer longitudinal and a thicker, inner circular layer. Myenteric plexi were observed between these layers in the intestine (Figure 3.1). No muscularis mucosae was observed in association with the intestine or caeca of these species.

The intestinal propria-submucosa in the winter flounder and yellowtail flounder consisted of an eosinophilic areolar connective tissue which became slightly more compacted and vascularized towards the mucosa (Figure 3.2A). The propria-submucosa in the halibut was composed of a dense irregular connective tissue which became a more aerolar and highly vascularized tissue close to the mucosa (Figure 3.2B). The propria-submucosa of the pyloric caeca was somewhat more dense in the halibut and yellowtail flounder than in the winter flounder.

The mucosa of the intestine in all three species was folded and covered with villi composed of an epithelial layer supported by connective tissue of the propria-submucosa (Figure 3.3A). The surface of the caecal mucosa was covered with villi but there was no folding of the mucosa (Figure 3.3B). Caecal villi were extremely long filling the lumen. The intestinal and caecal epithelial layer in all species consisted of simple columnar epithelium that was interspersed with goblet cells. The columnar cells possessed a basal nucleus, a very eosinophilic cytoplasm, and an apical brush border (Figure 3.4). Wandering cells of vascular origin, possibly lymphocytes, were noted in association with the basal region of the epithelium. The goblet cells exhibited a supranuclear region characterized by a swollen, distal region that contained a translucent cytoplasm and a basal region with associated nuclei.

#### 3.3.1.2 Rectum

Four distinctive layers were identified in cross-sections of the rectum: the outer serosa, the muscularis externa, the submucosa, and the mucosa. Exocrine pancreas was frequently associated with the rectal serosa in the winter flounder.

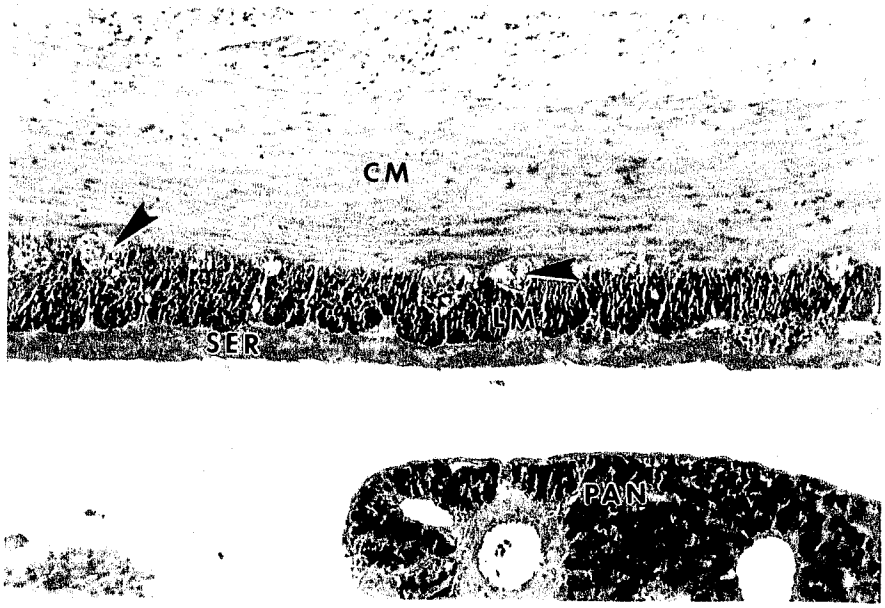
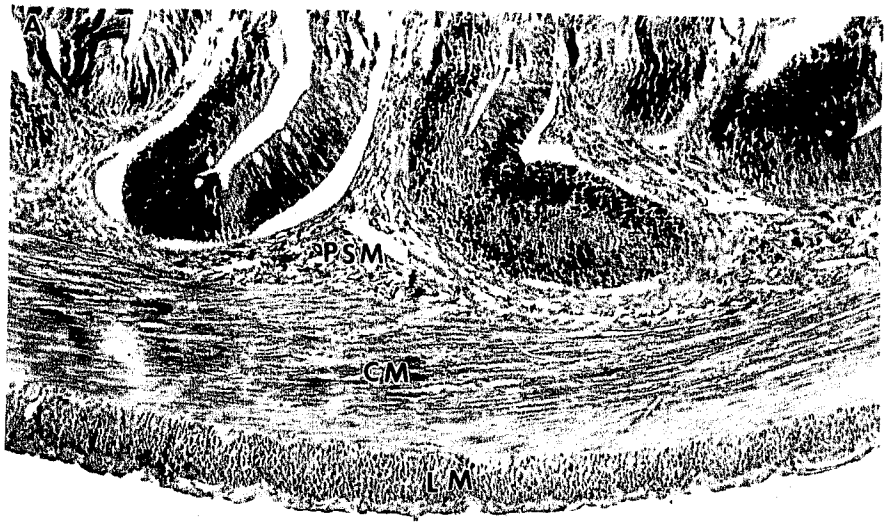
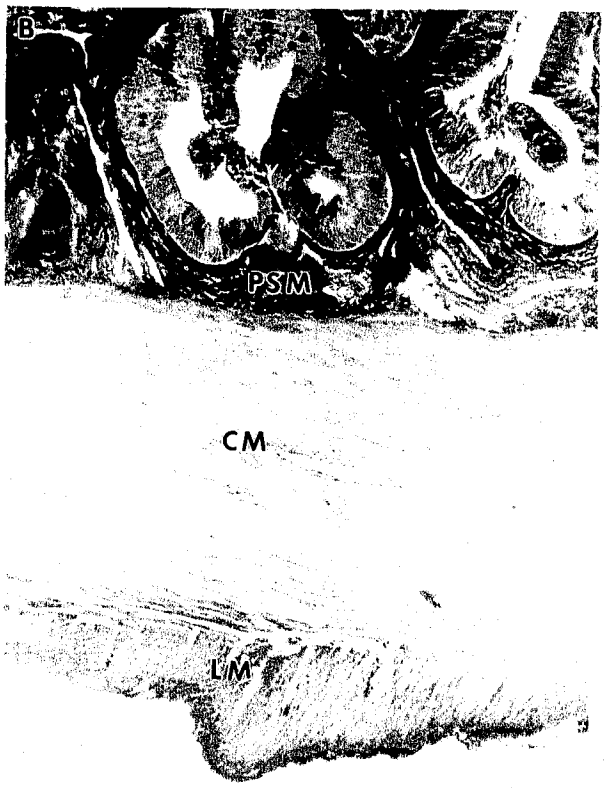



Figure 3.1. Detail of the muscularis externa from yellowtail flounder intestine, illustrating the circular (CM) and longitudinal (LM) smooth muscle layers, associated myenteric plexus (arrowheads) and serosa (SER). Note exocrine pancreatic tissue (PAN) adjacent to the serosa (X165).

Figure 3.2. Connective tissue layers from the intestine of (A) the winter flounder (X660) and (B) the Atlantic halibut (X100), illustrating differences in the density of the tissue forming the propria-submucosa (PSM). CM, circular muscle, LM, longitudinal muscle.


A
PSM
CM
LM


B
PSM
CM
LM

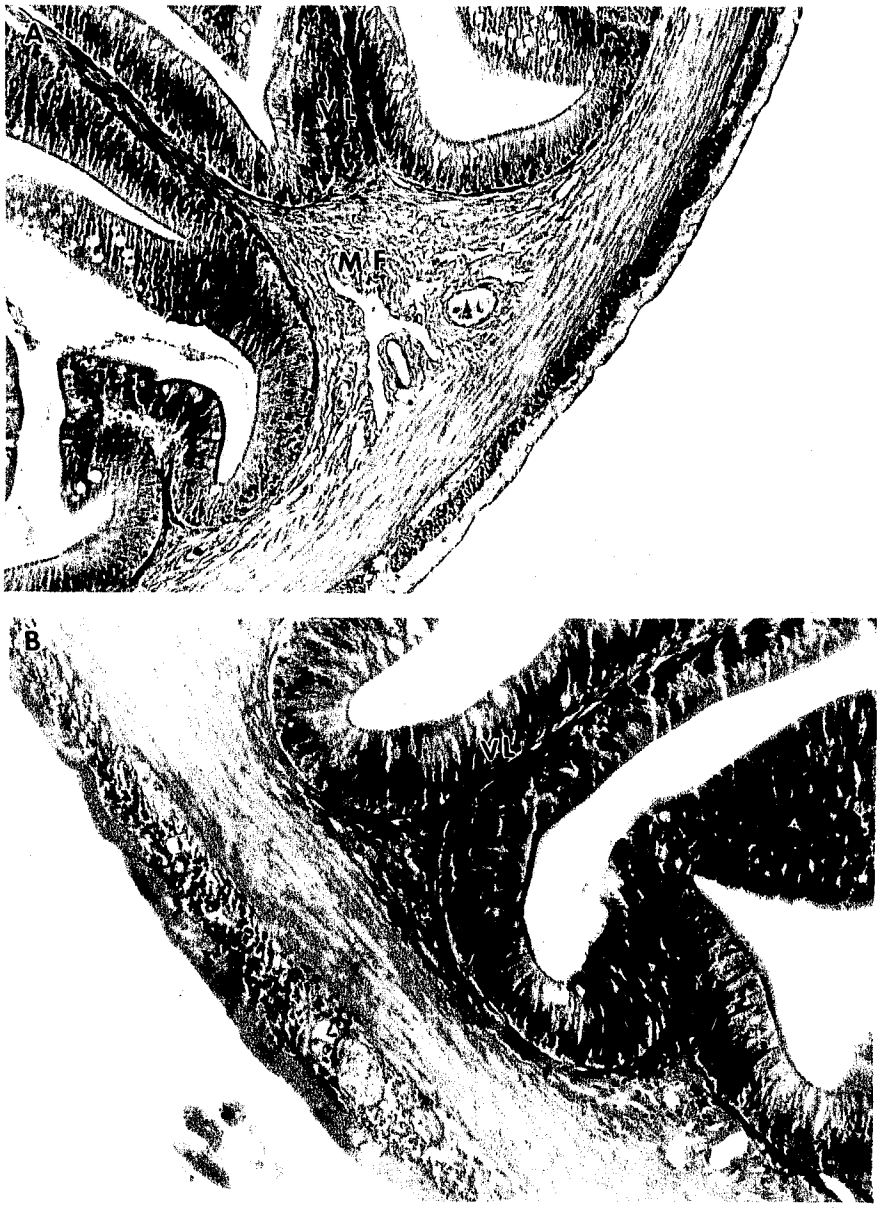



Figure 3.3. Transverse sections through the (A) intestine (X165) and (B) pyloric caeca (X250), from the yellowtail flounder to demonstrate the different degrees of mucosal folding in the respective regions. Note in the intestine, the mucosa consists of short folds (MF) which exhibit distinct branching villi (VL) whereas in the pyloric caeca the villi appear longer and there are no folds.

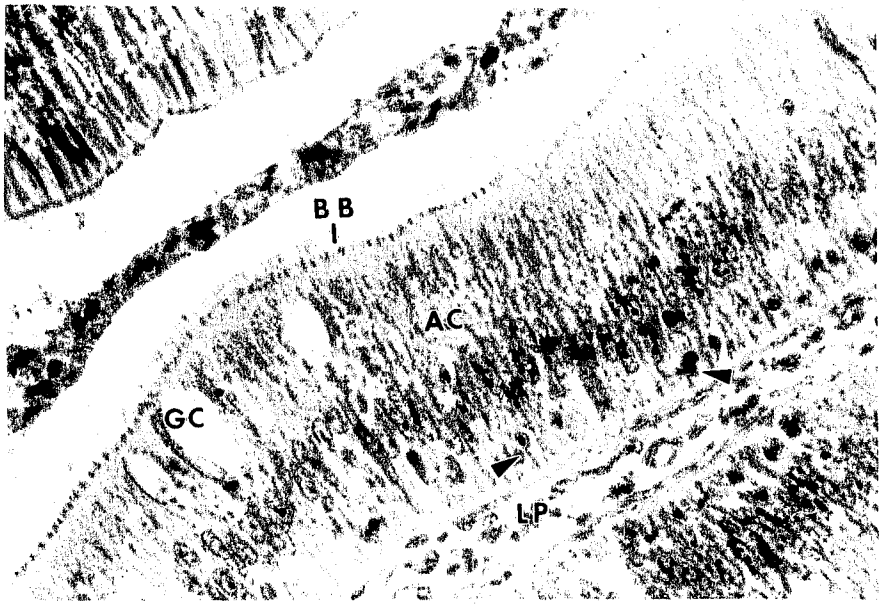



Figure 3.4. Detail of the Atlantic halibut pyloric caeca epithelium showing the columnar absorptive cells (AC) and goblet cells (GC) typical of the post-gastric mucosa stained with H&E. The columnar cells exhibit mid to basal nuclei and an apical brush border (BB) whereas the goblet cell is characterized by an apical translucent goblet structure and basal nuclei. Note the abundance of wandering cells (arrowheads) adjacent to the lamina propria (LP) (X660).

The muscularis externa was made up of two layers of smooth muscle, an inner thick circular layer and an outer somewhat thinner, longitudinal layer. This configuration was consistent between species, although the inner circular layer, in general, appeared thicker in the halibut rectum. The longitudinal layer of muscle also exhibited extensive folding, giving the outer surface of the rectum a ridged appearance in all species (Figure 3.5).

In the Atlantic halibut a prominent muscularis mucosae consisting of at least two layers of smooth muscle separated the areolar connective tissue of the submucosa from the lamina propria (Figure 3.6). The lamina propria adjacent to the muscularis mucosae consisted of a dense irregular connective tissue and became considerably more vascularized and cellular adjacent to the epithelium (Figure 3.7A and B).

An obvious muscularis mucosae was not evident in either flounder species. The areolar connective tissue of the propria-submucosa became slightly more dense in the region bordering the epithelium.

The surface of the rectal mucosa of all three species was arranged into short folds covered with villi (Figure 3.8). The epithelium was simple columnar and was interspersed with considerable numbers of goblet cells. The columnar cells exhibited basophilic basal nuclei, an eosinophilic apical cytoplasm and an apical brush border.

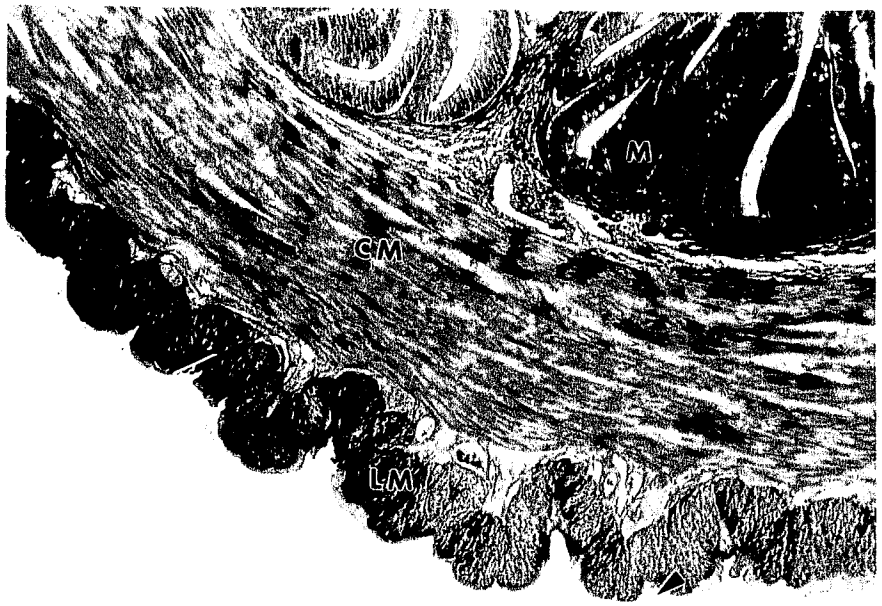



Figure 3.5. Rectum of the yellowtail flounder illustrating the characteristic folding of the longitudinal smooth muscle layer (LM) and adjacent serosa (arrowheads) asscciated with this region (X100). CM, circular layer, M, mucosa.

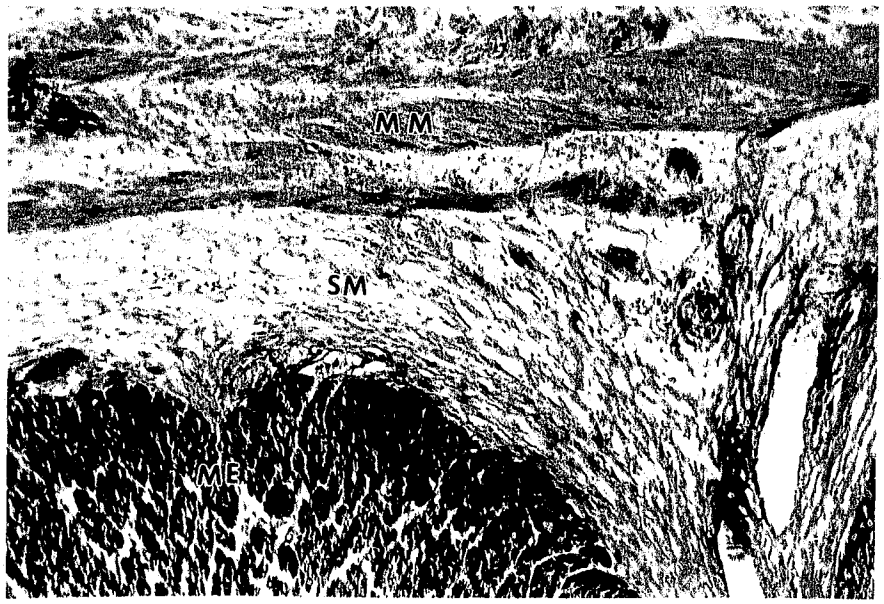



Figure 3.6. Muscularis mucosae (MM), submucosal connective tissue (SM) and adjacent muscularis externa (ME) from the rectum of the Atlantic halibut (X660).

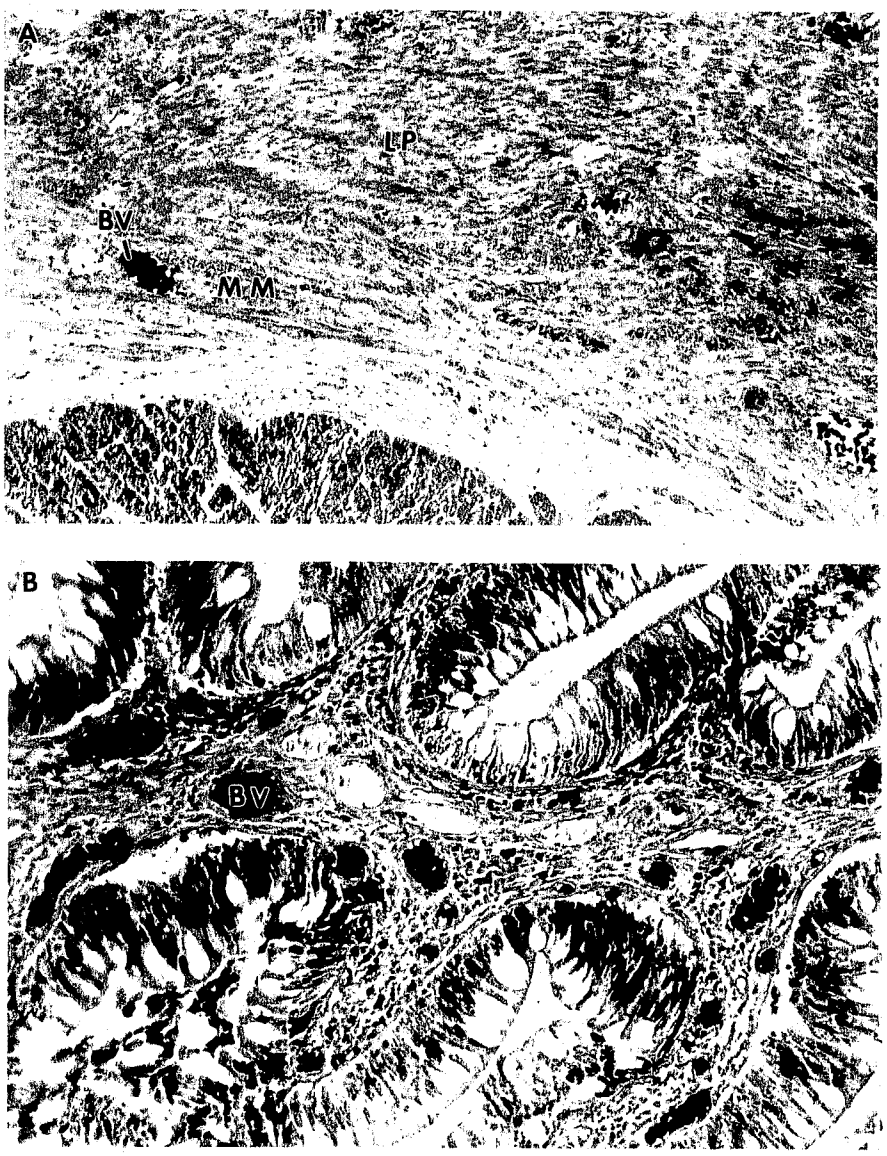



Figure 3.7. Detail of the lamina propria from the rectum of the Atlantic halibut demonstrating (A) the dense connective tissue of the lower lamina propria (LP) next to the muscularis mucosae (MM) with few blood vessels (BV) (X100) and (B) the extent of the vascularization in the lamina propria immediately adjacent to the epithelium (X165).

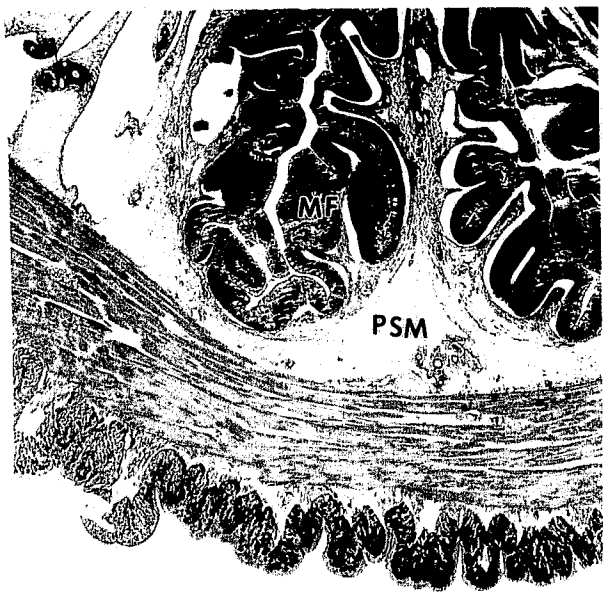



Figure 3.8. Transverse sectional overview of the rectum from the winter flounder demonstrating the characteristic villus branching from the mucosal folds (MF) and the presence of a lamina propria-submucosa (PSM) (X40).

### 3.3.2. Mucous histochemistry and goblet cell regional distribution

#### 3.3.2.1 Mucous histochemistry

##### 3.3.2.2.1 Intestine

The intestinal goblet cells (Table 3.1; Figure 3.9) show that the halibut contains only nonsulphated acid mucins and combinations with neutral mucins. The winter flounder intestinal goblet cells contain sulphated acid mucins and combinations with nonsulphated acid mucins (Figure 3.10). The yellowtail flounder intestinal goblet cells stained only as combinations of neutral mucins, nonsulphated acid mucins and acid mucins (Figure 3.11).

##### 3.3.2.2.2 Pyloric caeca

The caecal goblet cells (Table 3.2; Figure 3.12) stained primarily positive for nonsulphated acid mucins. Occasionally, however, some cells showed combination reactions for nonsulphated acid mucins, sulphated acid mucins, and neutral mucins. The goblet cells of the winter flounder pyloric caecae stained positive for sulphated acid mucins, nonsulphated acid mucins and combinations of both mucins (Figure 3.13). The yellowtail flounder caecal goblet cells stained only as mucin combinations. The reactions gave positive results for combinations of neutral mucins, nonsulphated acid mucins, and sulphated acid mucins (Figure 3.14).

##### 3.3.2.2.3 Rectum

The rectal goblet cells (Table 3.3; Figure 3.15) of the Atlantic halibut were positive for nonsulphated acid mucins and neutral mucins as well as within cell combinations of neutral, nonsulphated and sulphated acid mucus. The winter

TABLE 3.1. MUCOUS HISTOCHEMISTRY : INTESTINE

| | Atlantic halibut | Winter flounder | Yellowtail flounder |
|---|---|---|---|
| Neutral mucus | – | – | – |
| Non-sulphated acid mucus | + | – | – |
| Sulphated acid mucus | – | + | – |
| Cellular combinations | + | + | + |

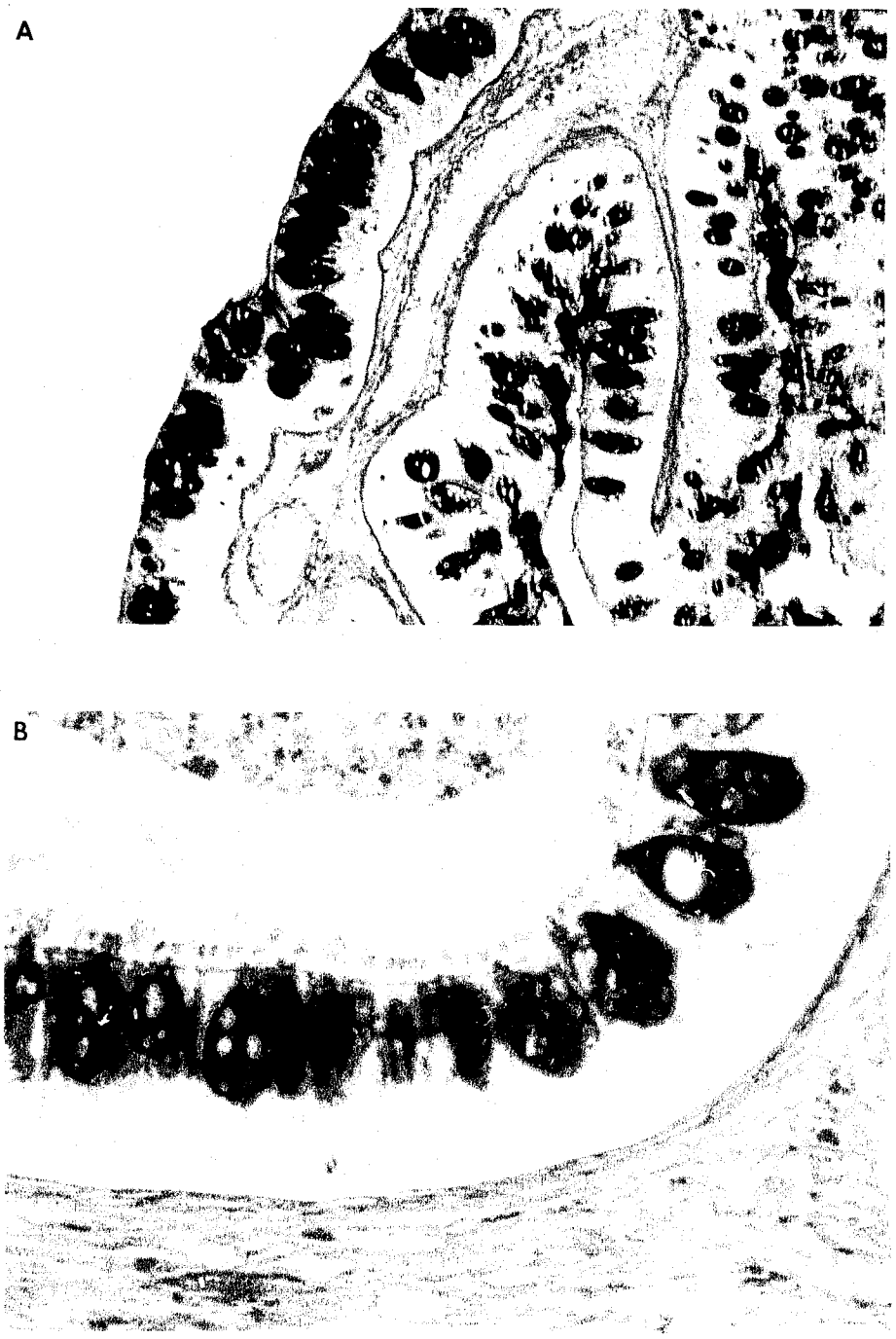



Figure 3.9. Alcian Blue/PAS differential staining of Atlantic halibut intestinal goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X660).

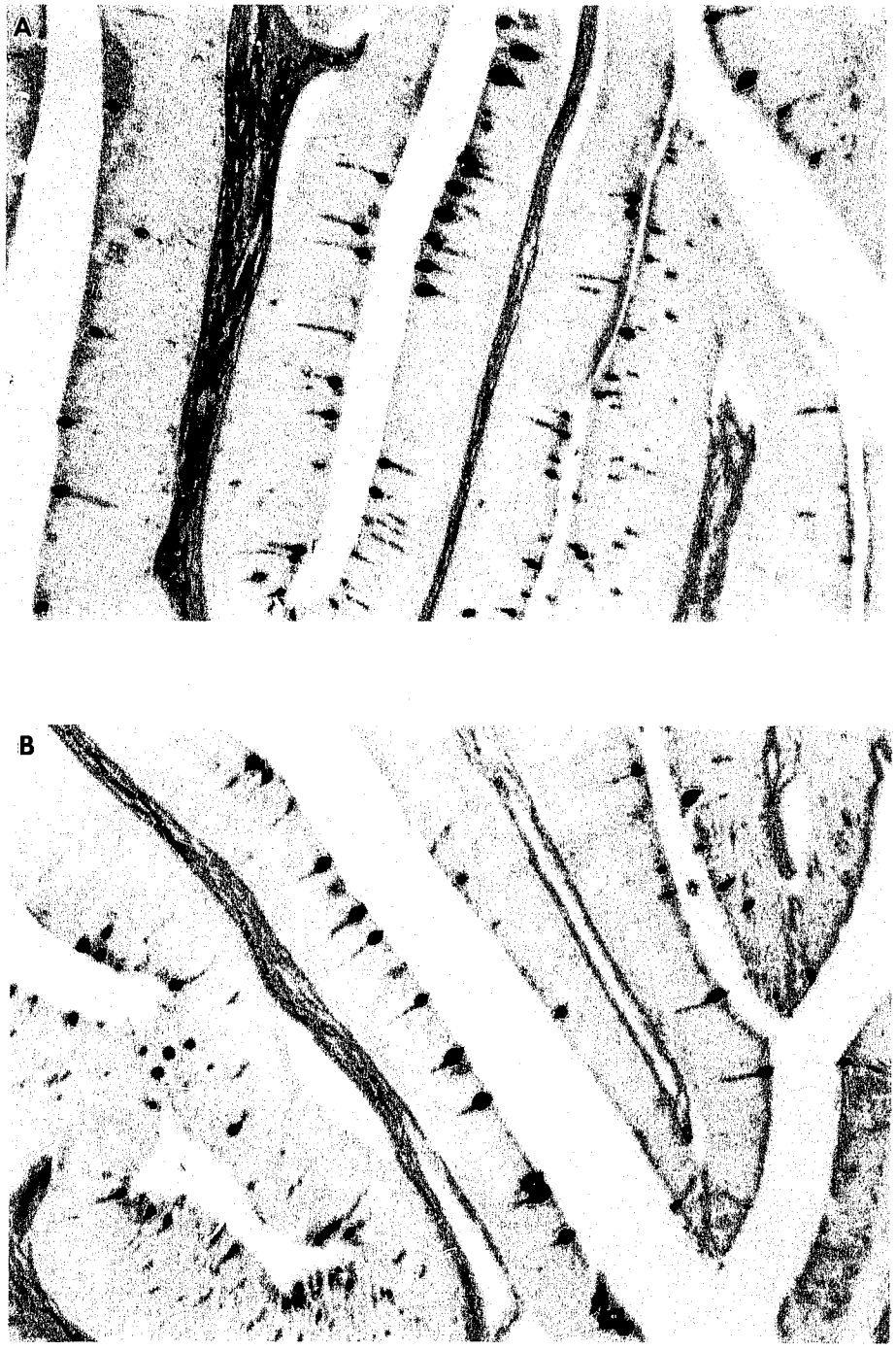



Figure 3.10. Alcian Blue/PAS differential staining of winter flounder intestinal goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

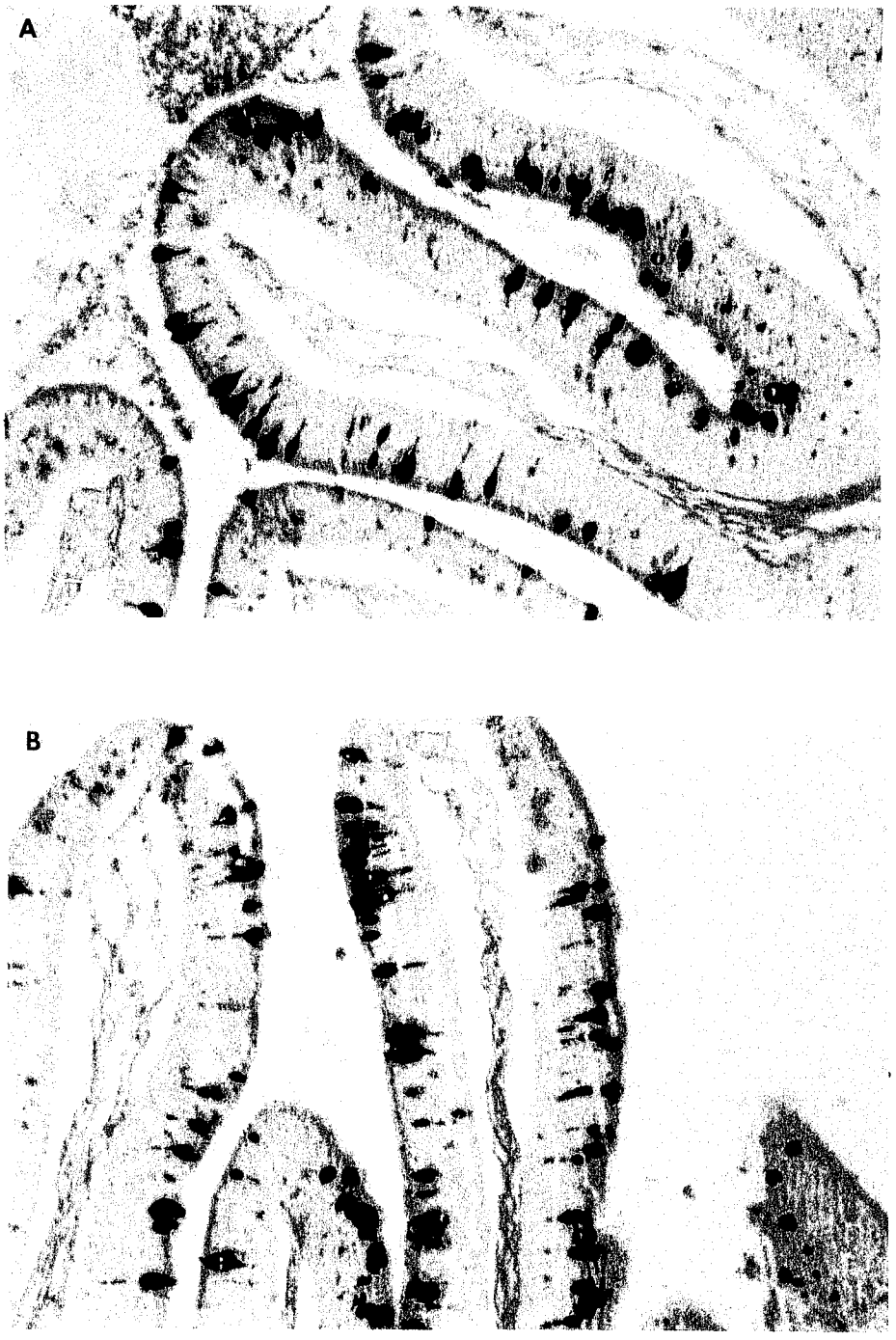



Figure 3.11. Alcian Blue/PAS differential staining of yellowtail flounder intestinal goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

TABLE 3.2. MUCOUS HISTOCHEMISTRY : PYLORIC CAECA

| | Atlantic halibut | Winter flounder | Yellowtail flounder |
|---|---|---|---|
| Neutral mucus | – | – | – |
| Non-sulphated acid mucus | + | + | – |
| Sulphated acid mucus | + | + | – |
| Cellular combinations | + | + | + |

TABLE 3.3. MUCOUS HISTOCHEMISTRY : RECTUM

| | Atlantic halibut | Winter flounder | Yellowtail flounder |
|---|---|---|---|
| Neutral mucus | + | – | – |
| Non-sulphated acid mucus | + | + | – |
| Sulphated acid mucus | – | + | – |
| Cellular combinations | + | + | + |

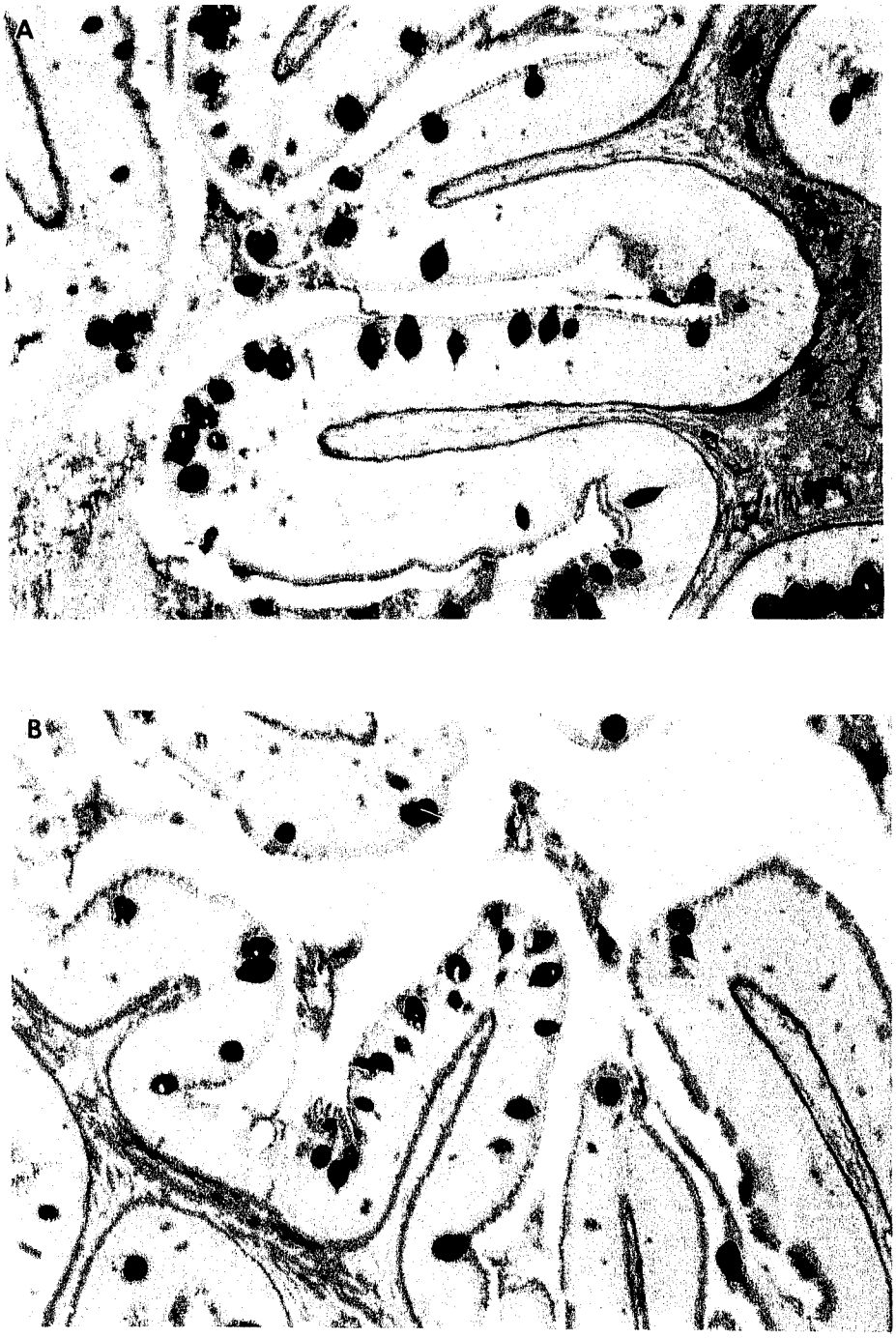



Figure 3.12. Alcian Blue/PAS differential staining of halibut pyloric caeca goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

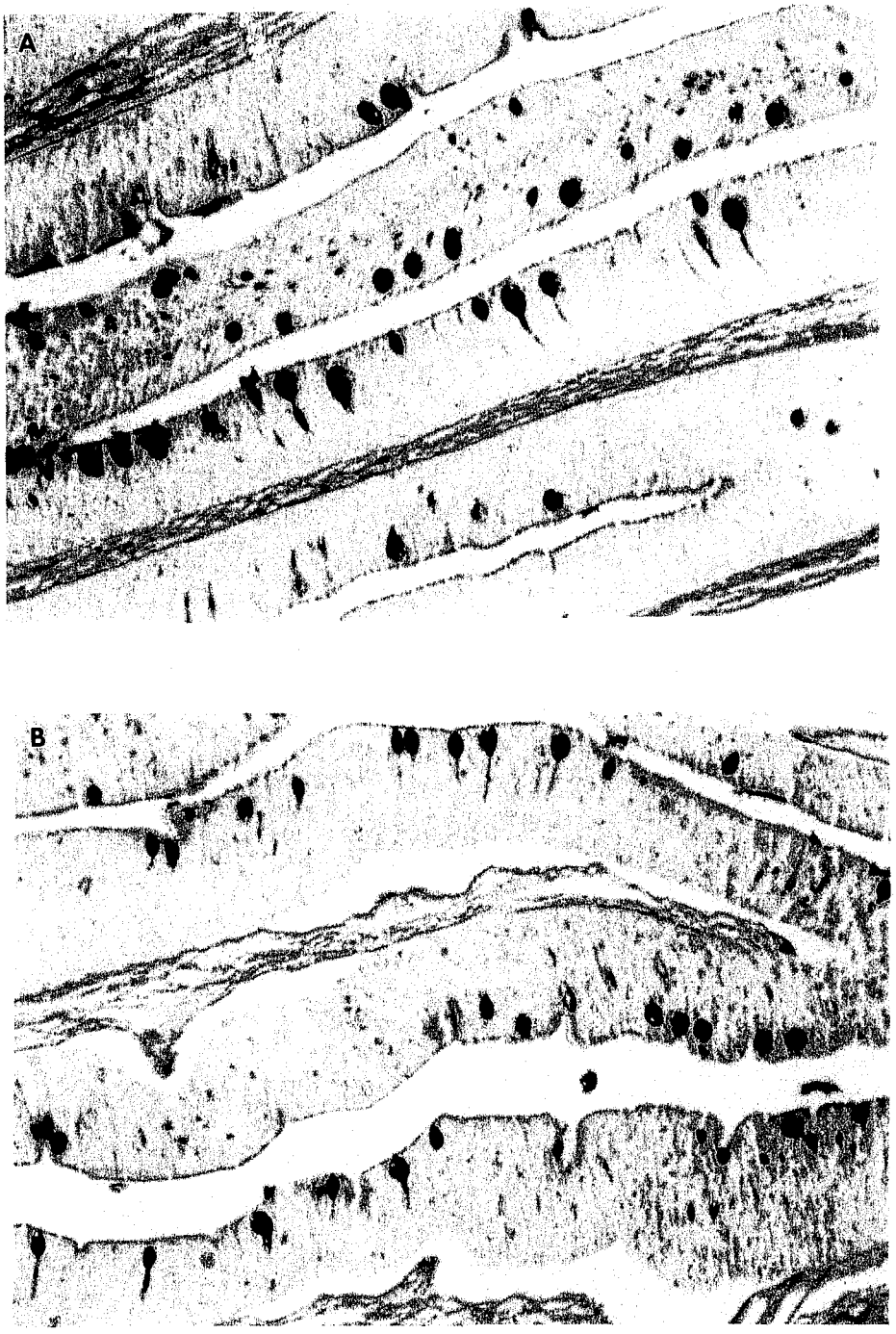



Figure 3.13. Alcian Blue/PAS differential staining of winter flounder pyloric caeca goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

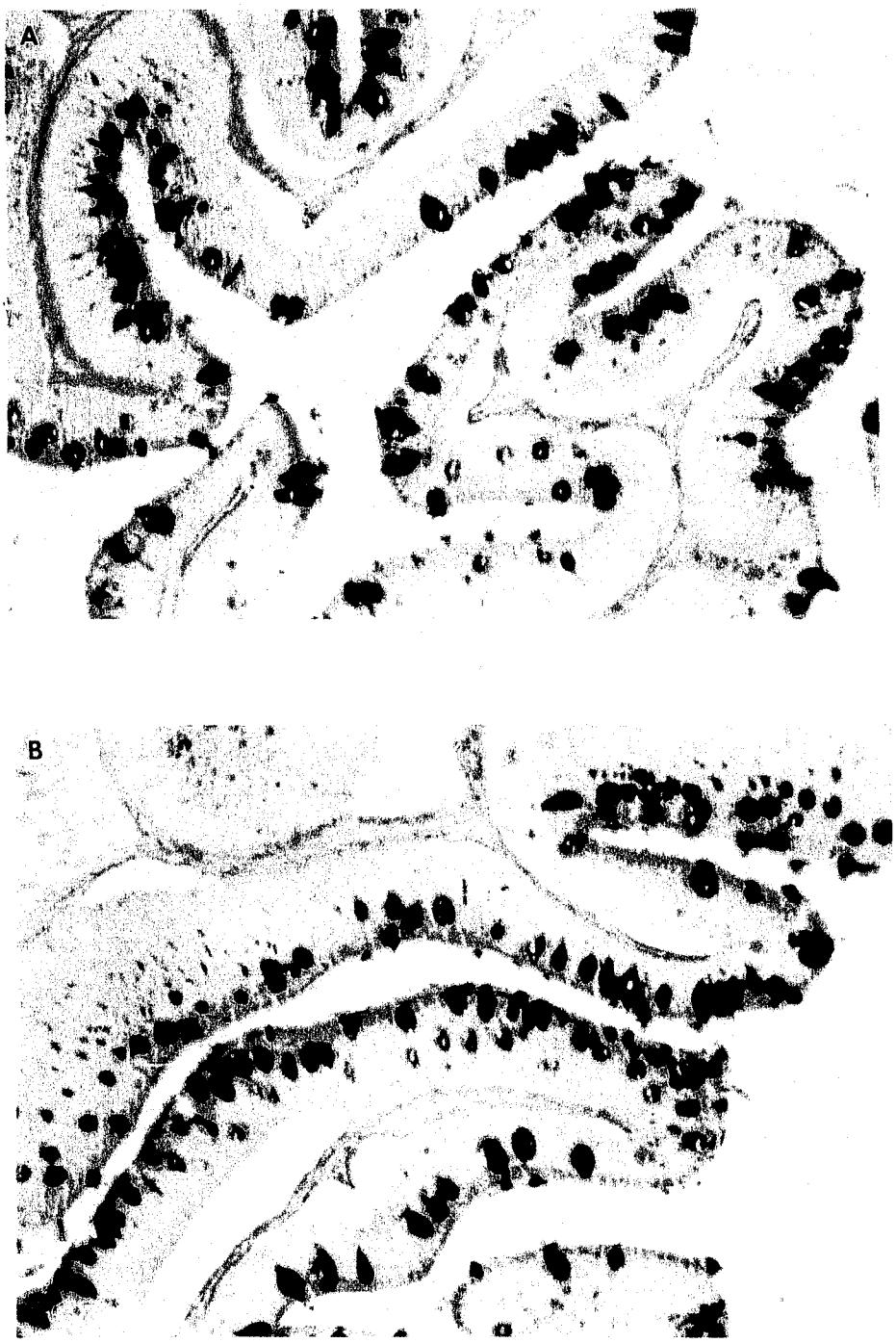



Figure 3.14. Alcian Blue/PAS differential staining of yellowtail flounder pyloric caeca goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

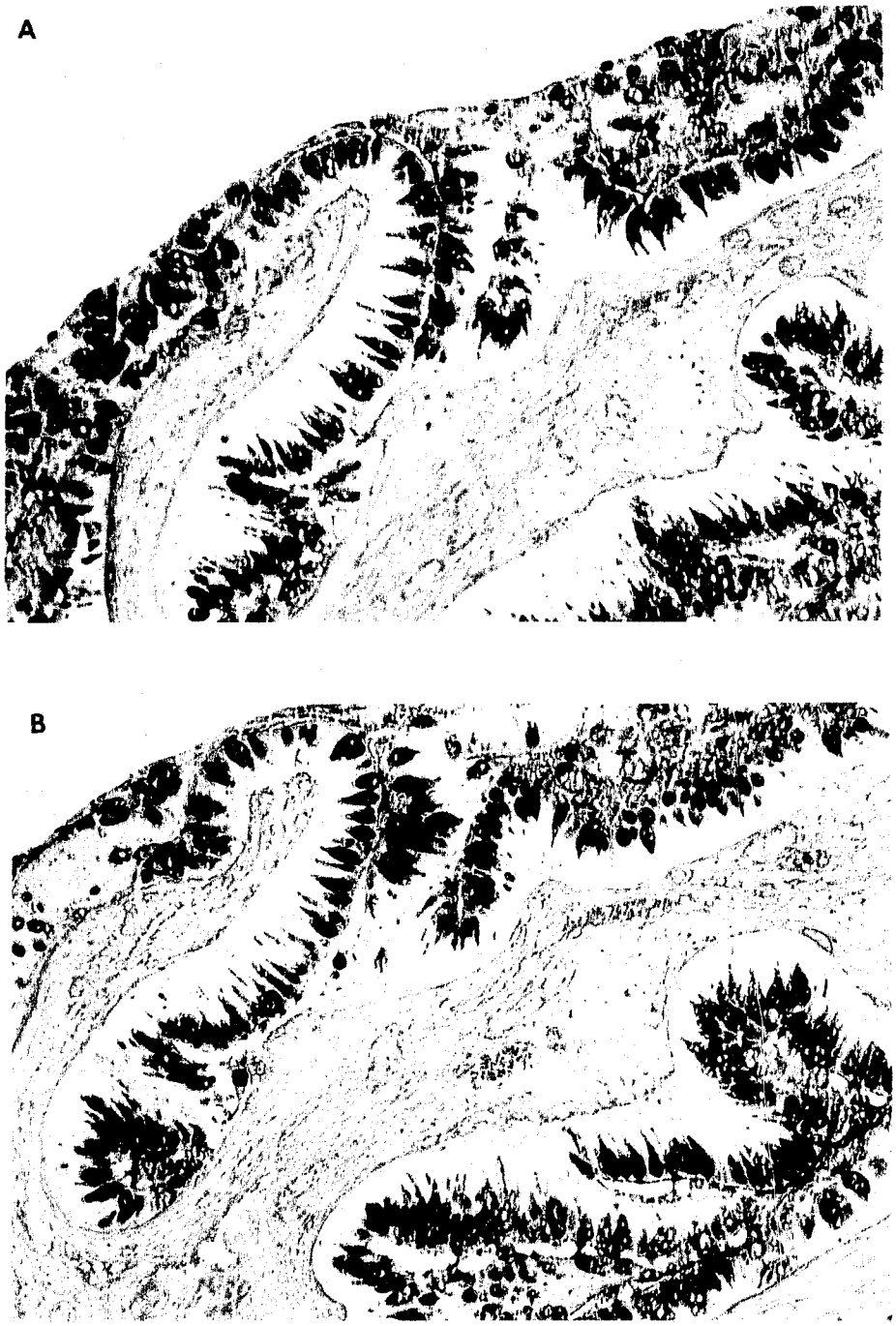



Figure 3.15. Alcian Blue/PAS differential staining of halibut rectal goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).


Figure 3.16. Alcian Blue/PAS differential staining of winter flounder rectal goblet cell mucus. (A) pH 2.5 (X250) (B) pH 1.0 (X250).

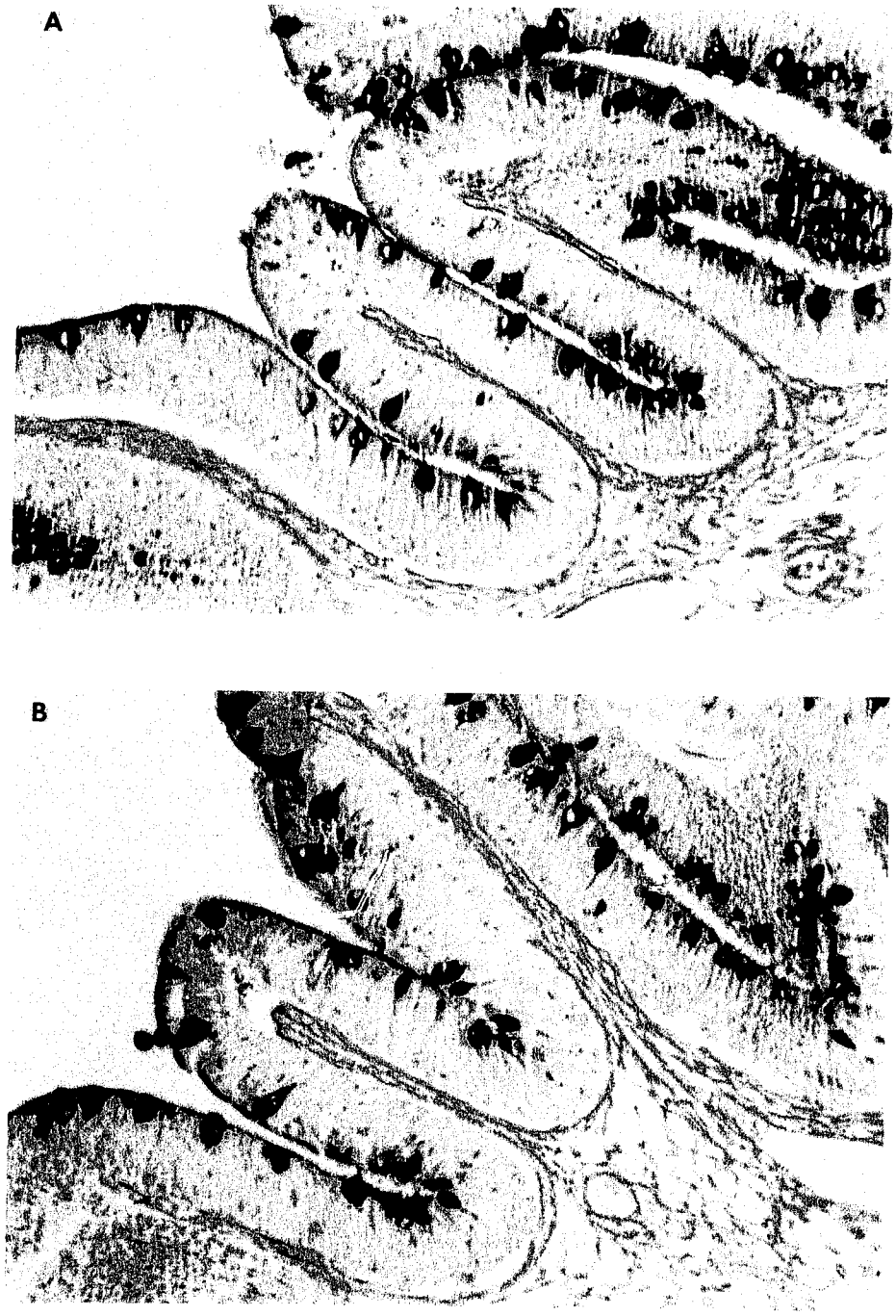



Figure 3.17. Alcian Blue/PAS differential staining of yellowtail flounder rectal goblet cell mucus. (A) pH 2.5 (B) pH 1.0 (X250).

flounder rectal goblet cells contain nonsulphated acid mucins, sulphated acid mucins and combinations of the two (Figure 3.16). Rectal goblet cells of the yellowtail flounder contained mucous chemo-type combinations comparable to those of the previous regions (Figure 3.17). These consisted of neutral mucins, nonsulphated acid mucins and sulphated acid mucins.

#### 3.3.2.1 Goblet cell dimensions and numerical distribution

Statistical analysis gave significant ($p < 0.05$) regional differences in mean goblet cell count per area, however, there was no significant difference ($p>0.05$) between number per area among species in the same regions (Figure 3.18). The number of goblet cells per area of gut increased significantly from the pyloric caeca/intestine through to the rectum ($p<0.05$).

Further observations on goblet cell size showed that the Atlantic halibut goblet cells were significantly larger ($p<0.05$), than those of the yellowtail flounder or the winter flounder whereas those of the latter were not significantly different ($p>0.05$) from one another (See Table 3.4).

#### 3.3.2.2 Estimated mucous volume fraction limits

Predicted limits suggest that the production of mucus in halibut increases linearly from the pyloric caeca/intestine to the rectum and that this mucous volume fraction range appears greater than that of the other species in all the regions. A similar trend was predicted for the winter flounder. The predicted trend for the yellowtail flounder suggests a quadratic relationship (Table 3.5; Figure 3.19).

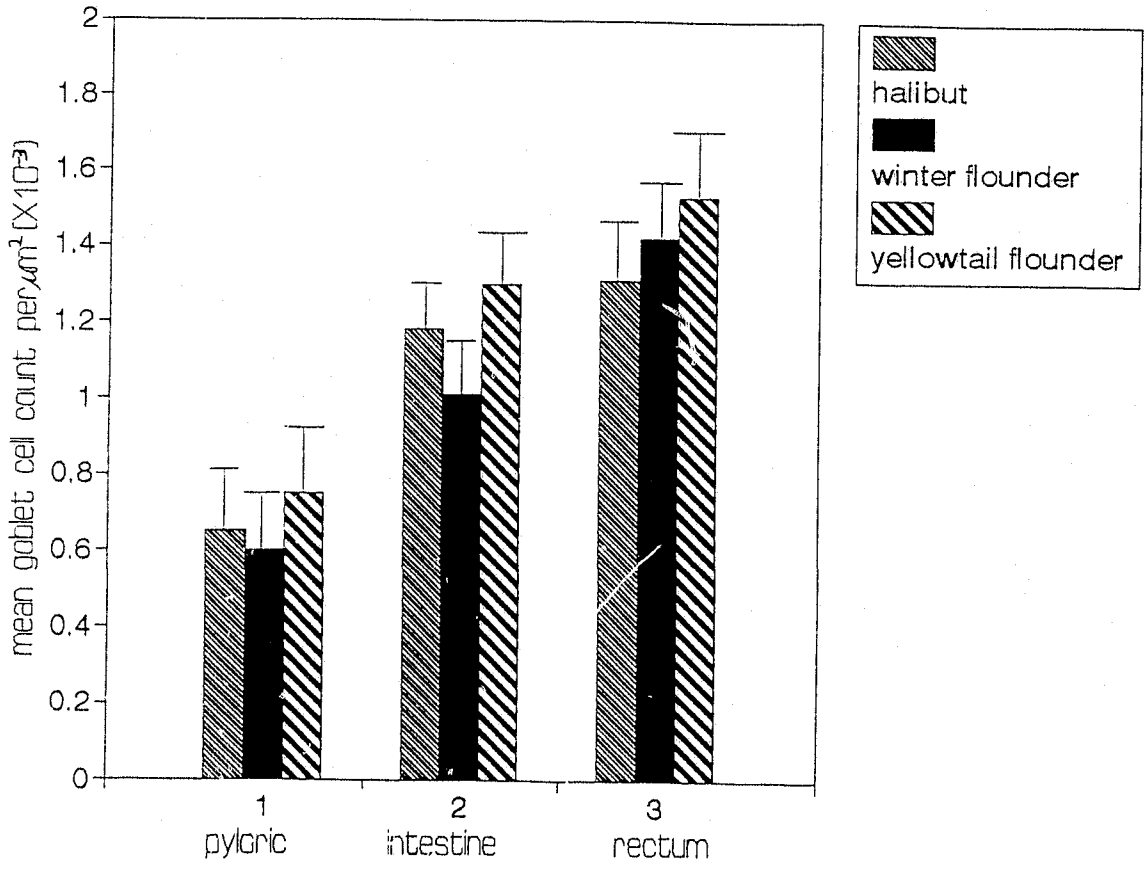



Figure 3.18. Plot of mean goblet cell count per $\mu m^2$ of epithelium, for the pyloric caeca, intestine and rectum in three species of pleuronectid.

TABLE 3.4. MEAN GOBLET CELL DIMENSIONS WITHIN POST GASTRIC REGIONS

| | DIMENSIONS ($\mu$m ± SE) N = 30 | PYLORIC CAECAE | INTESTINE | RECTUM |
|---|---|---|---|---|
| ATLANTIC HALIBUT | ↔ | 11 ± 0.56 | 12.53 ± 0.49 | 10.75 ± 0.65 |
| | ↕ | 19.6 ± 1.2 | 24.75 ± 1.0 | 22.75 ± 0.69 |
| WINTER FLOUNDER | ↔ | 10.4 ± 0.75 | 8.63 ± 0.48 | 7.81 ± 0.42 |
| | ↕ | 17.9 ± 1.4 | 14.5 ± 0.67 | 12.08 ± 0.80 |
| YELLOWTAIL FLOUNDER | ↔ | 8.4 ± 0.35 | 8.6 ± 0.33 | 8.95 ± 0.47 |
| | ↕ | 14.1 ± 0.65 | 13.64 ± 0.82 | 14.4 ± 0.95 |

TABLE 3.5 PREDICTED MUCUS VOLUME FRACTION RANGES ($\mu m^3/\mu m^3$)

| Location | Atlantic halibut | Winter flounder | Yellowtail flounder |
|---|---|---|---|
| Pyloric caeca | 0.096-0.091 | 0.051-0.045 | 0.051-0.045 |
| Intestine | 0.196-0.171 | 0.066-0.059 | 0.114-0.101 |
| Rectum | 0.315-0.267 | 0.081-0.067 | 0.104-0.094 |

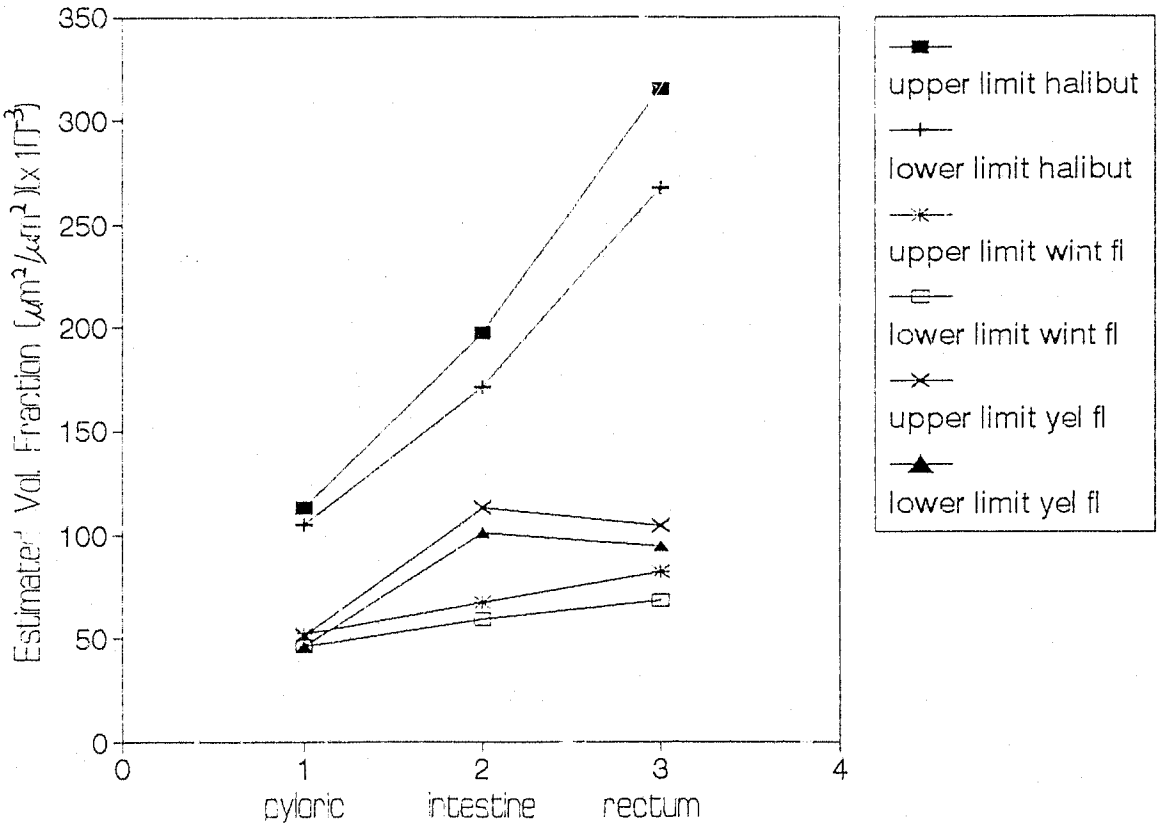



Figure 3.19. Plot of estimated volume fraction ranges of mucus produced in the pyloric caeca, intestine and rectum of three species of pleuron d.

### 3.3.3. Electron microscopy

General cell types

In all three species the post-gastric epithelium (intestine, pyloric caeca, and rectum) was defined by two main cell types, columnar epithelial cells and goblet cells. Variations between regions and species were defined with reference to the epithelial cells.

#### 3.3.3.1 Intestine and pyloric caeca

The lumenal surface of the intestinal and caecal epithelial cells was characterized by an apical plasma membrane folded into a series of microvilli which exhibited a filamentous core. The cytoplasm situated immediately beneath the microvilli was normally free of organelles but did exhibit vertical filamentous extensions from the microvilli as well as associated perpendicular cytoplasmic filaments. These were found to be associated with desmosomes which were identified with junctional complexes of the lateral membrane. This area corresponds with the terminal web of absorptive epithelial cells found in other vertebrates.

Generally, the apical cytoplasm exhibited abundant elongated mitochondria, occasional cisternae of RER, many elements (vesicles and saccules) of the smooth endoplasmic reticulum (SER), secondary lysosomes and a few multivesicular bodies (Figure 3.20).

The Atlantic halibut intestinal epithelium exhibited an apical cytoplasm further characterized by the presence of large spherical droplets of lipid that appeared non-membrane bound and free in the cytoplasm.

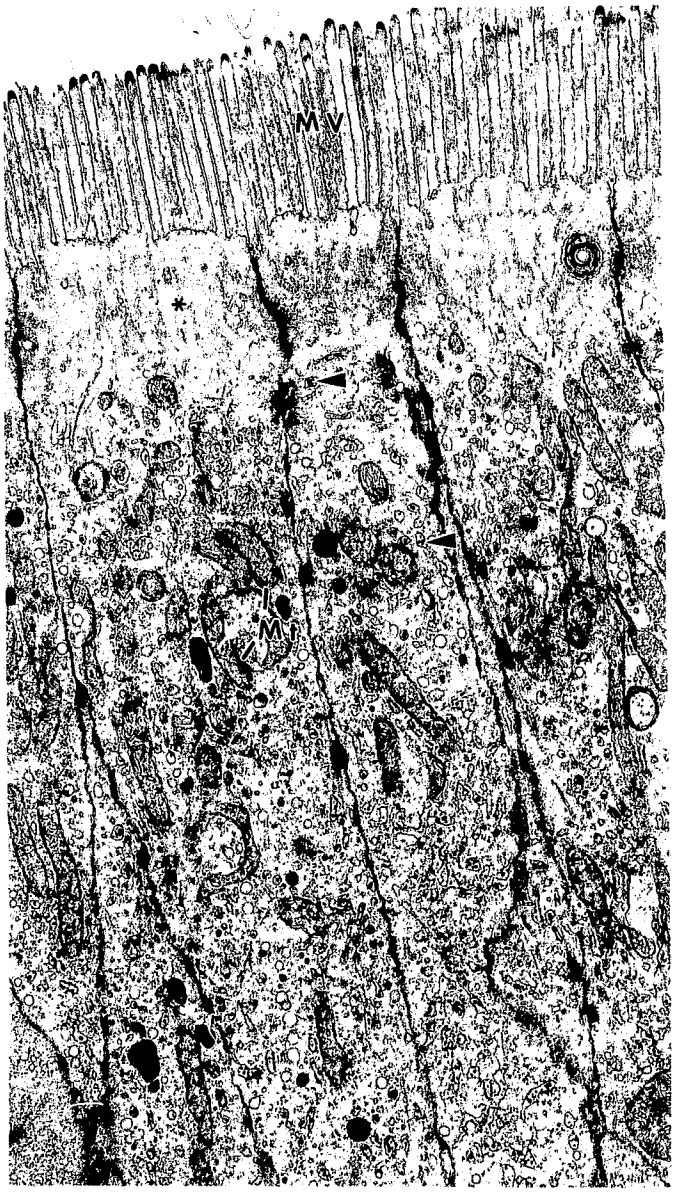



Figure 3.20. Electron micrograph of the apical portion of winter flounder pyloric caeca epithelial cells, demonstrating the general features exhibited by a typical post-gastric absorptive cell. Note the elongated apical microvilli (MV) with filamentous central cores (asterisk) extending into the terminal web, numerous mitochondria (Mt), and abundant smooth endoplasmic reticulum containing dense chylomicron particles (arrowheads) (X 11 500).

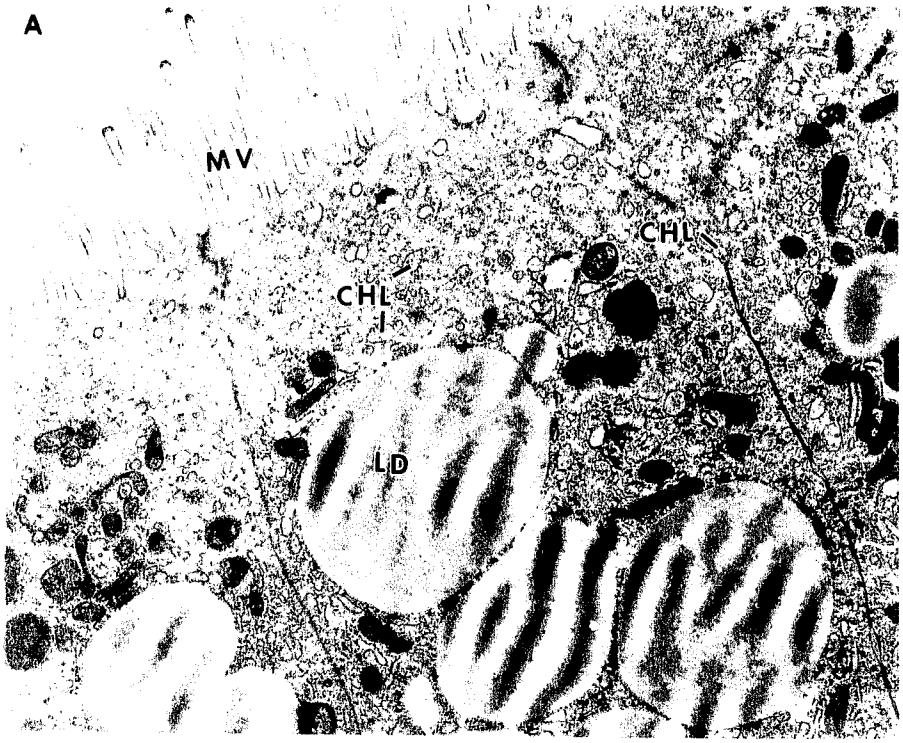
Smooth endoplasmic reticulum vesicles and saccules contained chylomicra (smaller lipid-based droplets of the same electon density and consistency as those free in the cytoplasm). Chylomicra were also observed in intercellular spaces (Figure 3.21A).

Large cytoplasmic lipid droplets were not observed in the apical cytoplasm of either the winter flounder or the yellowtail flounder. Small, very dense chylomicra droplets were noted, however, in the intercellular spaces and some SER elements of the winter flounder epithelium (Figure 3.21B). Similar dense chylomicra were observed within SER associated with the apical cytoplasm of the yellowtail flounder intestinal epithelium but not within intercellular spaces.

Bacteria and protozoan were found in close association with the apical membrane of both the intestinal and pyloric caecal epithelial cells of the winter flounder. The bacteria appeared to be seated deep within depressions of the membrane (Figure 3.22). Frequently, the underlying cytoskeleton was shown to bend, apparently due to the presence of bacteria. At no time however, did the membrane itself appear breached.

The basal cytoplasm of the intestinal and caecal epithelium in all three species was organized in a similar fashion. It was characterized by laminar basal infoldings of the plasma membrane with associated elongated mitochondria oriented perpendicular to the base of the cell (Figure 3.23). In the Atlantic halibut both smooth membrane bound chylomicra and non-membrane bound lipid droplets were frequently identified within the cytoplasm of the infoldings. These chylomicra and

Figure 3.21. (A) Apical portion of an Atlantic halibut intestinal epithelial cell showing the presence of large lucent non-membrane bound lipid droplets (LD) in the cytoplasm. Note the abundance of chylomicron particles (CHL) both within elements of the smooth endoplasmic reticulum and intercellular spaces (CHL). MV, microvilli. (X12 000). (B) Higher magnification of the more electron dense chylomicron particles (CHL) found associated with the intestinal epithelial cells of the winter flounder and the yellowtail flounder (X24 000).


A
MV
CHL
CHL
LD


B
CHL

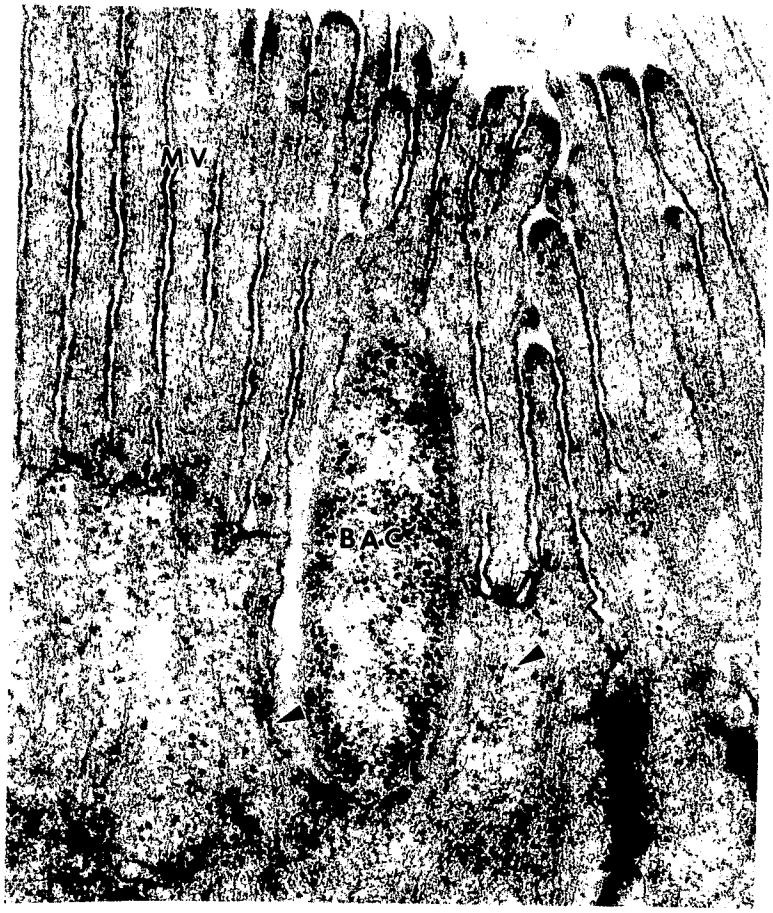



Figure 3.22. Electron micrograph illustrating the presence of a bacterium (BAC) in close association with the apical plasma membrane of winter flounder intestinal epithelial cells. Note that the bacteria is seated within a deep depression of the membrane resulting in an actual bending of components of the terminal web (arrowheads). MV, microvilli (X50 000).

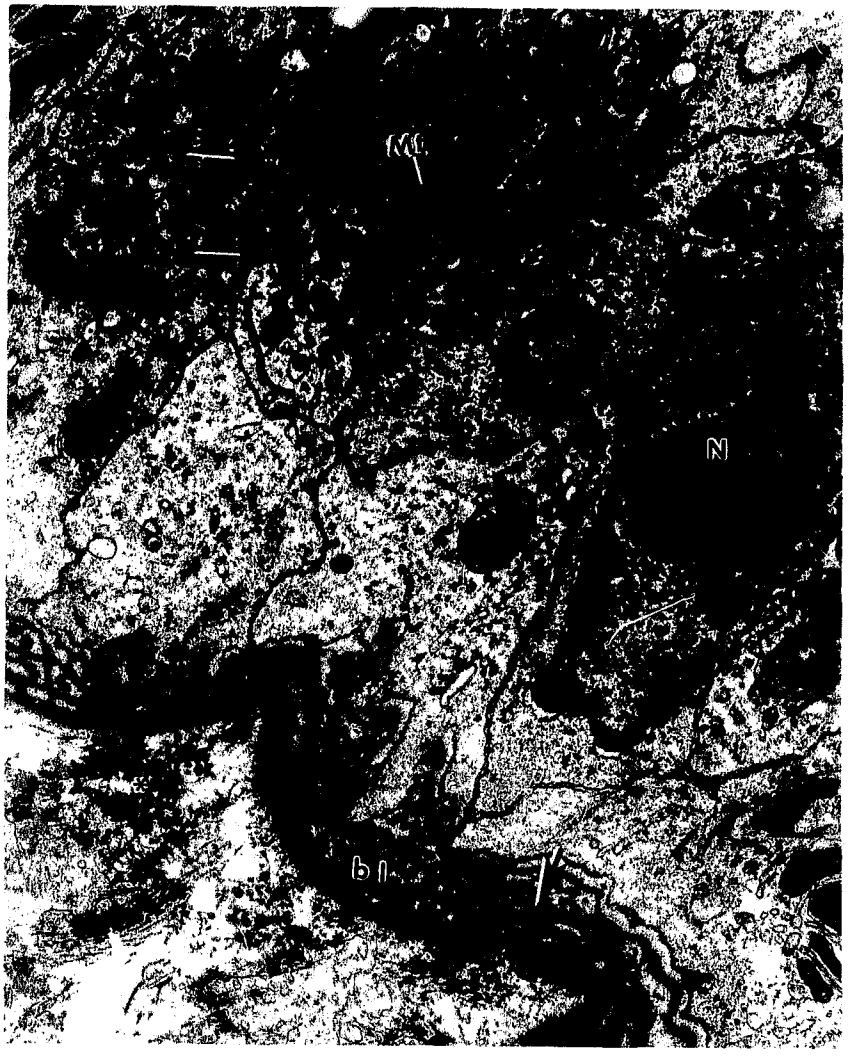



Figure 3.23. The basal portion of halibut intestinal epithelial cells showing the presence of laminar infoldings of the baso-lateral membrane with associated elongate mitochondria (Mt), a distinct reticulated basal lamina (bl) with dense bodies (arrows) and a wandering cell. Note also the association of membrane bound lucent chylomicra with the infoldings (arrowheads). Chylomicra are also observed within the extracellular matrix (asterisk). N, nucleus of wandering cell (X13 600).

lipid droplets were not found in the basal cytoplasm of the epithelium in the winter flounder or the yellowtail flounder. Wandering cells were common between epithelial cells but were observed most frequently between the basal regions of the cells for all three pleuronectid species (Figure 3.23). Occasionally, some of these wandering cells possessed electron-dense granules within their cytoplasm. It may be speculated that these cells could be lymphocytes or other wandering cells of vascular origin acting as part of the diffuse lymphoid system characteristic of most teleosts (Barrington, 1957).

The structure of the basal lamina associated with the intestinal and caecal epithelium of the Atlantic halibut demonstrated an unusual configuration (Figure 3.23). The lamina densa consisted of numerous reticulations and laminations that extended into the lamina lucida. The thickness of the basal lamina was up to 1 $\mu$m with an average of 620 $\pm$ 39 nm. Electron-dense bodies were commonly observed embedded within the reticulated matrix. The basal lamina identified with the winter flounder and the yellowtail flounder cells was normally uniform in nature with an average lamina densa thickness of 185 $\pm$ 16 nm (Figure 3.24).

Goblet cells appeared typical of those found in most vertebrates. The cells were joined to adjacent epithelial cells through junctional complexes and spot desmosomes. The apical cytoplasm was characterized by large mucous granules or droplets which appeared as attached compartments. The basal cytoplasm was defined by a euchromatic nucleus, RER and a well developed Golgi complex (Figure 3.25).


Figure 3.24. Electron micrograph of the basal lamina (bl) from a winter flounder intestinal epithelial cell showing the typical structure found in both the winter flounder and the yellowtail flounder. N, nucleus of fibroblast cell, CL, collagen fibrils (X28 900).

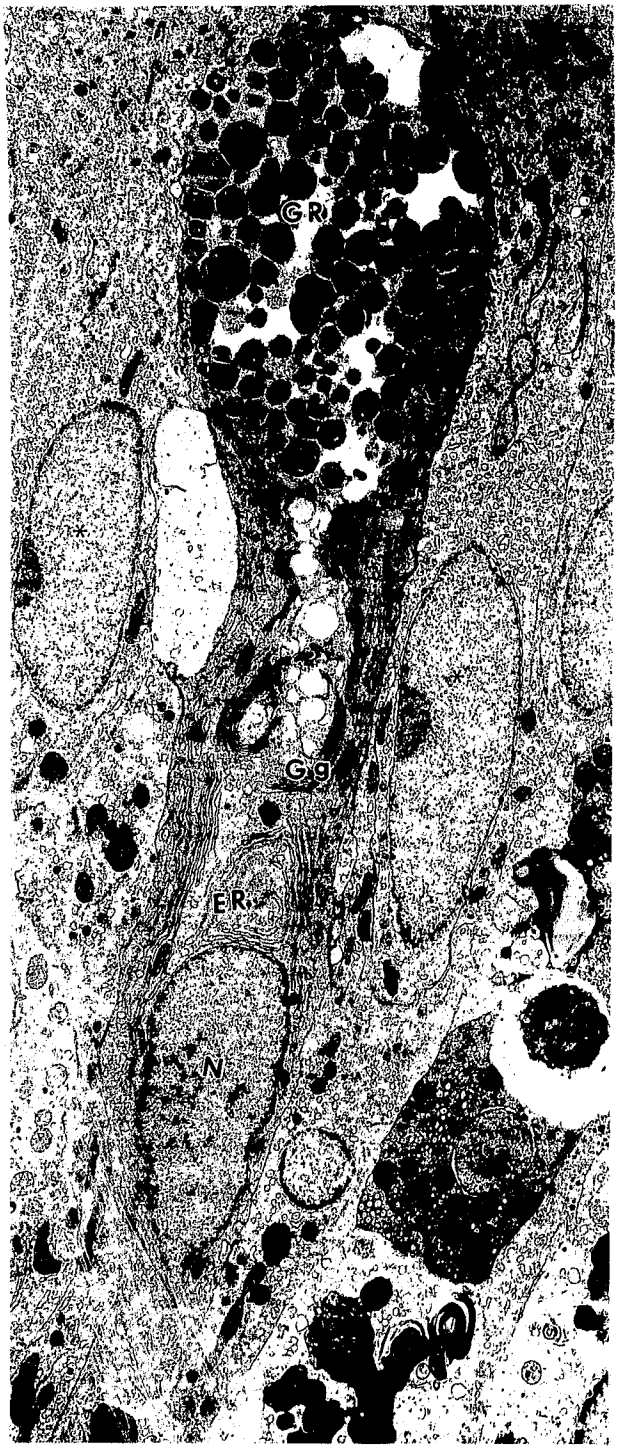



Figure 3.25. Electron micrograph of a typical post-gastric goblet cell showing the characteristic swollen goblet region with mucous granules (GR), basal nucleus (N), prominant Golgi complex (Gg), and abundant rough endoplasmic reticulum (ER). Note the nuclei from adjacent epithelial cells (asterisk) (X5 000).

Rodlet cells were observed primarily in the pyloric caeca of the Atlantic halibut, located among the epithelial cells. It was not uncommon to find them in the lumen or associated with microvilli. These cells were not commonly observed in the other species.

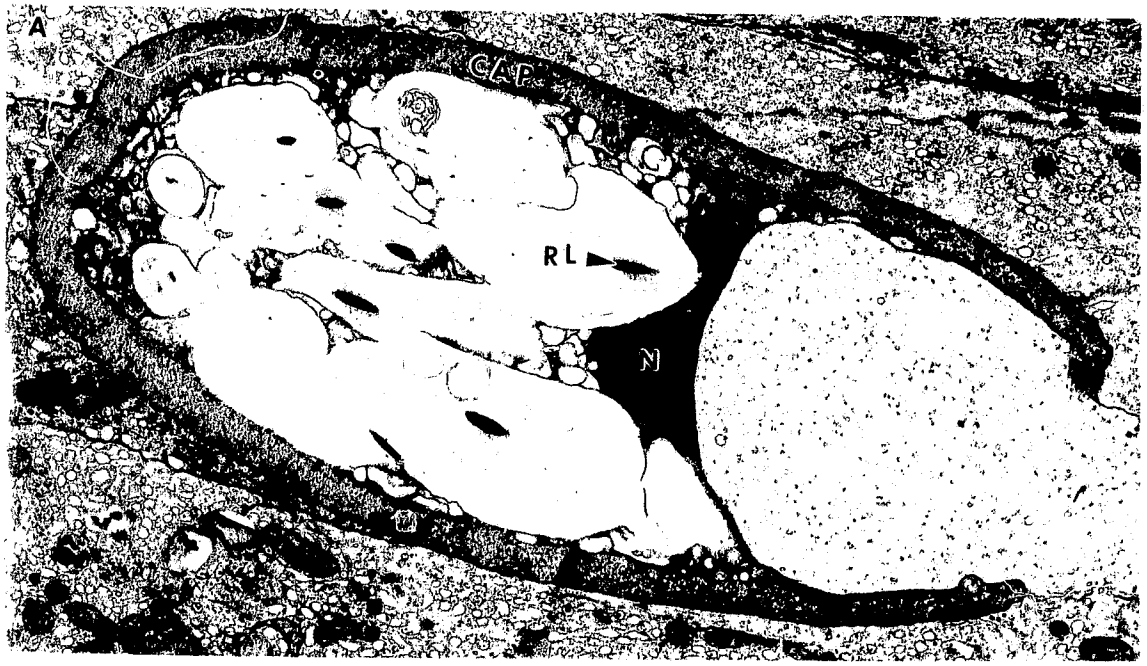
Generally, rodlet cells were characterized by a cytoplasm containing a peripheral filamentous zone, a pycnotic nucleus, and numerous cytoplasmic inclusions known as rodlets, which consisted of vesicle-like structures with a very dense cylindrical core surrounded by an area of flocculent material (Figure 3.26A). The vesicles were frequently coalesced with one another. In transverse section these rodlet structures were observed in a circular arrangement surrounding a central triplet (Figure 3.26B). The actual function of these cells is not known.

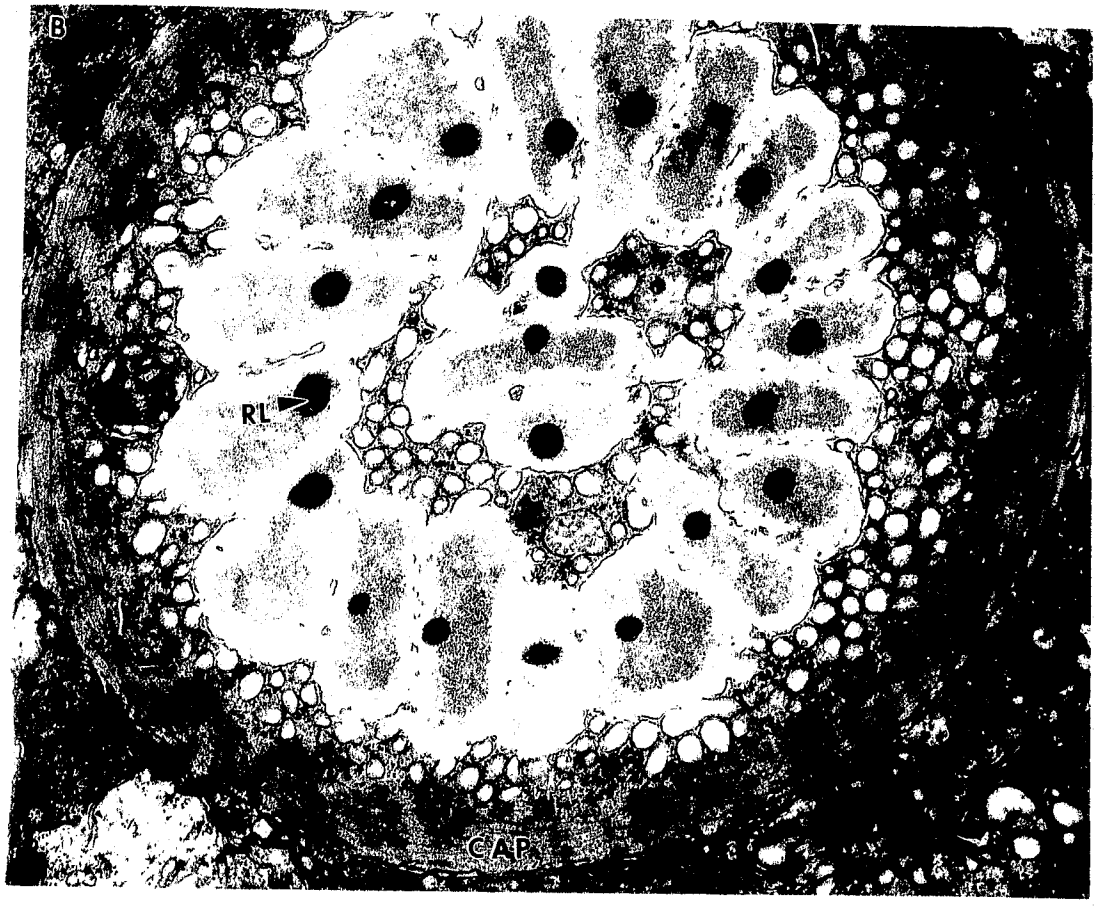
### 3.3.3.2 Rectum

The rectal epithelium was generally similar to that of the intestinal and caecal cells for all three species, however, the apical cytoplasm was characterized by numerous pinocytotic invaginations giving rise to a large population of small, smooth vesicles which appeared to traverse the terminal web. Species differences could be defined by the appearance of vesicular content.

In the Atlantic halibut the apical cytoplasm of the rectal epithelium was associated with numerous irregular shaped multivesicular bodies (MVBs), which were membrane bound cytoplasmic structures containing small, lucent vesicles (Figure 3.27A). Individual MVBs could contain up to 100 vesicles with an average diameter of 235 ± 7 nm. On occasion, laminations were also observed within the MVBs.

Figure 3.26. Rodlet cells associated with the absorptive epithelia of the Atlantic halibut. (A) longitudinal section through a rodlet cell showing the dense filamentous peripheral cytoplasm (CAP), electron dense nucleus (N), and the characteristic rodlets (RL) (X 8750) (B) Cross-section through a rodlet cell showing the unusual circular configuration of the rodlets (RL) around a central triplet and the circular orientation of cytoplasmic filaments (CAP) (X20 000).


A
CAP
RL
N


B
RL
CAP

In the winter flounder, vesicles containing an electron dense, flocculent material were consistently noted to accumulate in the supranuclear cytoplasm (Figure 3.27B). Frequently, these vesicles were observed to be in the act of fusing into larger bodies. Lamellar membranes were also seen to be a feature of the interior of many of these structures indicating that they may be a type of secondary lysosome (Figure 3.28).

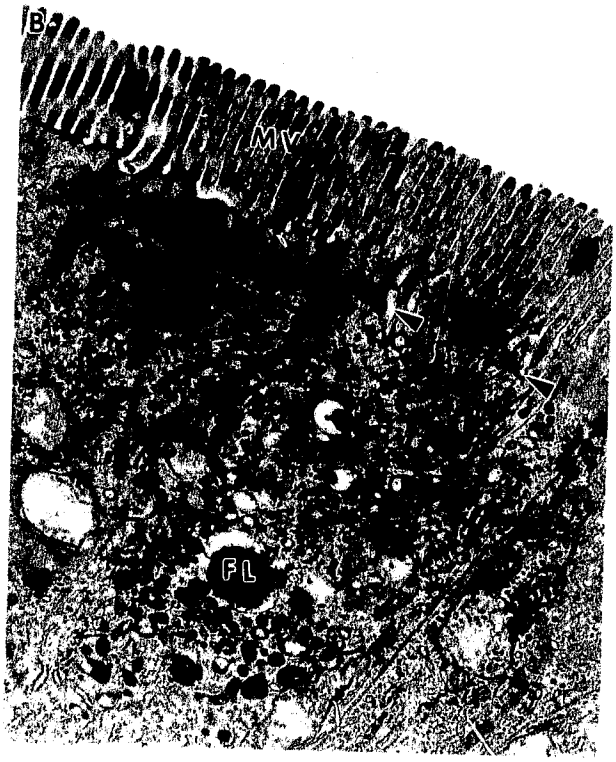
The yellowtail flounder rectal epithelium exhibited MVB's very similar to those observed in the Atlantic halibut, although of smaller dimensions. Irregularly shaped cytoplasmic inclusions were frequently observed in these cells. These inclusions were characterized by a fibrous internal substructure with the sporadic occurrence of membrane laminations, indication of a secondary lysosomal structure (Figure 3.29).

The basal or infra-nuclear region of the rectal epithelial cells in the yellowtail flounder and the halibut was characterized by numerous lamellar infoldings of the basal plasma membrane with associated elongate and occasionally branched mitochondria (Figure 3.30). Basal infoldings were not evident in similar areas of the winter flounder rectal epithelium.

The lamina densa of the basal lamina identified with the rectal epithelium of the Atlantic halibut was organized into a series of elaborate reticulations and laminations (Figure 3.31A). The overall mean thickness was found to be 850 ± 58 nm. The reticulations were noted to extend into the lamina lucida. The basal lamina associated with the epithelium of the yellowtail and the winter flounders showed no comparable configuration of the lamina densa (Figure 3.31B).

Figure 3.27. Electron micrographs demonstrating the species differences in cytoplasmic inclusions related to the pinocytosis of exogenous protein. (A) apical region of halibut rectal epithelial cells showing the presence of unusually large multivesicular bodies (MVB's). (X 9600). (B) apical region of a winter flounder rectal epithelial cell showing coated invaginations of the apical plasma membrane (arrowheads) and the presence of membrane bound vesicles containing an electron dense flocculent material (FL). MV, microvilli (X 12500).


A
MV
MVB


B
MV
FL

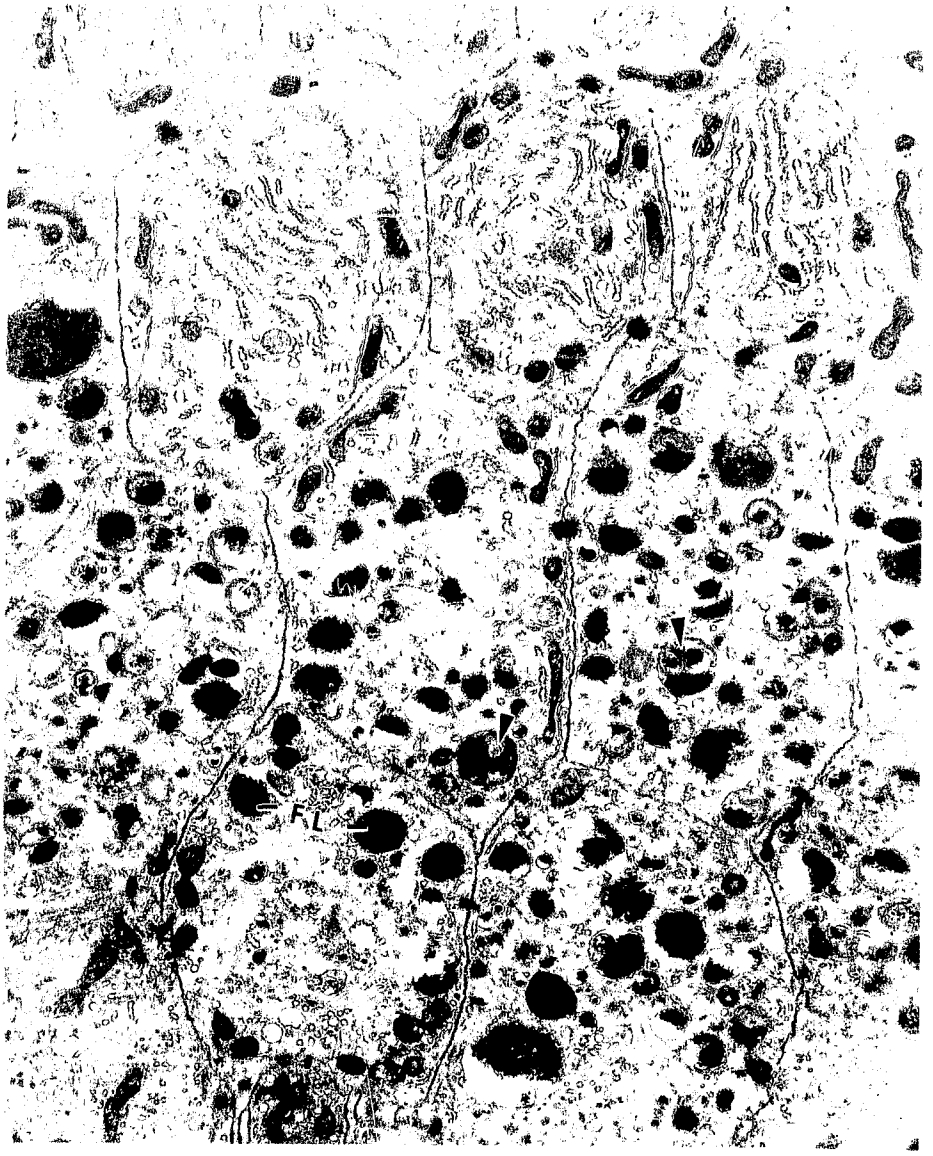



Figure 3.28. Cross-section through winter flounder rectal epithelial cells, showing the abundance of membrane bound vesicles containing flocculent material. Note the occurrence of laminar structures in some vesicles (arrowheads). FL, flocculent containing vesicles (X 11400).

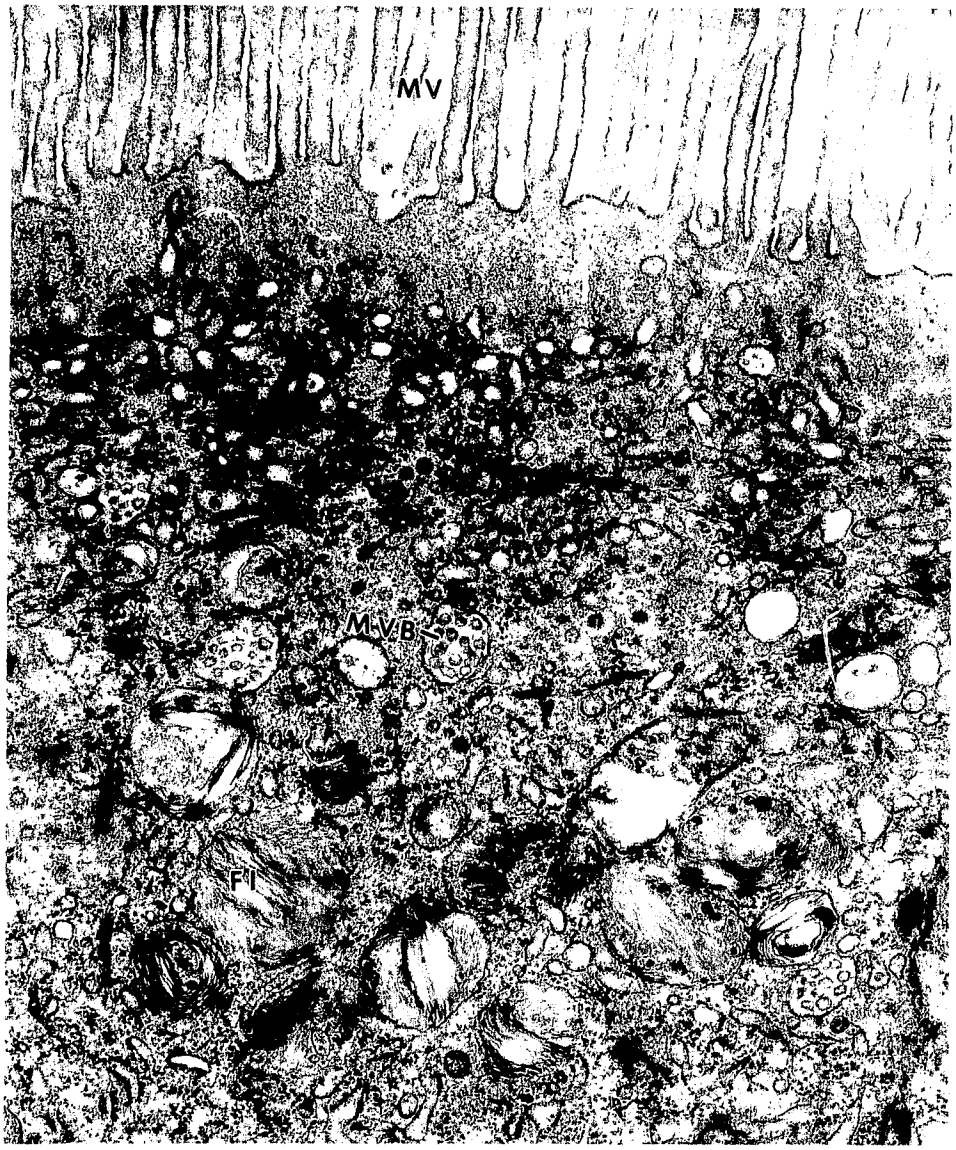



Figure 3.29. Electron micrograph from the apical region of a yellowtail flounder rectal epithelial cell showing an abundance of small multivesicular bodies (MVB) and irregularly shaped cytoplasmic inclusions characterized by a fibrous internal substructure (FI). MV, microvilli (X 32300).


Figure 3.30. Electron micrograph from the basal cytoplasm of yellowtail flounder rectal epithelial cells demonstrating the occurrence of laminar infoldings of the basolateral membrane. (arrowheads). Note the close association with numerous elongate mitochondria (Mt). BL, basal lamina (X 19000).

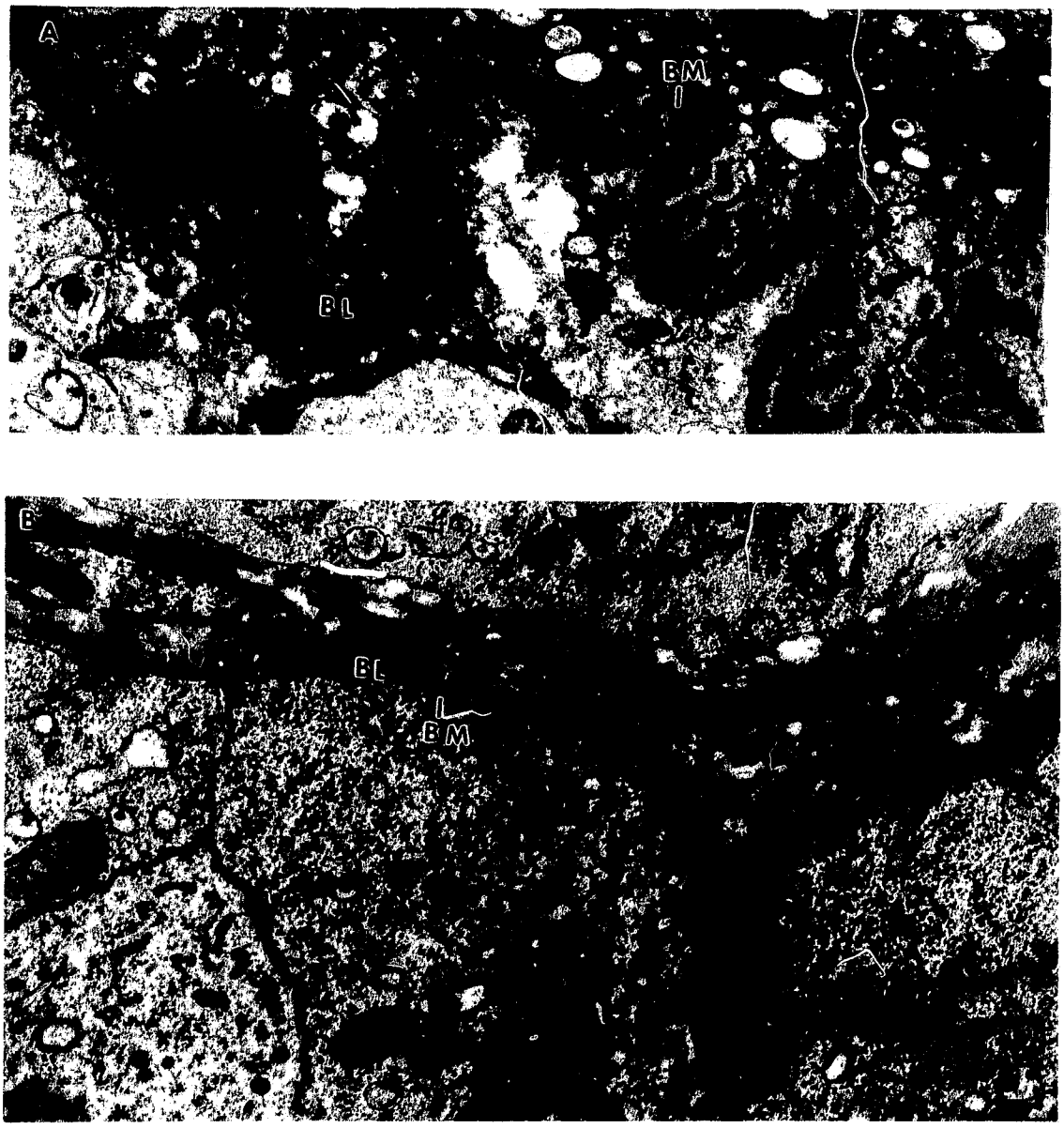



Figure 3.31. Electron micrographs demonstrating the differences in rectal epithelial cell basal lamina ultrastructure between the flounders and the halibut. (A) Basal lamina (BL) associated with rectal cells of the halibut exhibiting elaborate reticulations of the lamina densa (X 15200). (B) Basal lamina (BL) associated with the rectal cells of the yellowtail flounder that is devoid of the reticulations. BM, basal plasma membrane (X 32300).

Mean thicknesses (N=25) of the basal lamina ranged from 96 ± 4.2 nm for the yellowtail flounder to 284 ± 23 nm for the winter flounder.

### 3.4. Discussion

The basic tissue organization of the post gastric alimentary canal in the winter flounder, yellowtail flounder and the Atlantic halibut was consistent with that described in other species of fish (Barrington, 1957; Harder, 1972; Kapoor et al., 1975; Fänge and Grove, 1979), however, some histological variations were observed among species.

The rectum was distinguished macroscopically and microscopically from the main intestine in all three species by a deep folding of the outer longitudinal layer of muscle and the serosa, giving a distinctive ridged appearance externally. The thick inner circular layer of muscle was comparable to that observed in the American plaice, the glassy perchlet and the amberjack (Dawes, 1929; Marin and Blaber, 1984; Grau et al., 1992). This thickening of the muscularis has been correlated with the temporary storage and expulsion of fecal material in this area (Grau et al., 1992).

The occurrence of an obvious muscularis mucosae in association with the halibut rectum allowed for a clear demarcation between the dense irregular connective tissue of the lamina propria and the aerolar connective tissue of the submucosa. This was inconsistent with the winter flounder and the yellowtail flounder, which did not possess a muscularis mucosae in the rectum.

The dense connective tissue of the lamina propria adjacent to the muscularis mucosae in the halibut rectum is similar to the tissue type found associated with the stratum compactum of other teleosts, including the salmonids (Burnstock, 1959; Ezeasor and Stokoe, 1981; Bergeron and Woodward, 1982) and catfish (Krementz and Chapman, 1975). This layer in other species, however, is not observed as part of the lamina propria, but as a distinct collagen layer or stratum (Burnstock, 1959). The extensive nature of the compact tissue in the halibut may exempt it from being described as a stratum compactum as in species like the salmonids but instead as an unusually dense collagenous lamina propria.

The function of a tough collagenous layer adjacent to the epithelium, as observed in the halibut is extra epithelial support. The extra support in this species may reflect an adaptation to handle a diet of large fish and a deep water, high pressure habitat (Scott and Scott, 1988). Similarly, the absence of such a tissue in the winter flounder and the yellowtail flounder may reflect their relatively shallow water habitat and invertebrate diet (Scott and Scott, 1988). The occurrence of a deeply folded and highly vascularized mucosa in the rectum of all three species of pleuronectid suggests active absorption or other transport across the mucosa. Further, the presence of numerous large multivesicular bodies and electron dense flocculent structures, in association with smaller pinocytotic vesicles, suggests pronounced pinocytotic activity and intracellular digestion by the rectal cells from all three species. The paucity of these structures in the intestinal and caecal epithelia suggest that this process is specialized in rectal epithelia.

The pinocytotic uptake and subsequent intracellular digestion of exogenous proteins by rectal epithelia has been frequently documented in both larval fish and adult stomachless fish (Noaillac-Depeyre and Gas, 1976; Watanabe, 1981; 1984; Govoni et al., 1986; Caceci and Hrubec, 1990). It has been reported that in species lacking a functional stomach, the digestion of dietary protein occurs intracellularly in the epithelium of the hindgut as opposed to that in the lumen of the stomach (Govoni et al., 1986). More recently, authors have recorded similar occurrences in species of teleost possessing fully functional stomachs (Ezeasor, 1981; Morrison, 1987). In cases such as these the utilization of intracellular digestion may occur as a method of increasing protein digestion efficency. Noaillac-Depeyre and Gas (1979) suggested that the presence of undegraded proteins in the gut lumen may induce the ingestion of exogenous protein by the gut epithelium. Ezeasor (1981) theorized that due to physical limitations of the stomach as storage organ, when food is plentiful, gastric empting may be more frequent, thus resulting in incomplete hydrolysis of proteins. In cases such as these, the pinocytosis of proteins by the rectal cell would serve as a complementary mechanism to that of the stomach. The occurrence of pinocytotic inclusions in the rectal enterocytes of the pleuronectids from this study, provide evidence that intracellular digestion of protein may also be important in these fish species. The observed species variations in the structure of the pinocytotic cytoplasmic inclusions suggest different stages in the cellular digestion process and possibly species variations in rates of cellular metabolism.

The deep infoldings of the baso-lateral membrane of the rectal, intestinal and caecal enterocytes of both the halibut and the yellowtail flounder are similar to those described in the rainbow trout and the channel catfish (Krementz and Chapman, 1975; Ezeasor and Stokoe, 1981). The association of these infoldings with elongated mitochondria suggest that they may be involved in energy dependent activities such as osmoregulation (Ezeasor and Stokoe, 1981; Kirsch and Meister, 1982). The infrequency of these structures in the winter flounder suggests that this species may have an alternative method to carry out this physiological process.

The basic morphology of the intestine in all three species was similar to that observed in the rectum. The extent of serosal folding, however, was significantly reduced in comparison to the rectum, giving a relatively smooth serosal surface. Similarly, the circular layer of the muscularis externa appeared thinner than that in the rectum.

The dense irregular connective tissue of the propria-submucosa in the halibut intestine probably carries out the same supporting role as the lamina propria noted in the rectum, however, unlike the rectum no obvious muscularis mucosae was observed. In the region adjacent to the epithelium, the dense connective tissue gives way gradually to a loose, considerably more highly vascularized connective tissue. The extent of the vascularization in this area may be important in the receiving of and in transport of nutrients from the gut epithelia, although lacteals were not observed in the teleost intestine (Bauermeister et al., 1979). An areolar vascularized propria-submucosa was observed in the winter flounder and the yellowtail flounder.

Ultrastructurally, the intestinal epithelial cells of all three species demonstrated characteristic features which were associated with the cellular processing, storage and extracellular transport of lipid digestion products. The presence of chylomicra in both the SER and intercellular spaces was evidence of intracellular processing of lipid and extracellular transport in the intestinal epithelium of the winter flounder, yellowtail flounder and the halibut. Chylomicra are the transport form of lipid digestion products in the intestinal epithelium of vertebrates and are known to be assembled in vesicles of the Golgi complex and SER (Friedman and Cardell, 1972). The chylomicra observed within cytoplasmic vesicles of these pleuronectids were comparable to those observed in the intestinal epithelium of feeding rainbow trout (Bauermeister et al., 1979) and goldfish (Gauthier and Landis, 1971). The observed differences between the electron densities of the chylomicra in the species from this study may be reflections on the differing lipid compositions of the different prey species ingested. The occurrence of large cytoplasmic non-membrane bound lipid in the intestinal epithelia of the halibut implies that this species may have the ability to store excess lipid during digestion and absorption. During the process of lumenal digestion and cellular absorption of dietary fats, the limiting step may be the cellular conversion of fatty acids back into triglycerides as chylomicra. Similar theories have been proposed with regard to lipid digestion in the intestine of the tench, *Tinca tinca* L. and the perch, *Perca fluviatilis* L. (Noaillac-Depeyre and Gas, 1976; Noaillac-Depeyre and Gas, 1979). These workers have proposed that the formation of lipid droplets may be favoured by the cellular absorption of a quantity of lipid greater then

the capacity of the SER, or insufficient protein synthesis for the formation of chylomicra. The Atlantic halibut is known to feed at water temperatures of 5-6$^0$C (Bowering, 1986; Scott and Scott, 1988); thus the combination of an oily piscivorous diet and low water temperatures may allow for storage of excess lipid in the cell cytoplasm. The absence of free cytoplasmic lipid droplets in the winter flounder and yellowtail flounder may reflect dietary differences from that of the halibut, since these species depend on an invertebrate prey and were sampled at both summer and winter temperatures, respectively.

The pyloric caeca appear as blind sac extensions from the anterior intestine in many species of teleost (Barrington, 1957). Histologically, in comparison to the rectum and intestine, the pyloric caeca in all species had a thinner muscularis externa, and a mucosa which was not folded and had much longer villi. Ultrastructurally, the caecal epithelium of these species was similar to that of the intestine. Frequently the epithelia were observed with elements of SER containing chylomicra, giving further evidence that this area is important for lipid absorption in all three species. Comparable similarities have been observed in the rainbow trout and the Atlantic cod (Ezeasor, 1981; Morrison, 1987). Unlike the intestinal epithelium, the pyloric caeca of the halibut did not contain free lipid droplets, suggesting that the intestine is the primary area for lipid absorption and that the pyloric caeca may function as an area of supplementary absorption. Taking this into consideration, Buddington and Diamond (1986) proposed that, in general, pyloric caeca may be adaptations for increasing surface area without increasing the thickness or length of the intestine.

The presence of infoldings of the basal plasma membrane, similar to those observed in the intestine, may further indicate that this region is also important in ion transport.

While in most fish the pyloric caeca may appear to exist solely as an area for supplemental lipid absorption, in some species the caeca have shown considerable endocrine activity (Anderson, 1986; Beorlegui et al., 1992). Gland-like structures have been observed adjacent to the epithelia in the pyloric caeca of the cod, further suggesting an exocrine secretory function to this region (Morrison, 1987). Obvious evidence of secretory activity (endocrine or exocrine) was not observed in the pleuronectids of the present study.

An unusual feature of the post-gastric epithelia in the Atlantic halibut was a basal lamina, exhibiting a distinct reticulated and/or laminated lamina densa. Such an unusual structure was not associated with the basal lamina of either the yellowtail flounder or the winter flounder. In higher vertebrates, the occurrence of a laminated basal lamina is frequently correlated with epithelia experiencing abnormally high pressure, due to blood or other fluids, e.g. pathological conditions of the glomerular basement membrane (Ghadially, 1988). Occasionally, the condition may also be associated with aging in testis (Singh et al., 1993). The occurrence of a similar structure in association with the absorptive gut epithelia of the Atlantic halibut presented an unusual problem. It was reasoned that the development of a reticulated and laminated basal lamina in regions of abnormally high fluid pressure may be a cellular adaptation for increased epithelial support. The Atlantic halibut is a species

known to frequent extreme depths, but is also thought to move into relatively shallow water during foraging (~18 m) (G. Goff, personal communication). During vertical ascents from great depth the reticulated structure of the basal lamina in this species may aid in supporting the integrity of the epithelia during the periods of membrane equilibration required to withstand frequent and extreme changes in external pressure. This feature, combined with the unusually dense connective tissue observed in the gut, may very well be a species adaptation evolved to withstand the demanding environmental changes to which these fish are exposed.

An unusually thickened basal lamina associated with the intestinal mucosa of the perch and various species of centrarchids has been compared to the structure of the stratum compactum (Reifel and Travill, 1979). It has been proposed that a basal lamina such as this may be important in the structural support of the epithelium.

Rodlet cells were found in association with the epithelia of all regions of the post gastric alimentary canal in the halibut, however, they appeared most frequently in the pyloric caeca. The ultrastructure observed was consistant with previous observations of rodlet cells in other species (Morrison and Odense, 1978).

These distinct cells have produced considerable controversy. Numerous authors have classified them as parasites belonging to the Apicomplexa (Bannister, 1966; Iwai, 1968; Barber et al., 1979; Mayberry et al., 1979) whereas others, based on the species distribution and morphological features of these cells, consider them normal somatic cells performing a set function (Leino, 1974; Desser and Lester, 1975; Morrison and Odense, 1978; Mattey et al., 1979; Leino, 1982).

Evidence such as the formation of tight junctions between rodlet cells and typical epithelial cells (Mattey et al., 1979), the nonspecificity to tissue type, wide species and environmental distribution (Desser and Lester, 1975), and the lack of any obvious cellular or tissue necrosis in association with these cells (Mattey et al., 1979) have been suggested to indicate that these rodlet cells are normal somatic cells found in fishes.

Other workers have indicated that the absence of the cell in every species and in all members of a species of fish suggest that the rodlet cell should be classified as a parasite among the Apicomplexa (Mayberry et al., 1979). Reports demonstrating that the rodlets contain RNA indicate that the rodlets might form a transmissive or infective vehicle (Barber et al., 1979). Bannister (1966) reported the occurrence in rodlet cells of an unusual form of mitochondria which exhibited a long thin form with tubular cristae that were not similar to mitochondria found in surrounding cells.

Considering the strength of both sets of arguments one can see why the nature of these cells in fish is still up for debate. The occurrence of rodlet cells in the Atlantic halibut is a first observation for this species and the absence of them in the winter flounder and the yellowtail flounder post-gastric gut is consistent with a study of the distribution of rodlet cells in fish by Morrison and Odense (1978).

Bacteria were commonly observed in connection with the post-gastric mucosa of the winter flounder. The occurrence of microbes in association with the absorptive epithelium of fishes is not unusual and is frequently correlated with the presence of various digestive enzymes, for example, chitinase (Lindsay, 1984; Lindsay and

Gooday, 1985). In most cases, however, bacteria are found to be associated with a mucous biofilm and may not necessarily be in direct connection with the epithelium (G. Nganda, personnal communication). In rodents, a nonpathogenic, filamentous, Gram-positive organism has been found associated with the mucosa of the ileum and induced a host response defined by a "concentric cytoplasmic specialization" surrounding its attachment site (Madara, 1984). The bacteria observed in the winter flounder from the present study occurred in membrane depressions, but a similar host response was not observed. The intimate association of bacteria and epithelium in the fish suggests a closer functional symbiotic relationship that may aid in specific digestive processes, similar to that suggested by Lindsay and Gooday (1985). The absence of bacteria in association with the gut epithelium of the halibut and the yellowtail flounder may indicate that the presence of bacteria is a species specific phenomenon for this group.

Goblet cells are common components of the post-gastric regional mucosa in most species of fish, although few investigators have made attempts to quantify their distribution (Bucke, 1971; Groman, 1982). Goblet cell numbers per unit area in the present study showed no significant difference between species but revealed a gradual decrease in number from the rectum to the pyloric caeca. This distribution of goblet cells was similar to that described in the gut of the amberjack (Grau et al., 1992). The increased number of goblet cells in the rectal region would perhaps reflect the need for increased mucosa protection and lubrication for the expulsion of fecal material in all the three species.

The distributional trend in goblet cells is also reflected in the predicted mucous volume limits. By far the most dramatic effect is in the halibut, suggesting that the combination of cell number and large cell size results in a significant production of mucus across regions in this species. The significantly smaller cell volume in the winter flounder and the yellowtail flounder would explain the comparable difference in predicted mucous volume fraction between these species and the halibut, since there was no significant difference in the number per unit area between species.

Comparative studies of the mucous histochemistry of the alimentary canal goblet cells in teleosts are scanty. Reifel and Travill (1977; 1978; 1979) compared the gastro-intestinal mucous histochemistry of a number of species of freshwater teleosts. Bucke (1971) examined the mucous histochemistry and the regional distributions of goblet cells in the intestine and rectum of another freshwater fish, the pike. Similarily, Croman (1982) looked at mucous histochemistry in the gut of the striped bass. The present study is the first to compare both the regional distribution and the mucous histochemistry of cells in the alimentary canal epithelium across the three species of marine flatfish.

The distinct histochemical differences in mucus observed between these species were striking and suggested the possibility of species variations. Interestingly, the two closest taxonomic species, the winter flounder and the yellowtail flounder, have the most diversity in goblet cell mucous histochemistry. The winter flounder goblet cells consistently stained positive for only acid or sulphated acid mucins,

whereas those of the yellowtail flounder stained significantly more positive for combinations of neutral and variations of acid mucin. Some authors have stated that the coexistence of two or more types of mucins in a cell may indicate two or more levels of mucous maturation (Elbal and Agulleiro, 1986), with the final product in each goblet cell being the same. The uniformity of the mucous reactions between individual fish here indicate that the differences observed between species are based on the chemical nature of the mucus. The differences would indicate that the post-gastric regions in each species would demonstrate a different lumenal chemistry based on mucus. In contrast to the flounder species, the Atlantic halibut post-gastric regions exhibited a variation in goblet cell mucous histochemistry. This observation suggests different mucus chemistry for different regions. In general, the staining reactions for the halibut were reminiscent of a combination of both the winter flounder and the yeliowtail flounder.

Different types of mucosubstances have previously been correlated with assorted digestive functions. Neutral mucosubstances are combined with sites of alkaline phosphatase and together they assist in the digestion and emulsification of food into chyme (Clarke and Witcomb, 1980) Grau et al. (1992) further suggested that the presence of neutral mucins may indicate absorptive functions. Other investigators have suggested that mucosubstances may provide cofactors required for the enzymatic breakdown of food (Anderson, 1986). If one speculates that different cofactors become associated with different combinations of mucins, then distinct mucin differences between species may indicate differences in the amounts or kinds

of enzymatic cofactors available for digestion of a specific nutrient. This may reflect differences in dietary preference between species and in the case of the halibut may indicate a chemical division of labour between different regions of gut.

In conclusion, evidence from this study suggests that histologically the post-gastric regions of the alimentary canal in the winter flounder and the yellowtail flounder are similar and that both these species diverge in specific ways from that of the Atlantic halibut. From observations of the occurrence of specific kinds of cytoplasmic inclusions (such as lipid droplets, chylomicra, or multivesicular bodies), in the cells of specific regions, it can be concluded that the intestine and pyloric caecal epithelia function primarily in lipid absorption and transport and that the rectum is important for protein degradation.

Mucous histochemical evidence further alludes to differences in the chemical nature of the gut environment and/or the functional requirements for digestion in each species and thus maybe an important factor to consider when discussing digestive physiology, nutrition and diet development in these species. Further studies are required to define more fully the role of the different chemo-types of gut mucins in these species and the nature of their association with gut function.

# 4. GENERAL DISCUSSION

## 4.1. The Rationale of Studying the Fish Alimentary Canal

The primary objective of this work was to compare the histology of the alimentary canal in three species of cold water pleuronectid. These species are known to occupy different ecological niches in the wild and are currently being examined as candidates for cold water marine aquaculture. The rationale for this study is seated in both basic and applied science. Histological studies of gut have been useful in the past to assess disease problems as well as nutritional stress in aquaculture (Grau et al., 1992). Such studies can act as an information base to begin physiological studies of digestive function and nutrition (Dawes, 1929; Burnstock, 1959; Clarke and Witcomb, 1980). These investigations also provide a foundation for basic science studies in comparative feeding ecology or behavior as well as basic comparative anatomy and function (Martin and Blaber, 1984; Morrison, 1987).

The fact that these fish were sampled from wild populations permits one to utilize the data for both of the above purposes. First, it allows for a base line normal histology to be established for these species. These data can be used as reference in future culture situations, especially with regard to diet development or fish health requirements. Secondly, the knowledge of comparative alimentary canal histology between species of pleuronectid in the wild, helps one to understand the evolution of microanatomical adapations in gut structure that may correlate with feeding behavior or prey choice.

## 4.2. Significance of Species Differences

The pleuronectid species from this study have previously been divided into two groups as a result primarily of stomach anaylsis data based upon prey preference (De Groot, 1971; Libey and Cole, 1979; Scott and Scott, 1988). The yellowtail flounder and the winter flounder are defined as predominately invertebivorous, although water depths frequented do vary between species (Scott and Scott, 1988).

The winter flounder is known to prefer relatively shallow water zones whereas the yellowtail flounder, while occasionally observed in overlapping habitats with the winter flounder, is considered predominantly, a moderate depth species. In contrast, the Atlantic halibut is defined as a deep water piscivivore.

### 4.2.1. Morphological differences

Comparative alimentary canal histological data from the present study provides morphological evidence supporting adaptations for respective feeding ecologies and habitat preferences in these species.

The dense irregular connective tissue composing the lamina propria of the halibut gastric and rectal epithelium and the propria-submucosa of the pyloric caeca/intestinal epithelium, while not a true stratum compactum, does suggest that extra tissue support is required for the alimentary canal mucosa at the depths frequented by this species. This extra support may also be important, especially in the stomach, for handling a diet of large fish. The thick, reticulated basal lamina found associated with the post-gastric epithelia in this species maybe a further adaptation to rapid changes in depth distribution during foraging.

The presence of a gastric and post-gastric propria-submucosa composed of an aerolar connective tissue in both the winter flounder and the yellowtail flounder probably reflects the shallow water environment which these species are known to prefer with respect to the halibut. Similarly, the significantly smaller mouth size in the flounders and the resulting restriction in prey size would suggest a possible decrease in the extent of mechanical trauma to the alimentary canal, reducing the need for extra mucosal support thus providing an explanation for the observed differences between the flounders and the halibut.

While generally the morphology of the alimentary canal of the two flounder species was similar, but divergent from that of the halibut, variations in esophageal morphology between the winter flounder and the yellowtail flounder suggest inherent differences between these species. The denser nature of the connective tissue in the esophageal propria-submucosa in the winter flounder compared to that of the same area in the yellowtail flounder, coupled with the presence of prominent microridges in association with the apical plasma membrane of the esophageal surface secreting cells suggests that the winter flounder is generally more robust in this region and thus may be more specialized as an opportunistic feeder, and therefore, possibly a more flexible species.

### 4.2.2. Functional differences

Throughout this study, functional variations and similarities were inferred both between species and between regions of alimentary canal. All of these inferences are related to morphological or cellular differences within given regions.

Based upon cell type and ultrastructural characteristics, the alimentary canal of these pleuronectid species could be divided into four functional regions, esophagus, stomach, intestine/pyloric caeca, and rectum.

#### 4.2.2.1 Esophagus

The ESSCs described in the winter flounder and the yellowtail flounder, suggested a secretory function for these cells. Ultrastructurally, the granules in the winter flounder ESSCs were reminiscent of mucous-like secretion granules whereas those from the yellowtail flounder were more similar to an enzymatic-like secretion granule (chapter 2). A description of cells exhibiting granules with ultrastructural characteristics such as those in this region is a first for pleuronectids.

Authors have suggested that the esophagus in some teleosts may function in pregastric digestion (Reifel and Travill, 1977). The presence of ESSCs in these pleuronectids coupled with elaborate mucosal folding, apical membrane microridges, and a prominent circular layer of striated muscle provides evidence in support of this theory for these species.

The differences in ESSC granule morphology suggests species specific variations in mode of function. The mucus-like granules from the winter flounder ESSCs suggest a more viscous secretion as opposed to a serous-like secretion, indicated by the granule type in the yellowtail flounder. While different in morphology the functional nature of the cells may be similar, acting in the initiation of digestive processes. Differences in esophageal mucous chemotype (determined through histochemical staining) between species, as well as the above mentioned

species variations in ESSC granule morphology, suggests that if the secretory compounds are enzymatic in nature, each species type may require specific co-factors for enzyme operation. These proposed co-factors may well be associated with mucous chemistry and may be reflected through the observed species variations in the mucous chemical composition.

It is suggested from this study that since teleosts do not possess true salivary glands, the ESSCs in combination with the large numbers of goblet cells may be evolutionary precursors to salivary gland structures and thus perform a function similar to the salivary glands. More studies are required to further clarify the functional nature of both the mucus chemical differences and the ESSCs.

#### 4.2.2.2 Stomach

Information from this study showed that, similar to other stomach possessing teleosts, this region in pleuronectids is primarily important in the chemical digestion of food material and that this digestive process is carried out through the secretion of both proteinaceous enzymes and acid. These secretions appear to arise from a single cell type (oxyntico-peptic cell), observed to form simple tubular glands and which exhibit characteristics of both acid secreting cells and protein secreting cells.

While the species in this study all contained a glandular zone (Zone 3), the ability to divide the gastric epithelium into three zones based on cell morphology is not consistent with all species of teleost. The Atlantic cod and the rainbow trout are known not to possess mucous neck cells (Ezeasor, 1981; Morrison, 1987), whereas the American plaice and the freshwater perch have these cells (Dawes, 1929; Noaillac-

Depeyre and Gas, 1978). At present it is not clear as to why only some species have true mucous neck cells. In mammals, mucous neck cells are thought to produce a less viscous mucus which may aid in preventing the gland duct from autodigestion (Wheater et al., 1987). The mucus produced in mammalian neck cells is observed to be neutral in nature (Neutra and Padykula 1984). Histochemical staining from this study showed predominately neutral mucus to be present in the pleuronectid mucous neck cells, whereas, with exception of the winter flounder, the surface cells contained predominantly acid mucus (chapter 2). It is clearly evident that further work is required to determine why some species possess ultrastructurally distinct mucous neck cells whereas others do not and if present, whether they perform a similar function as suggested in higher vertebrates.

The most striking difference observed with reference to the stomachs in the pleuronectids was the interspecies variation in gastric mucous chemistry. As noted in chapter 1, gastric mucus has one main function, to protect the epithelium from acid and enzymatic digestion. This function is achieved through two variations on the same theme. The mucus can act as a mixing layer preventing the small amount of epithelial alkaline secretion from being immediately acidified by the comparatively large volume of acid but allowing for gradual and consistent neutralization. Secondly, the mucous layer can act as a renewable physical barrier to enzymatic digestion. While the degradation of the mucous layer is occurring at the lumen, a new supply is constantly being added, thus preventing the underlying epithelium from being digested.

The differences observed between the pleuronectids from this study suggest variations in the chemistry of the lumenal environment during digestion. The development of different chemo-types of mucus may be a species specific adaptation to chemical variations in the nature of the glandular secretions. Based upon what is known about the diets of these species one could suggest that these variations are diet related. Clearly, further work is necessary to shed more light on the functional significance of these differences.

#### 4.2.2.3 Post-gastric mucosa

The post-gastric mucosa observed in the pleuronectids from this study could be divided into two major regions based upon functional characteristics, the intestine/pyloric caeca and the rectum (chapter 3).

##### 4.2.2.3.1 Pyloric caeca/intestine

In all three species, the pyloric caeca/intestine had characteristics suggestive of lipid absorption, however, interspecies variations were evident. In the halibut, both regions presented characteristics of lipid absorption and processing, although the intestine further exhibited the presence of large lipid droplets within the cytoplasm. Based upon dietary conditions during sampling, this feature suggested a storage capability for these cells especially in times of excess lipid absorption and/or the saturation of the lipid processing cellular machinery. This storage feature could be a reflection of differences between fish versus invertebrate diets. The absence of large cytoplasmic droplets in the pyloric caeca, but the presence of smaller chylomicra within elements of the SER suggested that in this species the region could be

important as a secondary or supplementary area for lipid absorption. Within the yellowtail flounder and the winter flounder pyloric caeca/intestine, the absence of lipid storage droplets and the presence of smaller chylomicra within elements of the SER suggested that the general mechanism is similar across all three species. The differences in chylomicron electron density between the halibut and the flounders may also be a reflection of the composition of lipid available from different prey species (invertebrate versus fish).

The data obtained from this study allowed the development of a generalized scheme for lipid absorption and cellular processing in these species. Morphological evidence, such as the presence of cytoplasmic lipid droplets and chylomicra within elements of the SER, indicates that lumenally emulsified and digested fatty acids and monoglyercides diffuse across the apical plasma membrane of absorptive cells, where they are taken up by elements of the SER and Golgi complex or in the case of the halibut stored in large cytoplasmic lipid droplets for later utilization. From mammalian literature it is known that the fatty acids and monoglycerides are reassembled in the SER or Golgi complex, and coupled with apoprotein to form specialized transport molecules referred to as chylomicra (Kelly et al., 1984). After reassembly into the chylomicra, the molecules are transported to the basal or baso-lateral membranes where they are exocytosed into intercellular spaces for transport. Similarity between observations in this study and those carried out on mammals suggest, that analogous processes take place in the fish (Kelly et al., 1984).

#### 4.2.2.3.2 Rectum

Ultrastructural observations of the rectal epithelia in all three species indicated that the region appeared important in the uptake and intracellular digestion of proteinaceous material. This appears as a common feature of the hind-gut or rectal epithelia in many species of teleost including those with fully functional stomachs (Noaillac-Depeyre and Gas, 1976; Watanabe, 1981; 1984; Govoni et al., 1986; Caceci and Hrubec, 1990). Noaillac-Depeyre and Gas (1979) suggested that the presence of undegraded proteins in the gut lumen may induce the ingestion of exogenous protein by the gut epithelium. This process may be important as a method of increasing protein digestion efficiency especially in times of rigorous feeding.

The actual process which triggers the uptake and subsequent digestion of protein from the lumen is not known. In this study, ultrastructural characteristics indicated that considerable pinocytotic activity is occurring in these cells, more so than in any other region of the gut. Further, large multivesicular bodies and dense bodies are a common feature of the rectal cells in these species.

The exact process of how protein material is transported from a pinocytotic vesicle to the lysosomal membrane is still open for investigation. It seems probable that during pinocytosis the protein is bound to specific sites (coated pits) on the plasma membrane. The resulting pinocytotic vesicle is transported through the cytoplasm where it fuses with a lysosomal membrane and releases its contents within the vesicle. The original transport portion of the membrane is then evaginated toward the lumen of the lysosome forming an internal vesicle. A fusion of many

pinocytotic vesicles with subsequent membrane invaginations within the same lysosome may result in the formation of multivesicular bodies, as observed in the pleuronectids.

### 4.3. Significance to the Aquaculture of These Species

Histological studies of the gut of fish have been useful to assess disease problems, nutritional stress, and environmental toxicity (Grau et al., 1992). The information gained from the above studies have laid a framework for future research in digestive physiology as well as aspects of fish health.

The ultrastructural observations on lipid absorption and protein digestion enhance the present knowledge of digestive function in these species. The variability of mucous chemo-type across species and region of gut implies that the mucus is important in protection, but also suggests other functions with regard to digestive physiology. Finally, the morphological indications that the esophagus may have a function in the initiation of digestive processes in the winter flounder and the yellowtail flounder raises new questions as to where chemical digestion truly begins.

### 4.4. Conclusions

1. Based upon specific morphological and cellular charcteristics, the esophagus of the winter flounder and the yellowtail flounder may have a role in the initiation of chemical digestion in these species.

2. The stomach epithelia in the Atlantic halibut, the winter flounder and the yellowtail flounder, can be divided into three distinct vertical zones based upon ultrastructural differences in the cell type present. This is not a consistent feature in all species of teleost.

3. Variation in mucous histochemistry across species as well as between alimentary canal regions suggests that different chemo-types of mucus may be important for different digestive functions and that some variations may be species specific.

4. The post-gastric regions of the three pleuronectids from this study can be divided into two main areas based upon the ultrastructural features of the digestion and absorption of specific nutrients: lipid digestion is observed to occur in the intestine and pyloric caeca whereas the intracellular digestion of exogenous protein is observed to occur in the rectum. This is consistent with observations in other teleost species.

5. The numbers of goblet cells within post-gastric regions were not significantly different between species. Numbers were, however, significantly different between regions, with a trend suggesting an increase in goblet cell number and corresponding mucus production toward the rectum in all species. Goblet cell dimensions were also significantly larger in the halibut than in any of the other species.

### 4.5. Future Directions

The above studies have generated numerous questions with relation to the alimentary canal in these species of pleuronectid. Two of the most intriguing directions for future research on these animals are: A) the nature of the ESSCs and

their function as well as the chemical content and mode of action of the granules from the different species, and B) the significance of different mucous chemo-types within different regions of the alimentary canal and in the different species.

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APPENDIX A

PRIMARY FIXATIVES USED IN TISSUE SAMPLING PROTOCOL:

| ELECTRON MICROSCOPY FIXATIVES | LIGHT MICROSCOPY FIXATIVES |
|---|---|
| A. 2.5% Glutaraldehyde and 4.0% Paraformaldehyde in 0.06M Sodium Cacodylate buffer, pH 7.2 (modified Karnovsky's). | A. Bouin's Fluid:<br>Picric acid, sat. aqueous sol. 750 ml<br>37-40% formalin 250 ml<br>Glacial acetic acid 50 ml |
| B. 2.5% Glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH 7.3. | B. 10% Neutral buffered formalin. |
| C. 2.5% Glutaraldehyde in 0.06M Sodium Cacodylate buffer, pH 7.2. | |

## APPENDIX B

HISTOLOGICAL AND HISTOCHEMICAL STAINS FOR PARAFFIN SECTIONS:

| STAIN | REACTION |
|---|---|
| Haematoxylin and Eosin (H & E) | Stains basophilic structures (i.e. nuclei) blue and acidophilic components orange or red. |
| Periodic acid-Schiff (PAS) reagent | Produces an insoluble magenta coloured compound upon reaction with carbohydrates |
| Alcian blue (AB): pH 2.5 | Stains sulphated and nonsulphated acid glycosaminoglycans blue |
| Alcian blue (AB): pH 1.0 | Stains only sulphated acid glycosaminoglycans blue |

APPENDIX C

TABLE OF MORPHOLOGICAL DATA:

| | Mean Forklength (cm ± SE) | Mean Stomach length (cm ± SE) | Mean Pyloric caecae length (cm ± SE) | Mean Intestine length (cm ± SE) | Mean Rectum length (cm ± SE) |
|---|---|---|---|---|---|
| Atlantic halibut | 83.25 ± 2.9 n = 4 | 19.25 ± .85 | 6.42 ± .13 | 52.5 ± 3.1 | 5.4 ± .14 |
| Winter flounder | 25.0 ± 3 n = 8 | 4 ± .28 | 2.5 ± .10 | 29.25 ± 2.8 | 2.67 ± .28 |
| Yellowtail flounder | 28.2 ± 2 n = 6 | 3.7 ± .19 | 2.23 ± .24 | 32 ± 2.2 | 2.1 ± .17 |