THE HISTOCHEMISTRY OF THE GASTROINTESTINAL TRACT OF COD (GADUS MORHUA L.)

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THE HISTOCHEMISTRY OF THE GASTROINTESTINAL TRACT OF COD (*GADUS MORHUA* L.)

By

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A Thesis

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ABSTRACT

This study was undertaken to correlate the histological features of the gastrointestinal tract of the cod with the occurrence of some of the hydrolytic enzymes and periodic acid-Schiff (PAS) reactive compounds. Representative tissue of nine regions of the tract (cardiac, fundic and pyloric stomach, pylorus, caeca, anterior loop of small intestine, posterior loop of small intestine, rectum and liver) were fixed in cold acetone, Lison's fluid and Baker's formol calcium for the histology and the localization of alkaline phosphatase, acid phosphatase, esterase and PAS-positive substances.

The distribution of alkaline phosphatase was mainly extracellular within the lamina propria and muscularis mucosae in the stomach region and in the striated border of the small intestine, caeca and rectum. The muscular layers, especially the outer longitudinal, indicated a very high activity of alkaline phosphatase. The acid phosphatase and esterase were intracellular and concentrated at the distal portion of the surface epithelium in the stomach and intestine. A high concentration of PAS-positive substances was observed in the gastric surface epithelial and intestinal goblet cells. It was not investigated whether or not a correlation exists between enzyme activity and age, sex of the animals or the season.

ACKNOWLEDGEMENTS

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Sincere thanks are offered to Dr. F. A. Aldrich and Dr. G. Moskovits for their kind advice and suggestions, and to Dr. W. Threlfall for the identification of the parasites. A number of fishermen aided to obtain living specimens: Mr. James Wells, Mr. John Wells and Mr. Otto Wells.

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INTRODUCTION

The cod is one of the most important commercial fish in the boreo-arctic region. Its taxonomy is quite complex with several synonyms applicable to the same species. According to Svetovidov, (1948) Gadus morhua morhua applies to the Atlantic cod, and morhua callarias to the Baltic cod. The world's cod fisheries are located between the 35th and 80th parallels of north latitude (mostly north of 43° N) at varying distances from land, depending on the width of the continental shelf, from the surface down to at least 250 fathoms, but mostly at depths from 20 to 150 fathoms.

The cod-fishing industry is an important economic branch in Newfoundland and Labrador. According to statistical records of the International Commission for the Northwest Atlantic Fisheries the annual catch of cod was 222 thousand metric tons in 1963, whereas the total landing of all species was only 297 thousand metric tons. The ecology, life history and population dynamics of the fish, which are of immediate economic value, have been intensively studied, but very little has been done on chemical and microscopic levels. Ege and Obel (1935) demonstrated that the proteolytic enzymes of the stomach of cod are active at a higher pH than that of mammals. Labarre and Tremblay (1951) studied the proteolytic digestive enzymes of the stomach of the cod. Reznik (1958) has made a comparative study of the histology of the digestive organs in some representatives of the family Gadidae, and recently

Bishop and Odense (1966 a and b) described the morphology of the digestive tract of the cod and the ultrastructure of the epithelial cells of the intestine.

It is the purpose of this thesis to further investigate the histology in correlation with the localization of some of the hydrolytic enzymes (alkaline phosphatase, acid phosphatase, esterase) and periodic acid-Schiff (PAS) reactive compounds. No attempt was made to correlate this enzyme activity with sex, age or season.

The results of this thesis have been organized in two parts; the first describes the histological features of the gastrointestinal tract which is in agreement with the findings of Bishop and Odense, needed as a basis for the account of the histochemical findings reported in the second part.

METHODS

From May 1965 to January 1966 samples of one to four fish were collected at about monthly intervals in the vicinity of St. John's, Newfoundland (Table 1). From May to September 1965 living fish were provided by the Fisheries Technological Unit at St. John's, and from October to January 1966 dissection and fixation of the material was performed immediately after capture on the fishing boat.

Fixation and treatment of tissue for the histology of the gastrointestinal tract

Small pieces of tissue of the nine regions of the gastrointestinal tract (cardiac stomach, fundic stomach, pyloric stomach, pylorus, caeca, anterior loop of small intestine, posterior loop of small intestine, rectum and liver) were fixed in Lison's fluid, dehydrated and embedded in paraffin. The methods employed are indicated and discussed in Appendix 1. Sections were cut with a standard rotary microtome at 7 to 8 µ and stained by Mallory's triple stain and Fraenkel's Orcein method (see Appendix 2).

Fixation and treatment of tissue for the histochemistry

The enzymes were located in paraffin as well as in cryostat frozen sections. Better results for the enzyme activities were obtained in frozen sections. For a discussion of the different results obtained by these two methods, the

Table 1

Samples of cod collected from May 1965 to January 1966

Sample No.	Date Collected	Weight of Fish	Length of Fish	Sex of Fish
1	May 5, 1965	2268 g	60 cm	Male
2	June 14, 1965	2268 g	49 cm	Female
3	June 14, 1965	2268 g	50 cm	Male
4	June 23, 1965	680 g	40 cm	Male
5	June 23, 1965	998 g	46 cm	Female
6	Sept. 14, 1965	1905 g	57 cm	Female
7	Sept. 14, 1965	1134 g	53 cm	Male
8	Sept. 25, 1965	2948 g	70 cm	Male
9	Sept. 25, 1965	2495 g	66 cm	Female
10	Oct. 26, 1965	1814 g	62 cm	Female
11	Oct. 26, 1965	1361 g	56 cm	Female
12	Oct. 26, 1965	3402 g	69 cm	Female
13	Oct. 26, 1965	1361 g	57 cm	Male
14	Nov. 27, 1965	4763 g	75 cm	Female
15	Dec. 1, 1965	2041 g	62 cm	Female
16	Dec. 1, 1965	1134 g	50 cm	Female
17	Jan. 14, 1966	5443 g	87 cm	Female
18	Jan. 14, 1966	4763 g	77 cm	Female

reader is referred to Appendix 3. It is probable that each of the alkaline phosphatase, acid phosphatase and esterase are composed of a spectrum of enzymes (Pearse, 1961). The tests employed in this study characterize the non-specific enzymes of alkaline phosphatase, acid phosphatase and esterase.

The tissues for paraffin infiltration were fixed in cold acetone for 24 hours at 4° C, and embedded in 1% celloidin and paraffin in vacuo (see Appendix 1). Sections were cut at 7 to 8 μ on a standard Spensor microtome and mounted on slides with egg albumin. The paraffin was removed with light petroleum and acetone and passed to water before incubating in specific substrates.

Tissues from all the regions of the tract except the caeca were fixed in Baker's formol calcium for 24 hours at 4° C and then sectioned in the cryostat (Lab-Tek) at -30° C The sections were transferred to water and then passed into the appropriate media and incubated. After completion of the staining the sections were floated onto slides and mounted with glycerol jelly.

Alkaline Phosphatase. The enzyme was histochemically demonstrated by the Gomori's calcium-cobalt method (1946). Sections were incubated in a medium containing calcium chloride and sodium B-glycerophosphate. In a positive test for alkaline phosphatase, the phosphate of the substrate was liberated by the enzyme to form a black precipitate with the calcium ions.

This precipitate remained within the tissue at sites of the occurrence of the enzyme, and presented the diagnostic features of localizing alkaline phosphatase. The optimum incubating time was determined at 2½ hours for the frozen sections; however, a longer incubation (5½ hours) was used for paraffin sections. Control sections were incubated in a medium without sodium β-glycerophosphate so that no black precipitate was formed in the sites of the enzyme concentration. The methods are indicated and discussed in Appendix 3.

Acid Phosphatase. The enzyme was demonstrated by Gomori's lead nitrate method (Gomori 1950). The incubating medium contained sodium β-glycerophosphate as the substrate and lead nitrate instead of calcium chloride. Here, in a positive test for acid phosphatase, the phosphate ions liberated by the enzyme combined with the lead ions to form the black precipitate at the sites of occurrence of the enzyme within the tissue. Control sections were also employed and incubated without the sodium β-glycerophosphate. The method is listed and discussed in Appendix 3.

Esterase. The enzyme was demonstrated by Gomori's α-naphthyl acetate method (Gomori 1953, see Appendix 3). The medium contained α-naphthyl acetate and Fast B Salt. The principle of the method is the liberation of naphthyl ions by the esterase which is immediately coupled with the Fast B Salt to form a brownish precipitate in the tissue. Control sections were incubated in the same medium without the

α-naphthyl acetate.

PAS Reactive Compounds. The tissues were fixed in Lison's fluid and stained by Hotchkiss PAS-technique (Hotchkiss 1948). Some were counter-stained by Harris-Hematoxylin and Fast Green. Control sections were acetylated with a mixture of pyridine and acetic anhydride for 24 hours at room temperature before staining (see Appendix 3).

RESULTS

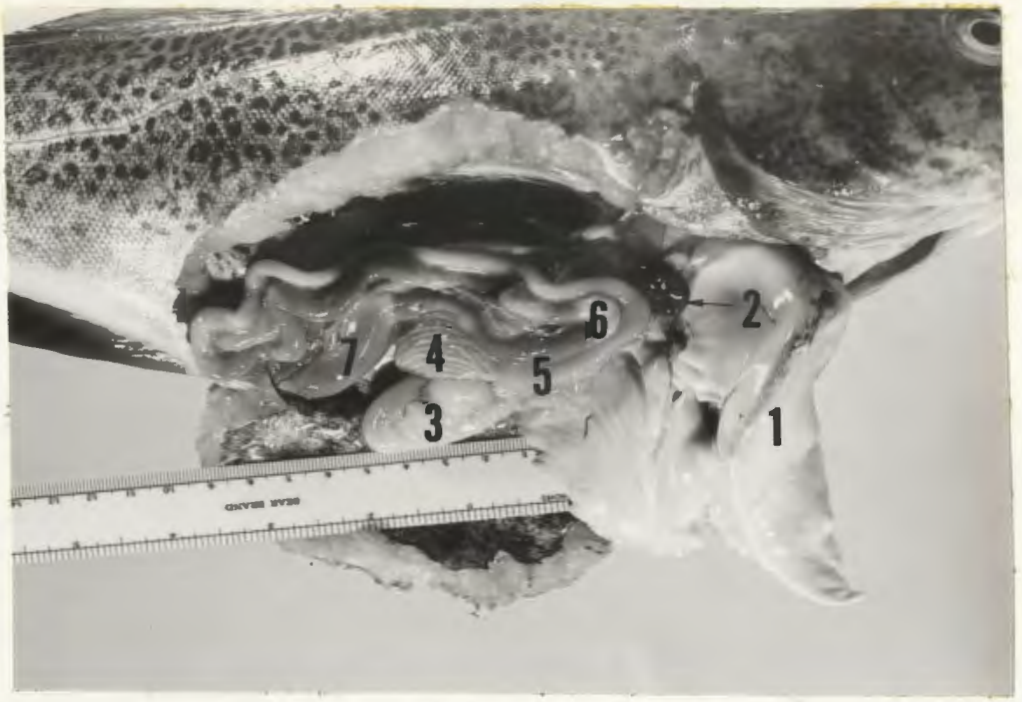
Histological features of the gastrointestinal tract of the cod

Gross Anatomy. The digestive organs are shown in Figure 1 as they lie in the abdominal cavity when gonads and liver are removed. These organs are best shown when removed from the body cavity as indicated in Figure 2. The portions of this gastrointestinal tract used for sectioning are illustrated in Figure 3. As obvious from these illustrations the esophagus is a wide but short tube, not distinctly separated from the entrance of the stomach. The stomach is a thick-walled and elongated pouch with a wide anterior cardiac region and a narrow pyloric region which enters the small intestine. In the undisturbed position the stomach is largely concealed by the liver. Several bundles of pyloric caeca join and enter the anterior portion of the small intestine through several openings. The entrance of the common bile duct is just posterior to the openings of the pyloric caeca. The small intestine forms a loop distally then turns forward, forms a second loop and widens at its transition to the rectum which is a short wide tube (Figure 1 and 2).

Microanatomy. The gastrointestinal tract of vertebrates is classically divided into four layers: mucous membrane, submucosa, muscularis externa and serosa. The mucous membrane is further subdivided into surface epithelium, lamina propria

Fig. 1. The gross anatomy of the cod showing the original position of the gut: liver (1), gall-bladder (2), stomach (3), pyloric caeca (4), anterior loop of small intestine (5), posterior loop of small intestine (6) and rectum (7).

Fig. 2. The gastrointestinal tract of cod after removed from the body cavity: esophagus (1), cardiac stomach (2), fundic stomach (3), pyloric stomach (4), pylorus (5), anterior loop of small intestine (6), pyloric caeca (7), posterior loop of small intestine (8), rectum (9), anus (10) and gall-baldder (11).



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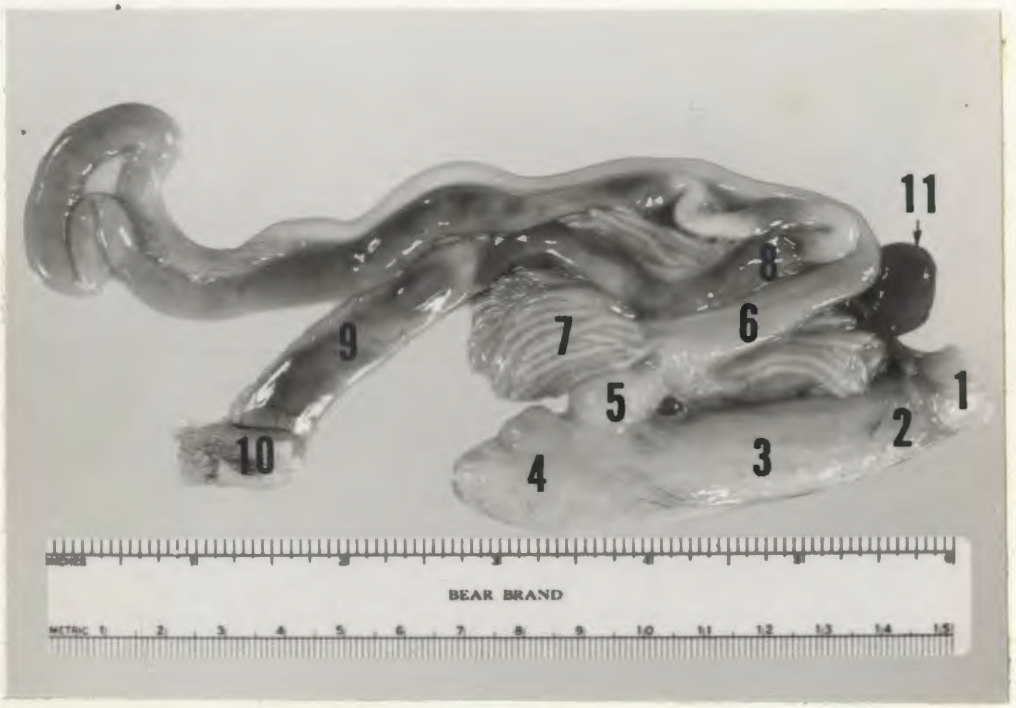

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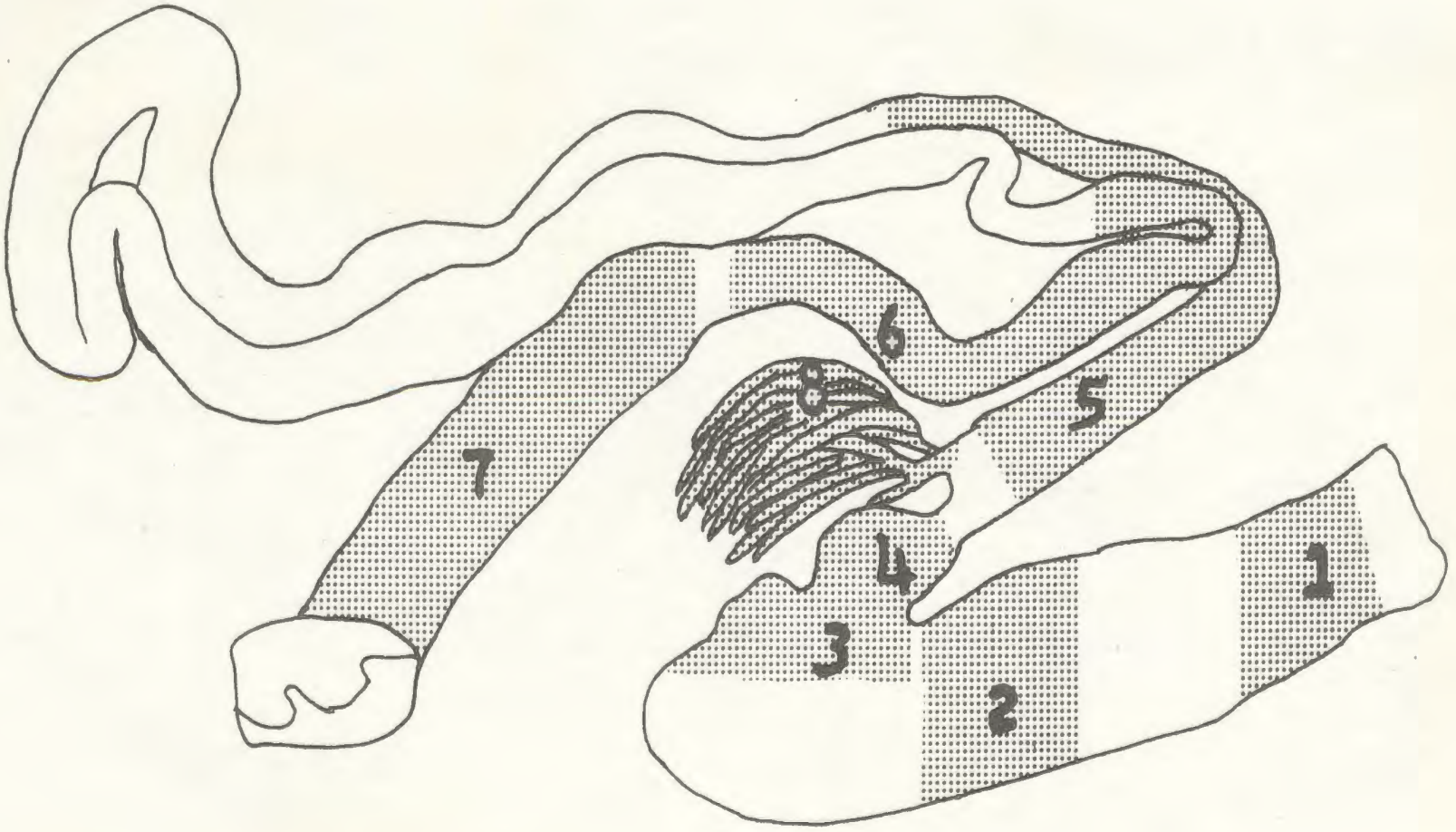


Fig. 3. The gastrointestinal tract of cod; the stippled areas indicate the parts of the this tract investigated. (1) cardiac stomach, (2) fundic stomach, (3) pyloric stomach, (4) pylorus, (5) anterior loop of small intestine, (6) posterior loop of small intestine, (7) rectum, (8) caeca

and a muscularis mucosae. These layers are modified within the various parts of the tract in keeping with their specific function. To described the histology of the gastrointestinal tract in cod, its various parts are analyzed with reference to these layers.

Stomach. The tall columnar cells that line the stomach mucosa continue into the gastric pits which lead into the branched simple tubular gastric glands (Figure 4B). The surface epithelial cells have a homogeneous border which has an affinity for eosin (Figure 4B); after the PAS technique these cells display a mass of granules in the border as well as in the distal portion (Figures 5A-D). The lamina propria consists of a compact network of collagenous fibers. The gastric glands beneath the lamina propria consist of one type of secretory cells (Bishop and Odense, 1966 a) with dense accumulations of eosinophilic granules.

There is a gradual change in the size of the gastric glands and the gastric pits from the cardiac to the pyloric regions of the stomach. The gastric glands are often very long in the cardiac region and may diminish in length to two-third of the original size in the fundus. In the pyloric region these gastric glands are further reduced, although their pits are considerably deeper and in the pylorus they disappear (Figures 5A-D). The muscularis mucosae is composed of longitudinal smooth muscle fibers widely scattered beneath the gastric glands. It is most prominent and compact in the pylorus.

Fig. 4A. Cardiac stomach: surface epithelium (E), lamina propria (LP), gastric glands (GG), muscularis mucosae (MM), submucosa (SB), inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 9, Mallory's triple stain, X50).

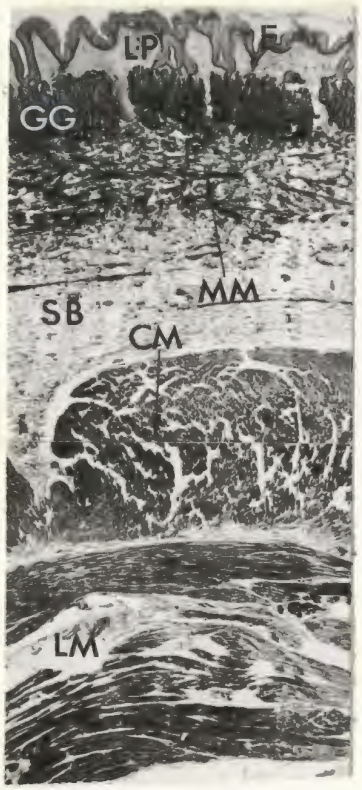
Fig. 4B. Cardiac stomach: eosinophilic border (EB) of the surface epithelium, basal nuclei (BN) of the surface epithelium. (Sample 9, Mallory's triple stain, X250).

Fig. 5A. Cardiac stomach: surface epithelium (E), lamina propria (LP), basement membrane (BM), gastric glands (GG), muscularis mucosae (MM). (Sample 6, PAS technique, X250).

Fig. 5B. Fundic stomach: (Sample 6, PAS technique, X250).

Fig. 5C. Pyloric stomach: (Sample 6, PAS technique, X250).

Fig. 5D. Pylorus : (Sample 6, PAS technique, X250).



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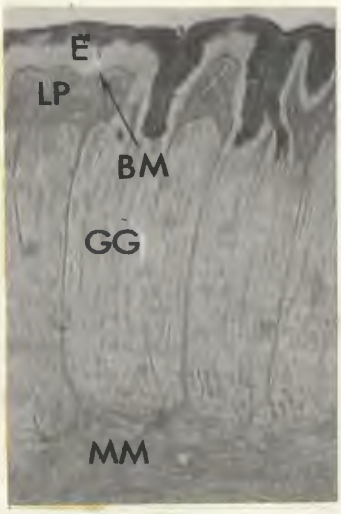
4 B



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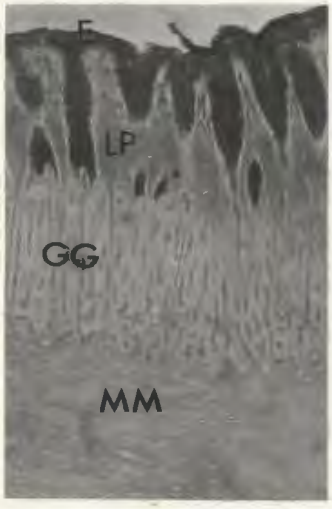

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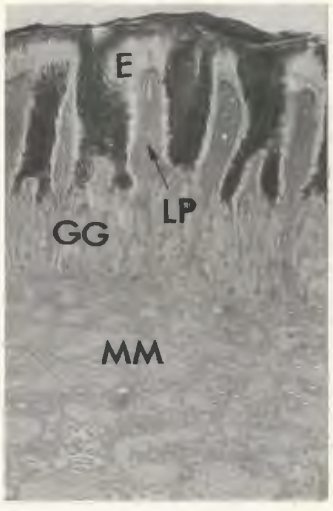

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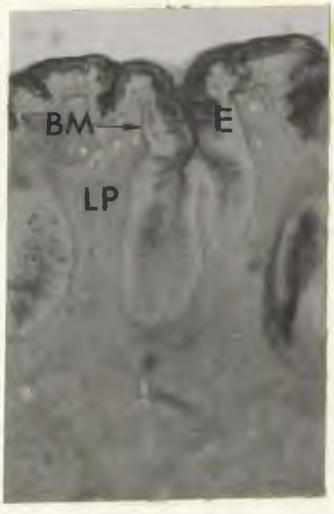


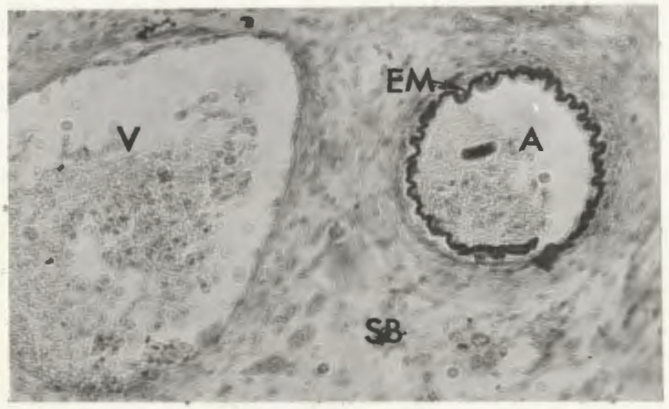
5 D

The submucosa consists of loosely arranged collagenous fibers; elastic tissue is present only in the walls of arteries in this region (Figure 6). The muscularis externa consists of two layers, the inner circular and outer longitudinal muscle layers. While in the esophagus these two muscle layers are composed of striated muscle, in the most anterior region of the cardiac stomach they are replaced by smooth muscle (Figure 7). The inner circular muscle widens in the pyloric region to form a sphincter.

Intestine and pyloric caeca. The small intestine, caeca and rectum have very similar histological features (Figures 8, 9, 10, and 11). The cells lining the intestinal and caecal villi are simple columnar with a prominent striated border at the free end. Bishop and Odense (1966b) have shown that the striated border consists of microvilli of much greater length than found in other vertebrates; the longest are in the caeca. The lamina propria is composed of a delicate network of collagenous fiber less compact than in the stomach. Intestinal glands are present in the small intestine, caeca and in the rectum, but their size varies in the different regions. They are lined by cuboidal cells which stain more deeply with azocarmine than the columnar cells of the villi. Goblet cells are distributed throughout the intestine, in the glands and among the epithelial cells of the villi but are less numerous in the caeca. They are as tall as the epithelial cells and are expanded distally (Figures 36A and 46A).

Fig. 6. Fundic stomach: elastic tissue in the submucosa (SB); artery (A), internal elastic membrane (EM), vein (V). (Sample 9, Fraenkel's Orcein stain, X450).

Fig. 7. Junction of esophagus and cardiac stomach: the skeletal muscles extending from the esophagus into the cardiac stomach; circular skeletal muscle (CK), longitudinal skeletal muscle (LK), circular smooth muscle (CM) of the cardiac stomach. (Sample 3, PAS technique, X500).



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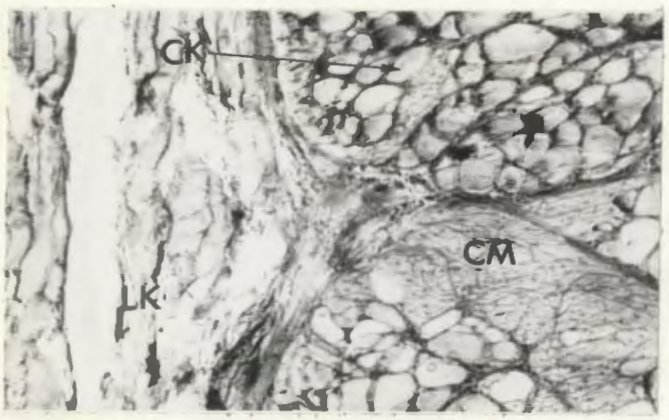

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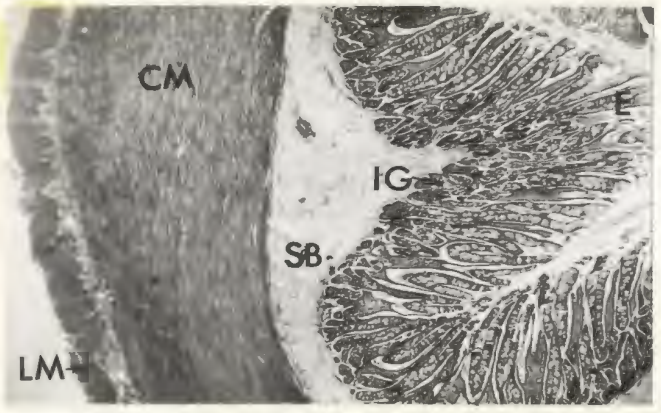
Fig. 8. Anterior portion of the small intestine: surface epithelium (E), intestinal glands (IG), submucosa (SB), inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 9, Mallory's triple stain, X50).

Fig. 9. Posterior portion of the small intestine: (Sample 9, Mallory's triple stain, X50).

Fig. 10. Rectum: (Sample 9, Mallory's triple stain, X50).

Fig. 11. Caeca: (Sample 9, Mallory's triple stain, X50).

Fig. 12. Anterior portion of the small intestine: elastic tissue in the submucosa (SB); artery (A), internal elastic membrane (EM). (Sample 9, Fraenkel's Orcein stain, X1100).



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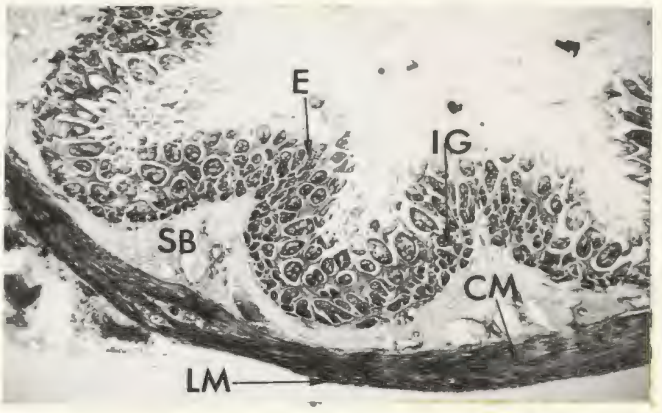

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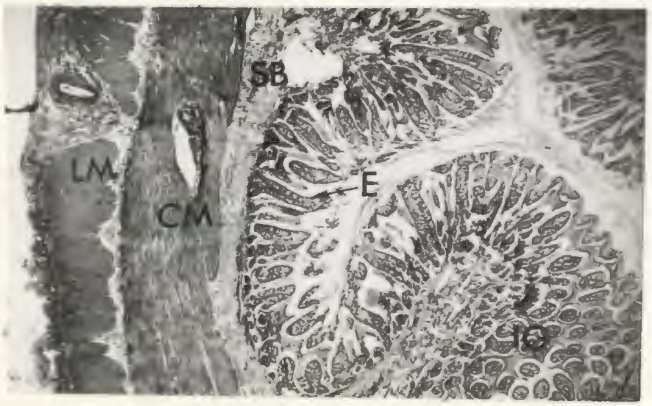

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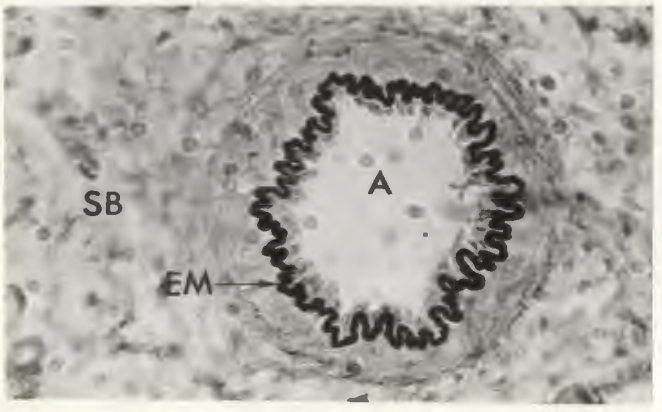

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The "pear-shaped" cell described by Al-Hussaini (1949), also noted in cod by Bishop and Odense (1966a) was not observed in this study. The muscularis mucosae is absent in the small intestine, caeca and rectum. The lamina propria merges with the submucosa, which is particularly narrow in the caeca. The connective tissue in this region is loose collagenous. Elastic tissue is present only in the wall of arteries in submucosa (Figure 12). The inner circular muscle is wider than the outer longitudinal muscle. In the posterior loop of the small intestine and the caeca the longitudinal muscle coat becomes very thin (Figures 9 and 11).

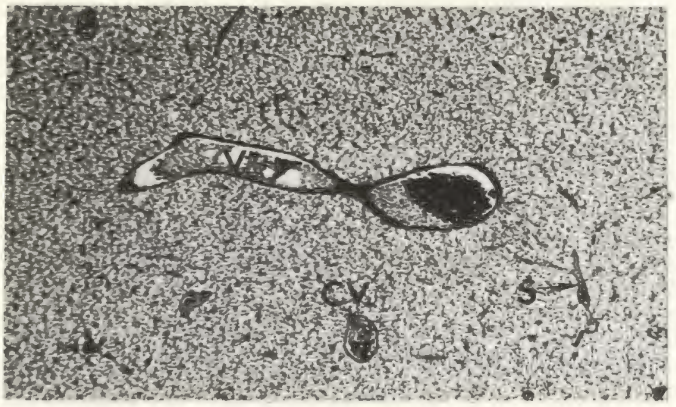
Liver. The organ is composed of laminae of hepatic cells, as in other fishes (Elias and Bengelsdorf, 1950), which are separated by blood sinusoids. The interlobular connective tissue is sparse in cod and the lobular investment is incomplete; as a consequence the central veins are rather irregularly located (Figure 13). After Mallory's triple stain no granules are noted in the hepatic cells (Figure 14). The bile ducts in the portal canal are surrounded by a thick connective tissue coat and are lined by columnar epithelium with a prominent basement membrane (Figure 15).

Parasites. Many of the specimens of cod investigated exhibit a fairly heavy infestation of parasites, mainly nematodes (Class Nematode, Phylum Aschelminthes) and tapeworms (Class Cestoda, Phylum Platyhelminthes). The intestinal lumen was

Fig. 13. Liver: portal vein (V), central vein (CV), sinusoid (S). (Sample 9, Mallory's triple stain, X120).

Fig. 14. Liver: central vein (CV), sinusoid (S). (Sample 9, Mallory's triple stain, X500).

Fig. 15. Liver: artery (A), bile duct (BD), epithelial lining of bile duct (E), basement membrane of the epithelium of bile duct (BM). (Sample 3, PAS technique, X250).



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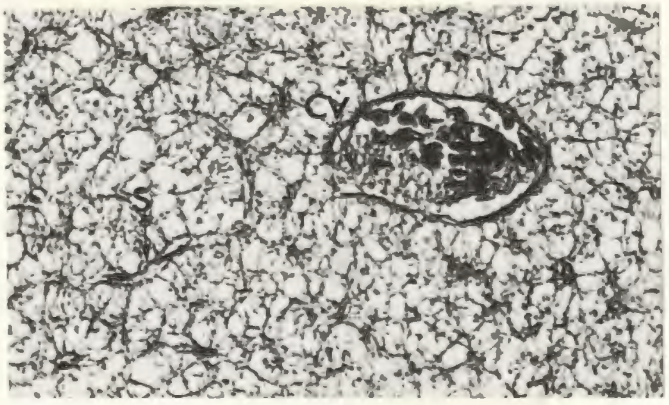

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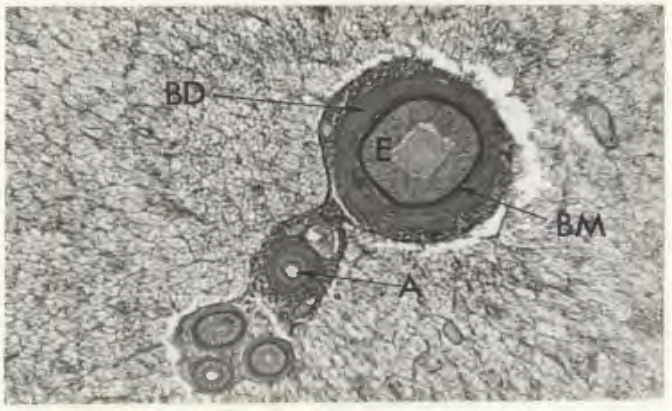

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often filled with adult nematodes, with the larva encysted within the muscular wall (Figure 17), or adhering to the surface epithelium (Figure 16). Cross sections of adult nematodes are seen in the lumen of the intestine (Figures 18 and 19). It appears that tapeworms are restricted to the caeca only (Figure 20), while nematodes are noted within all portions of the tract. Both types of parasites show an intense PAS-positive reaction.

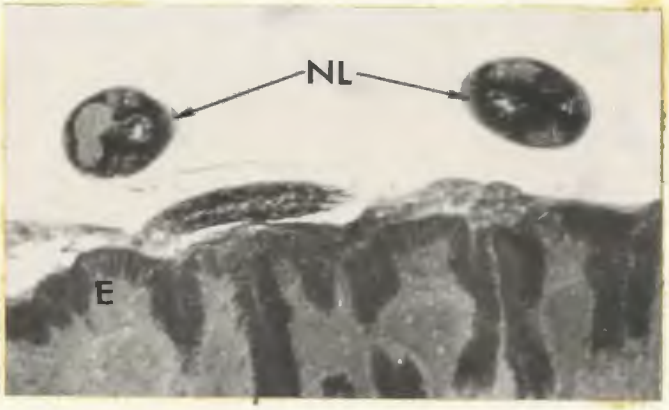
Fig. 16. Fundic stomach: surface epithelium (E), nematode larva (NL). (Sample 3, PAS technique, X350).

Fig. 17. Small intestine: nematode larva (NL), inner circular muscle layer (CM), outer longitudinal muscle layer (LM), submucosa (SB), intestinal glands (IG). (Sample 6, PAS technique, X100).

Fig. 18. Cross section of an adult nematode (NA) in the lumen of small intestine. (Sample 11, PAS technique, X300).

Fig. 19. Cross section of an adult nematode (NA) in the lumen of rectum. (Sample 18, Gomori's calcium-cobalt method, X320).

Fig. 20. Caeca: head portion of a tapeworm (T), goblet cells (G). (Sample 11, PAS technique, X200).



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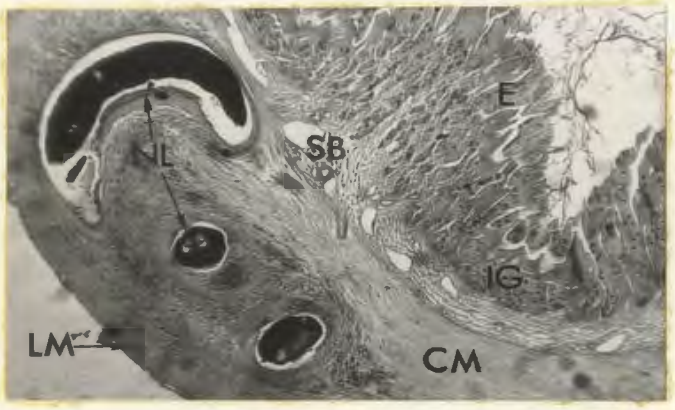

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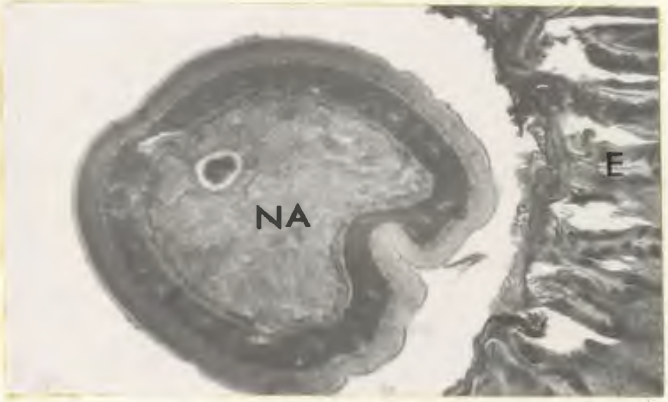

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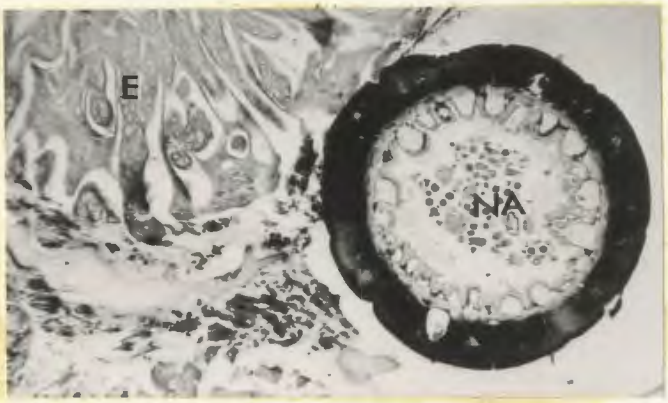

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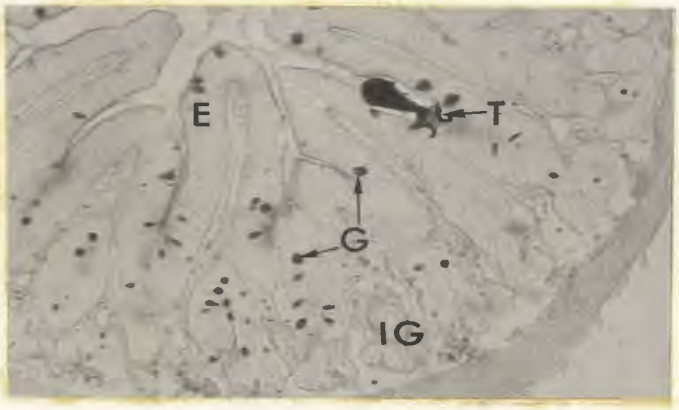

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The occurrence of the hydrolytic enzymes and the PAS-positive substances

The occurrence of the hydrolytic enzymes and PAS-positive substances is considered for each portion of this tract and is described and illustrated within the mucosa, submucosa and muscularis externa. Reference is made to control sections for the substantiation of the histochemical results. The distribution is shown in Table 2, 3 and 4 for specimens 17 and 18 from the total of eighteen investigated. Samples one to sixteen were analyzed incompletely, and the results obtained from them were used to validate the procedures employed. The problems encountered regarding the inactivation of the enzymes due to fixation, paraffin infiltration are discussed in Appendix 3.

Stomach. The distribution of the hydrolytic enzymes in frozen sections, and of PAS reactive compounds in paraffin sections is shown in Table 2.

Alkaline Phosphatase. No enzyme activity could be observed in the distal border of surface epithelium in paraffin or frozen sections, some black precipitate is apparent within the proximal portion of the epithelial cells (Figure 23A) but it could not be resolved whether or not it is an artefact. Intense reaction of the enzyme is noted in the lamina propria and muscularis mucosae throughout the entire stomach. This is particularly well shown in the thin paraffin sections (Figures 21A-B, 22A-B

Table 2. The distribution of alkaline phosphatase, acid phosphatase, esterase and PAS reactive compounds in paraffin and frozen sections of the stomach.

Sample		Cardiac					Fundic					Pyloric					Pylorus				
		Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)	Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)	Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)	Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)
Dist. part of surface epi.	17	-	-	-	++	++	-	-	-	++	++	-	-	-	++	+++	-	-	+	++	+++
	18	-	-	++	+	++	-	-	+	++	++	-	-	++	++	+++	-	*	++	++	++
Prox. part of surface epi.	17	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-
	18	+	+	+	-	-	-	++	+	-	-	+	-	+	-	-	+	*	+	-	-
Lamina propria	17	++	++	-	+	+	+	-	-	-	+	+	+	-	-	+	++	++	-	-	+
	18	++	++	-	+	+	++	++	-	-	+	++	-	-	-	+	++	*	-	-	+
Gastric glands	17	-	-	-	+	+	-	-	-	-	+	-	-	-	+	+					
	18	-	-	++	+	+	-	+	+	+	+	-	-	+	+	+					
Muscularis mucosae	17	++	++	-	+	+	++	+	-	+	+	+	+	-	+	+	++	++	-	-	+
	18	++	++	-	*	+	++	++	-	*	+	++	-	-	*	+	++	*	-	-	+
Submucosa	17	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	18	-	-	-	+	+	-	-	-	+	+	-	-	++	++	+	-	*	+	+	+
Circular muscle	17	++	++	-	-	+	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+
	18	+	++	-	-	+	+	+	-	-	+	+	-	-	-	+	+	*	-	-	+
Longitudinal muscle	17	+++	+++	-	-	+	+++	+	-	-	+	++	++	-	-	+	+++	++	-	-	+
	18	+++	+++	-	-	+	+++	+++	-	-	+	+++	++	-	-	+	+++	*	-	-	+

Significance of figures employed in this Table:

(P) = paraffin section
(F) = frozen section
\- = negative
\+ = slight
++ = moderate
+++ = intense

Alk = alkaline phosphatase
Acid = acid phosphatase
Es = esterase
PAS = PAS reactive compounds
* = no data could be obtained due to loss of tissue

Fig. 21A. Fundic stomach: alkaline phosphatase in the lamina propria (LP), muscularis mucosae (MM), inner circular muscle layer (CM), outer longitudinal muscle layer (LM); surface epithelium (E), gastric glands (GG), submucosa (SB). (Sample 17, frozen section, Gomori's calcium-cobalt method, X60).

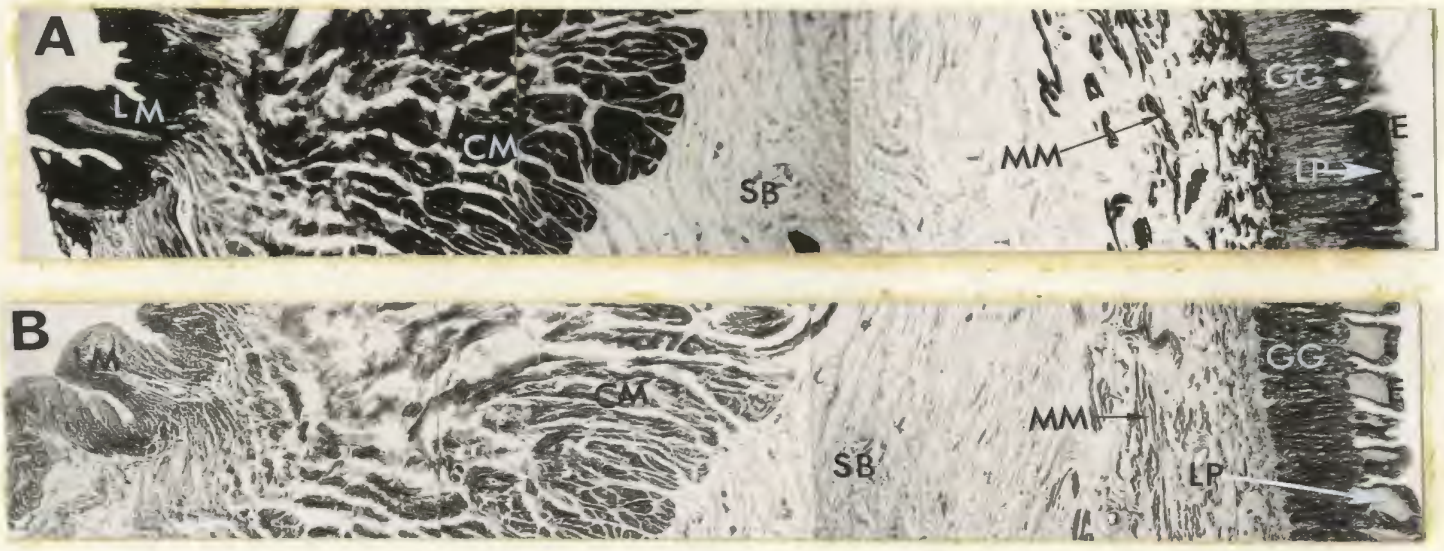
Fig. 21B. Fundic stomach: control frozen section for Sample 17, (X60).

Fig. 22A. Cardiac stomach: alkaline phosphatase in paraffin section. (Sample 17, Gomori's calcium-cobalt method, X45).

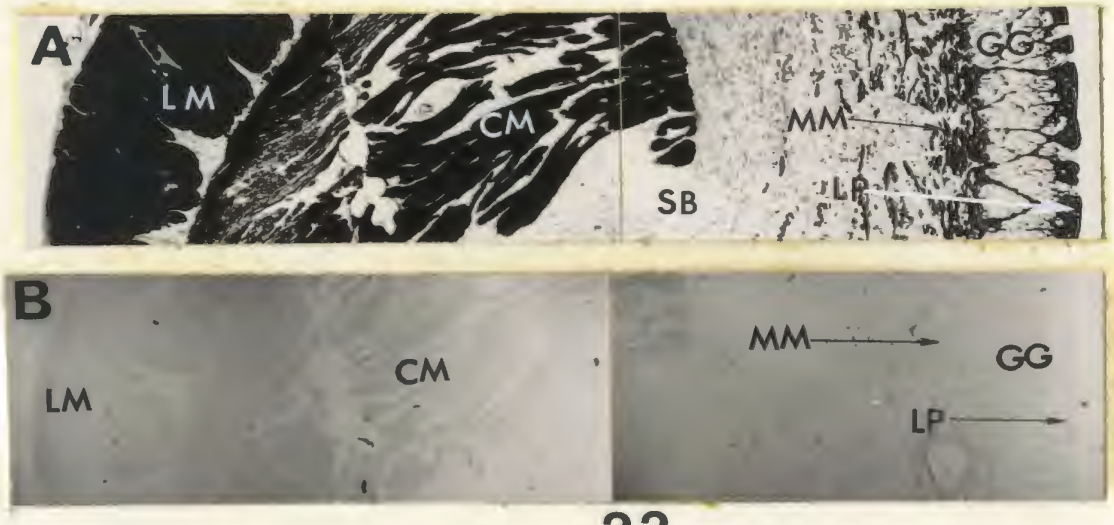
Fig. 22B. Cardiac stomach: control paraffin section for Sample 17, (X45).

Fig. 23A. Fundic stomach: alkaline phosphatase in frozen section. (Sample 17, Gomori's calcium-cobalt method, X250).

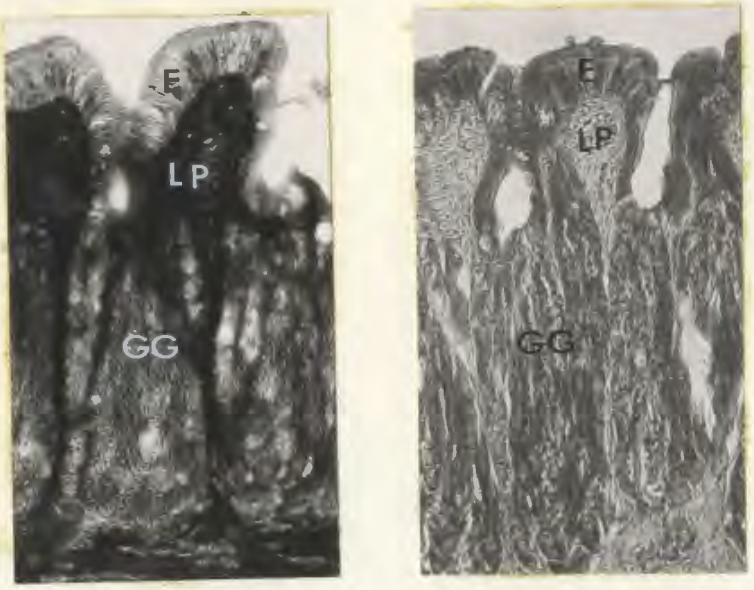
Fig. 23B. Fundic stomach: control frozen section for Sample 17, (X250).


A
LM
CM
SB
MM
GG
E
LP
B
LM
CM
SB
MM
GG
E
LP

21


A
LM
CM
SB
MM
GG
LP
B
LM
CM
MM
GG
LP

22


E
LP
GG
E
LP
GG

23 A

23 B

and 23A-B). In the frozen control sections the lamina propria shows as a clear area, but a darker tone is observed within the muscularis mucosae which coincides with the pattern of the alkaline phosphatase (Figure 21B). This is also observed in the muscularis externa. The paraffin control sections (Figure 22B) are quite clear in all these regions. A possible explanation of this anomaly may be the thickness of the frozen section, or that minute amounts of phosphate occur within the tissue, which might react with the enzyme to produce a slight degree of activity. The latter possibility was brought up by Danielli (1945) who reported that non-enzymic calcium phosphate has a strong tendency to be precipitated at the sites of alkaline phosphatase activity; this tendency would only be compounded by the bulk of frozen sections.

No alkaline phosphatase activity was apparent in the gastric glands in paraffin or frozen sections, but the connective tissue strands connecting the lamina propria with the muscularis mucosae are strongly positive (Figures 22A-B and 23A-B). The submucosa is largely negative (Figures 21A-B and 22A-B).

The muscularis externa, shows a varying degree of enzyme activity. The inner circular layer reacts moderately while the outer longitudinal layer is extremely rich in alkaline phosphatase (Figures 21A-B and 22A-B). Very intense enzyme reaction is apparent within the outer longitudinal muscle

layer in the entire stomach and in the entire gastrointestinal tract.

Acid Phosphatase. The distribution of acid phosphatase in the stomach does not correspond to that of alkaline phosphatase. It is confined to the surface epithelium and the gastric glands (Figures 24A-B, 25A-B and Table 2).

The strongest activity of acid phosphatase is found within the distal portion of the surface epithelium; a slight nuclear activity is also noted within the proximal area of these cells. However, it is difficult to determine critically the nuclear activity of acid phosphatase since it is weak and only occasionally noticeable. The gastric glands display a slight to moderate activity. The enzyme was not seen in the distal portion of the surface epithelium and in gastric glands of specimen 17 (Table 2); this failure was possibly due to inactivation prior to incubation as all the portions of the tract could not be treated at the same time. The control sections show no enzyme activity within the areas mentioned (Figures 24B and 25B).

Esterase. The esterase is found within most layers of the stomach. In the surface epithelium enzyme activity is concentrated in the distal border (Figures 26A-B, 27A-B and Table 2). The lamina propria is slightly reactive only in the cardiac stomach; whether or not this type of activity is an artefact or is due

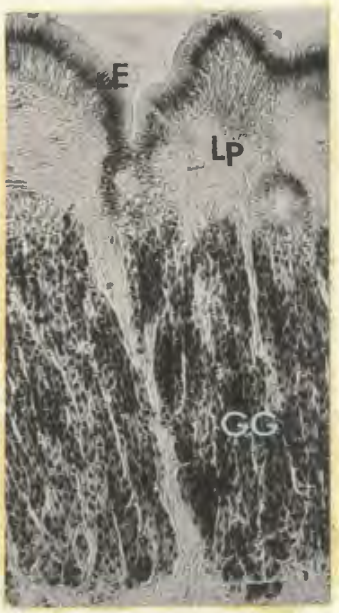
Fig. 24A. Cardiac stomach: acid phosphatase in the surface epithelium (E) and gastric glands (GG); lamina propria (LP), submucosa (SB), inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 18, frozen section, Gomori's lead nitrate method, X50).

Fig. 24B. Cardiac stomach: control frozen section for Sample 18, (X50).

Fig. 25A. Cardiac stomach: acid phosphatase in the distal portion of the surface epithelium (E) and gastric glands (GG). (Sample 18, frozen section, Gomori's lead nitrate method, X250).

Fig. 25B. Cardiac stomach: control frozen section for Sample 18, (X250).

24



25 A

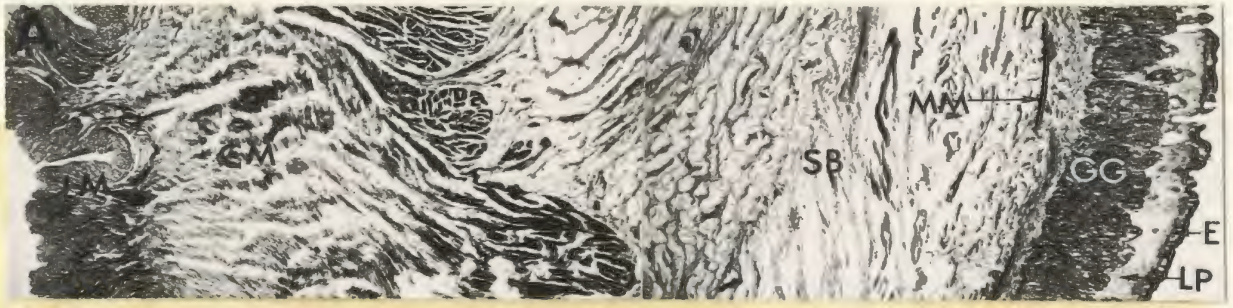
25 B

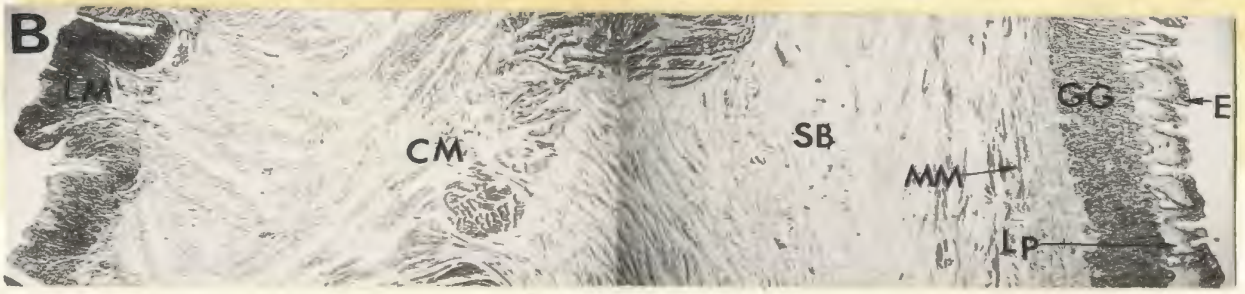
Fig. 26A. Fundic stomach: esterase in the surface epithelium (E), lamina propria (LP), gastric glands (GG), muscularis mucosae (MM), submucosa (SB), inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 18, frozen section, α-naphthyl acetate method, X50).

Fig. 26B. Fundic stomach: control frozen section for Sample 18, (X50).

Fig. 27A. Fundic stomach: esterase in the surface epithelium (E), lamina propria (LP), gastric glands (GG). (Sample 18, frozen section, α-naphthyl acetate method, X250).

Fig. 27B. Fundic stomach: control frozen section for Sample 18, (X250).





27 A

27 B

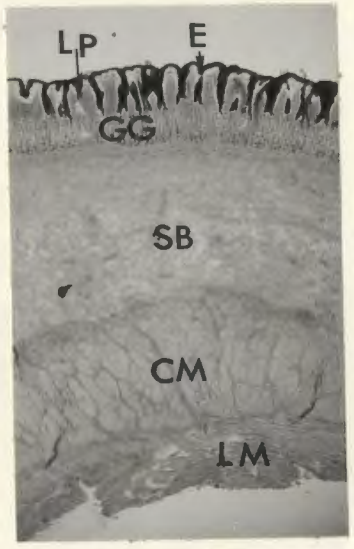
to diffusion of the enzyme from neighboring areas remains unknown.

The gastric glands and muscularis mucosae show moderate enzyme activity (Figures 26A-B). Within the submucosa some scattered brownish precipitate is attached to the collagenous fibers, and this was also noted within the media of arteries within the submucosa. The muscularis externa generally shows no esterase activity except in the fundic region (Figures 26A-B). The control sections show no enzyme activity within the areas mentioned (Figures 26B and 27B).

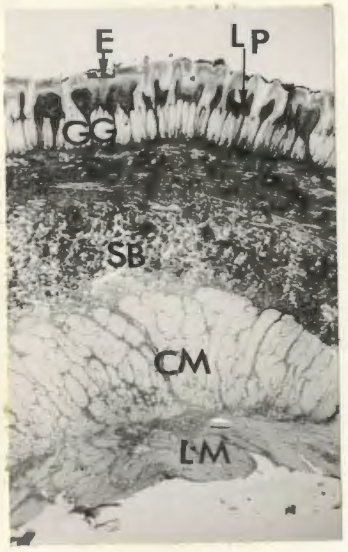
PAS Reactive Compounds. In the stomach the most intense PAS staining is seen in the surface epithelium, particularly in the distal border which contains numerous and tightly packed purple granules (Figures 5A-D, 28A and Table 2), while only some scattered granules are noted in the proximal part. The basement membrane is well-defined by the PAS reaction (Figures 5A and 5D).

A weak reaction is noted within the connective tissue of the lamina propria and submucosa. The cells of the gastric glands reveal a moderate degree of staining (Figures 5A-C), concentrating in the distal part of the cells. In the muscularis mucosae and muscularis externa the periphery of muscle fibers shows PAS staining (Figure 7).

The control section (Figure 28B) is unstained in areas corresponding to the PAS-positive structures. The dark appearance in the lamina propria and submucosa is due to the counter-staining by Fast Green (FCF).



28 A



28 B

Fig. 28A. Fundic stomach: PAS-positive compounds in the surface epithelium (E), lamina propria (LP), gastric glands (GG), submucosa (SB), inner circular muscle layer (CM) and outer longitudinal muscle layer (LM). (Sample 11, paraffin embedded section, PAS technique, X60).

Fig. 28B. Fundic stomach: control paraffin section for Sample 11, (X60).

Intestine and pyloric caeca. The distribution of alkaline phosphatase, acid phosphatase, esterase and PAS-positive compounds in the small intestine, caeca and rectum is summarized in Table 3.

Alkaline Phosphatase. Strong enzyme reaction is noted in paraffin and frozen sections within the striated border of the surface epithelium (Figures 30A, 37A and 40A). The control sections show no reaction in the corresponding site (Figures 30B, 37B and 40B). The proximal region of the surface epithelial and intestinal cells contains black granules which characterize the enzyme activity (Figures 30A-B and 40A-B). The lamina propria shows slight to moderate activity. Alkaline phosphatase could not be demonstrated in both paraffin and frozen sections of some samples possibly due to delays resulting from handling a large number of tissues at one time (Table 3). There is no enzyme activity in the submucosa, although some black precipitate was observed in the paraffin and frozen sections of some samples, whether this was the result of diffusion could not be determined (Figure 39A and Table 3).

The muscularis externa shows as intense enzyme activity as observed in the stomach: the inner circular layer only a weak to moderate, and the outer longitudinal layer a very intense activity. The inner circular muscle layer of the caeca shows more intensive enzyme reaction than that of the small intestine and rectum (Figures 29A, 37A and 39A).

Table 3. The distribution of alkaline phosphatase, acid phosphatase, esterase and PAS reactive compounds in paraffin and frozen sections of the small intestine, caeca and rectum.

		Small intestine										Caeca	
		Anterior					Posterior						
	Sample	Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)	Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)	Alk (P)	PAS (P)
Striated	17	++	++	-	-	++	++	++	-	-	++	++	+
border	18	++	+	-	-	++	++	++	-	-	+++	++	+
Surface	17	*	+	++	++	-	*	+	++	++	-	+	-
epithelium	18	*	-	++	++	-	*	+	++	++	-	+	-
Goblet	17	-	-	-	-	++	-	-	-	-	++	-	++
cells	18	-	-	-	-	+++	-	-	-	-	+++	-	+
Lamina	17	+	++	+	+	-	-	+	+	-	-	+	-
propria	18	+	-	+	+	-	+	+	+	+	-	+	-
Intestinal	17	+	+	++	+	++	+	+	+	+	++	+	+
glands	18	+	+	++	+	++	+	+	+	+	++	+	+
	17	-	-	-	+	-	-	-	-	+	-	-	-
Submucosa	18	-	+	-	+	-	-	-	-	+	-	-	-
Circular	17	+	+	-	-	+	+	++	-	-	+	++	+
muscle	18	-	-	-	-	+	+	+	-	-	+	+	+
Longitudinal	17	++	++	-	-	+	+++	+++	-	-	+	+++	+
muscle	18	+++	+	-	-	+	+++	++	-	-	+	++	+

Significance of figures employed in this Table:			
(F)	= frozen section	Alk	= alkaline phosphatase
(P)	= paraffin section	Acid	= acid phosphatase
-	= negative	Es	= esterase
+	= slight	PAS	= PAS reactive compounds
++	= moderate	*	= no data could be obtained due to loss of tissue
+++	= intense		

Fig. 29A. Small intestine: alkaline phosphatase in the surface epithelium (E), intestinal glands (IG), submucosa (SB), inner circular muscle layer (CM) and outer longitudinal muscle layer (LM). (Sample 17, paraffin section, Gomori's calcium-cobalt method, X60).

Fig. 29B. Small intestine: control paraffin section for Sample 17, (X60).

Fig. 30A. Small intestine: alkaline phosphatase in the striated border (ST), lamina propria (LP) and intracellular distribution (IA) in the proximal portion of the surface epithelium and intestinal glands; goblet cells (G) (Sample 17, paraffin section, Gomori's calcium-cobalt method, X250).

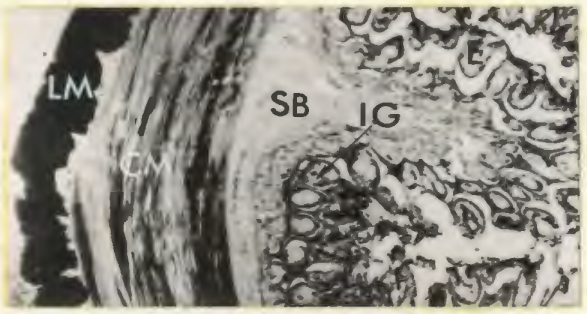
Fig. 30B. Small intestine: control paraffin section for Sample 17, (X250).

Fig. 31A. Small intestine: acid phosphatase in the surface epithelium (E) and intestinal glands (IG). (Sample 18, frozen section, Gomori's lead nitrate method, X70).

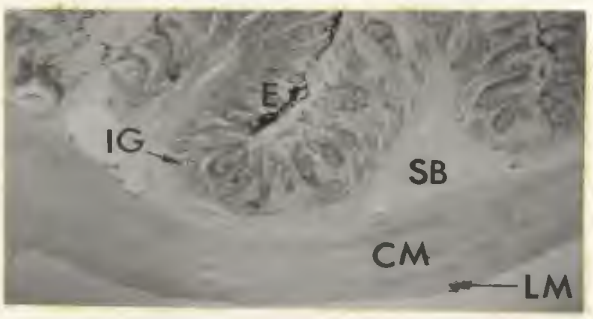
Fig. 31B. Small intestine: control frozen section for Sample 18, (X70).

Fig. 32A. Small intestine: acid phosphatase in the surface epithelium (E), lamina propria (LP) and intestinal glands (IG). (Sample 18, frozen section, Gomori's lead nitrate method, X250).

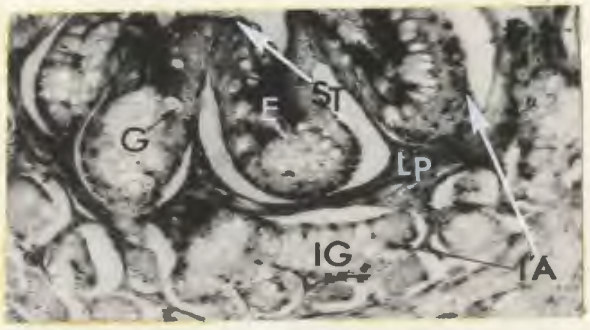
Fig. 32B. Small intestine: control frozen section for Sample 18, (X250).



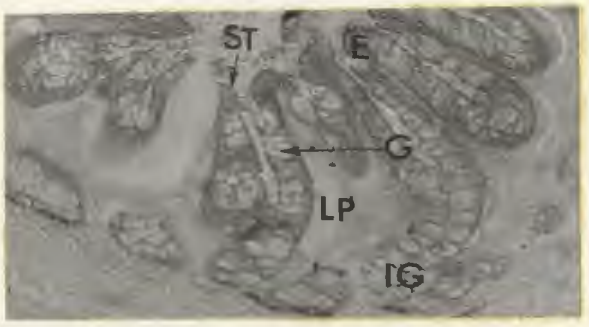
29 A



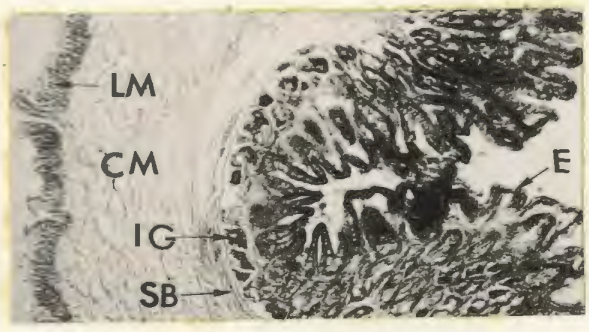
29 B



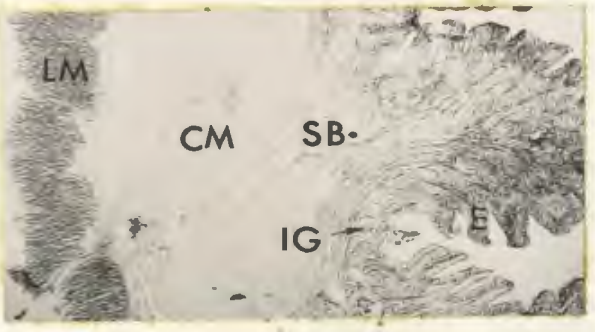
30 A



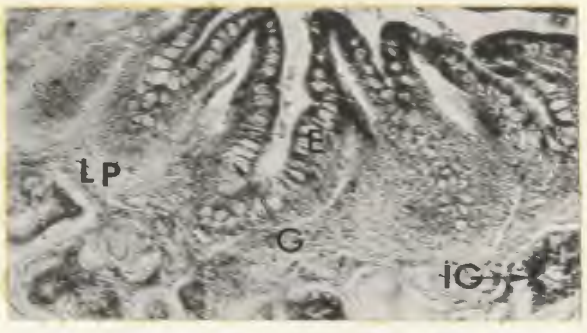
30 B



31 A



31 B



32A

32 B

Acid Phosphatase. It is mainly confined to the surface epithelium and the intestinal glands of the small intestine and rectum. The striated border and goblet cells do not exhibit any enzyme activity. In the surface epithelium, the enzyme is concentrated in the distal part of the cells (Figures 31A-B, 32A-B, 41A-B, 42A-B and Table 3), and a similar particulate activity is also noted within the cells of intestinal glands. A weak enzyme activity is observed within the reticuloendothelial system of the lamina propria; whether or not this is due to diffusion or the so-called "random precipitate" has not been determined (see Appendix 3). The submucosa and muscularis externa of the small intestine and rectum do not show any activity of acid phosphatase.

Esterase. The striated border and goblet cells of the surface epithelium are negative while the distal portion contains dense accumulations of the brownish precipitate which characterizes esterase activity (Figures 34A-B, 44A-B and Table 3). The reticuloendothelial system within the lamina propria indicates a weak and diffuse enzyme activity which may be the result of diffusion from other areas. In the cells of the intestinal glands a moderate amount of brownish precipitate is noted (Figures 34A-B and 44A-B). Within the submucosa some scattered brownish granules are seen to adhere to the collagenous fibers, probably a result of random precipitate. The muscularis externa is usually negative, but for a random occurrence of non-specific

Fig. 33A. Small intestine: esterase in the surface epithelium (E), intestinal glands (IG), submucosa (SB); inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 17, frozen section, α-naphthyl acetate method, X60).

Fig. 33B. Small intestine: control frozen section for Sample 17, (X60).

Fig. 34A. Small intestine: esterase in the surface epithelium (E), lamina propria (LP), intestinal glands (IG); goblet cells (G). (Sample 17, frozen section, α-naphthyl acetate method, X250).

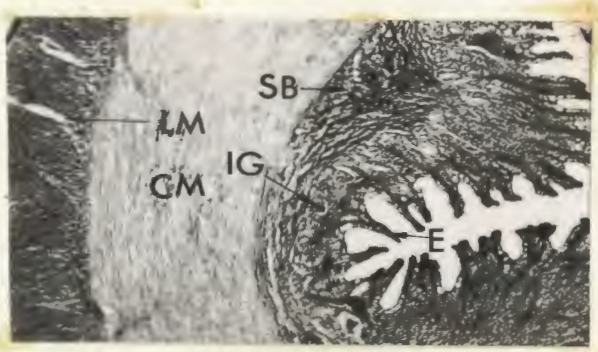
Fig. 34B. Small intestine: control frozen section for Sample 17, (X250).

Fig. 35A. Small intestine: PAS-positive compounds in the striated border (ST), surface epithelium (E), intestinal glands (IG), submucosa (SB), body wall of the adult nematode (NA). (Sample 11, paraffin section, PAS technique, X70).

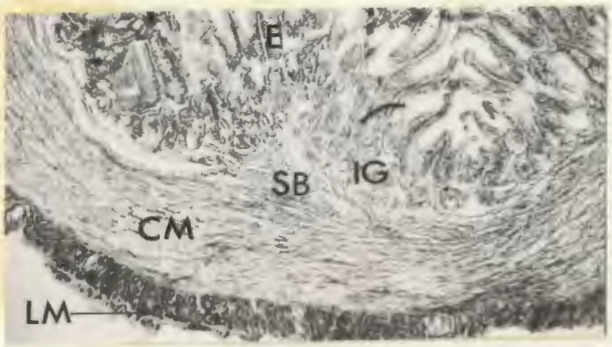
Fig. 35B. Small intestine: control paraffin section for Sample 11, (X70).

Fig. 36A. Small intestine: PAS-positive compounds in the striated border (ST) and goblet cells (G) of the surface epithelium. (Sample 11, paraffin section, PAS technique, X250).

Fig. 36B. Small intestine: control paraffin section for Sample 11, (X250).

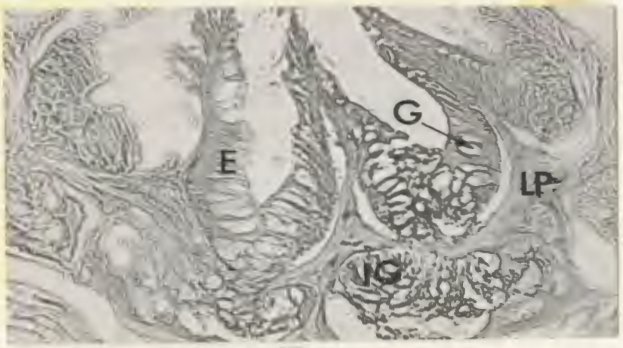


33 A



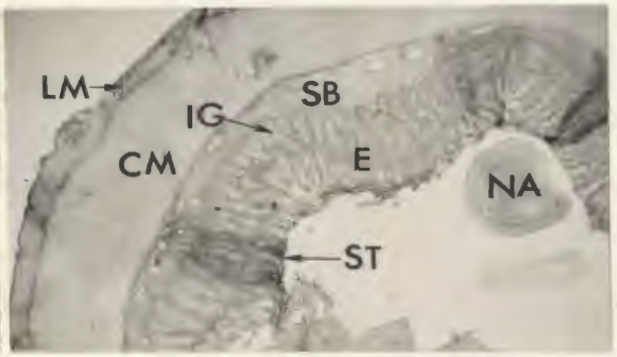
33 B

34 A

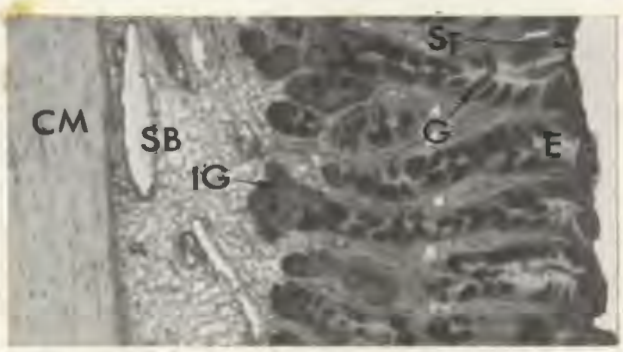


34 B

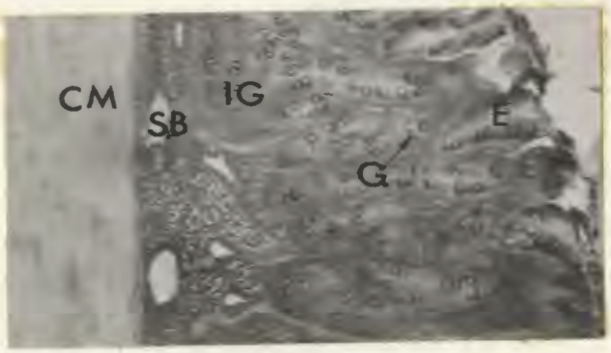
35 A



35 B



36 A



36 B

precipitate which is also seen in the control sections (Figures 33A-B and 43A-B).

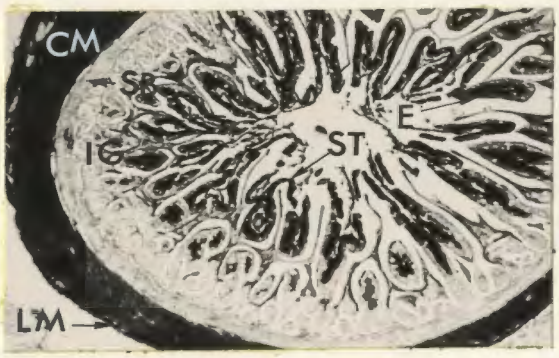
PAS Reactive Compounds. The goblet cells of the surface epithelium and intestinal glands show intense staining with the PAS (Figures 35A-B, 36A-B, 38A-B, 45A-B, 46A-B and Table 3), and some purple granules are scattered within the epithelial cells (Figure 46A). The striated border of the epithelial cells is PAS-positive, and there is an accumulation of mucous secretion at the free end of the surface epithelium which reacts intensely (Figures 35A-B, 45A-B and 46A-B). The basement membrane is also PAS-positive (Figures 38A and 46A). The number of goblet cells in the surface epithelium and intestinal glands of the caeca appears to be smaller than in the small intestine and rectum. The lamina propria and submucosa are not stained. The muscularis externa shows a similar pattern as seen in the stomach: the edges of muscle fibers have a border of low PAS-positive intensity.

Fig. 37A. Caeca: alkaline phosphatase in the striated border (ST), surface epithelium (E), intestinal glands (IG), submucosa (SB), inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 17, paraffin section, Gomori's calcium-cobalt method, X60).

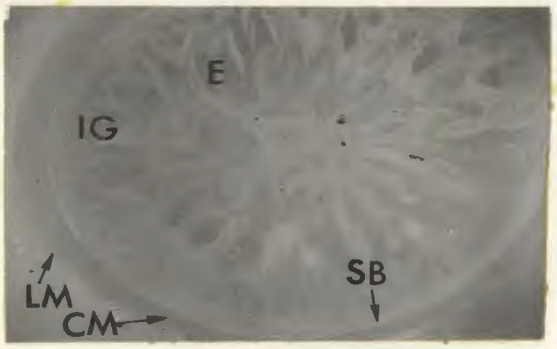
Fig. 37B. Caeca: control paraffin section for Sample 17, (X60).

Fig. 38A. Caeca: PAS-positive compounds in the striated border (ST), basement membrane (BM), goblet cells (G) of the surface epithelium and intestinal glands. (Sample 11, paraffin section, PAS technique, X200).

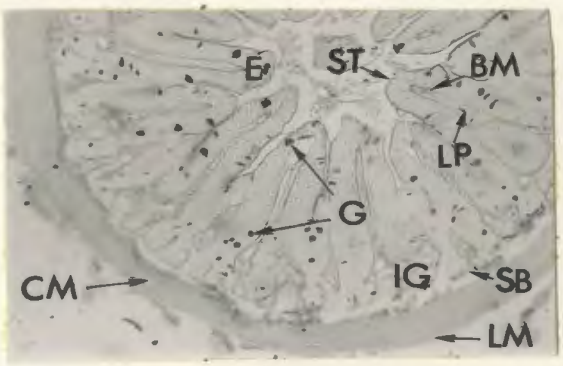
Fig. 38B. Caeca: control paraffin section for Sample 11, (X200).



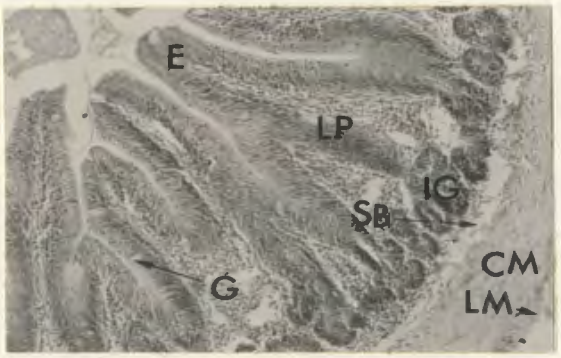
37 A



37 B



38 A



38 B

Fig. 39A. Rectum: alkaline phosphatase in the surface epithelium (E), intestinal glands (IG), submucosa (SB), inner circular muscle layer (CM), outer longitudinal muscle layer (LM). (Sample 18, paraffin section, Gomori's calcium-cobalt method, X50).

Fig. 39B. Rectum: control paraffin section for Sample 18, (X50).

Fig. 40A. Rectum: alkaline phosphatase in the striated border (ST), intracellular distribution (IA) in the proximal region of the surface epithelium (E) and intestinal glands (IG); goblet cells (G). (Sample 18, paraffin section, Gomori's calcium-cobalt method, X250).

Fig. 40B. Rectum: control paraffin section for Sample 18, (X250).

Fig. 41A. Rectum: acid phosphatase in the surface epithelium (E), intestinal glands (IG). (Sample 18, frozen section, Gomori's lead nitrate method, X50).

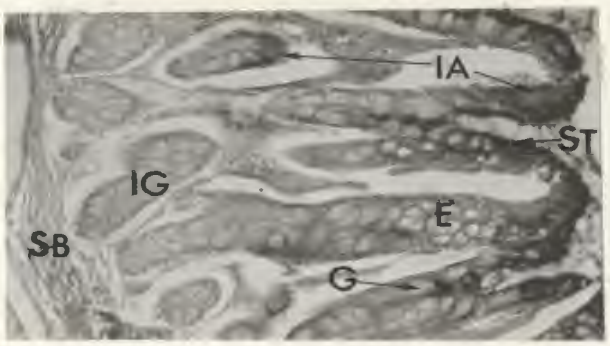
Fig. 41B. Rectum: control frozen section for Sample 18, (X50).

Fig. 42A. Rectum: acid phosphatase in the surface epithelium (E), intestinal glands (IG). (Sample 18, frozen section, Gomori's lead nitrate method, X250).

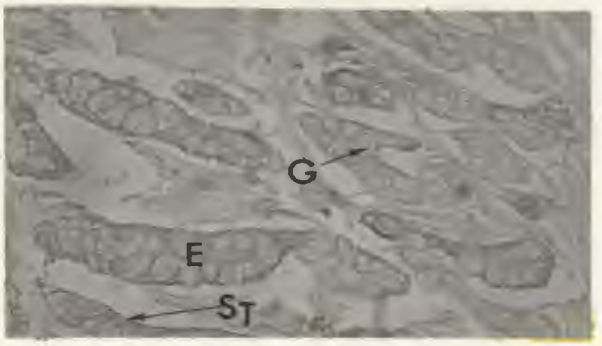
Fig. 42B. Rectum: control frozen section for Sample 18, (X250).

39 A

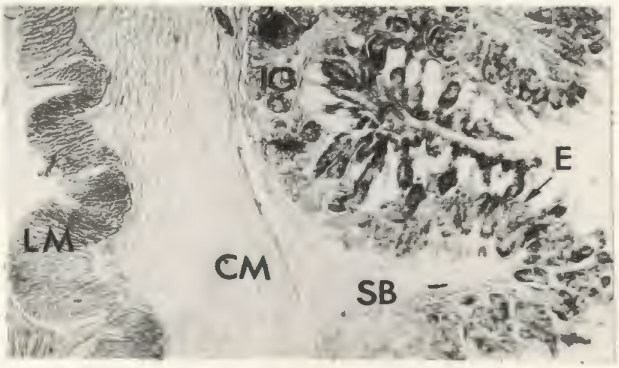
39 B



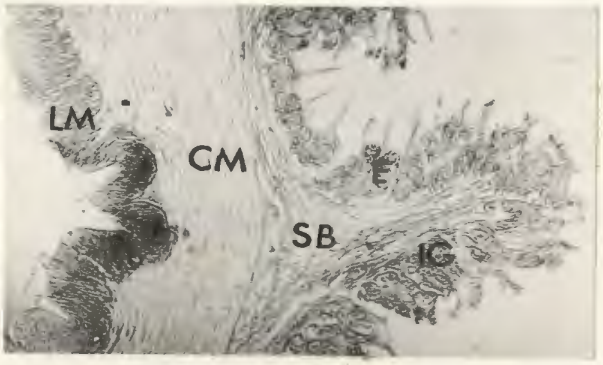
40 A



40 B

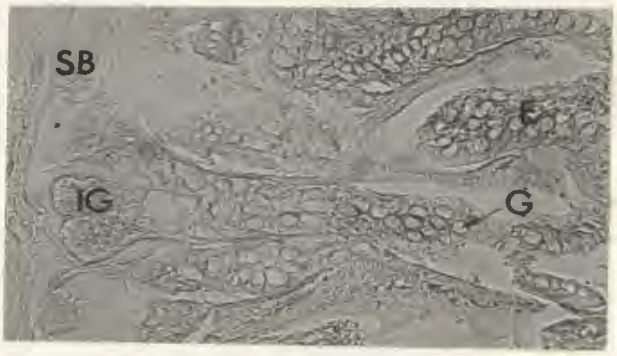


41 A



41 B

42 A



42 B

Fig. 43A. Rectum: esterase in the surface epithelium (E), intestinal glands (IG), submucosa (SB); inner circular muscle layer (CM), outer longitudinal muscle layer (LM), pancreas (P). (Sample 18, frozen section, α-naphthyl acetate method, X50).

Fig. 43B. Rectum: control frozen section for Sample 18, (X50).

Fig. 44A. Rectum: esterase in the surface epithelium (E), intestinal glands (IG), submucosa (SB). (Sample 18, frozen section, α-naphthyl acetate method, X250).

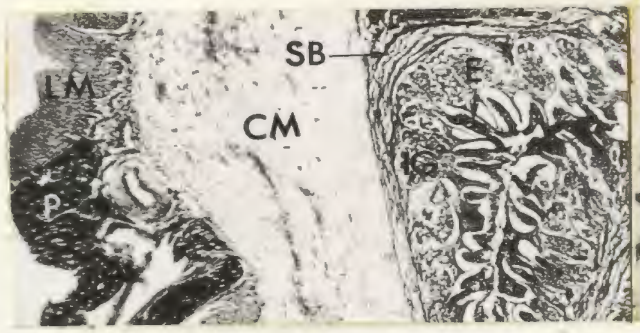
Fig. 44B. Rectum: control frozen section for Sample 18, X250).

Fig. 45A. Rectum: PAS-positive compounds in the striated border (ST), surface epithelium (E), intestinal glands (IG). (Sample 11, paraffin section, PAS technique, X60).

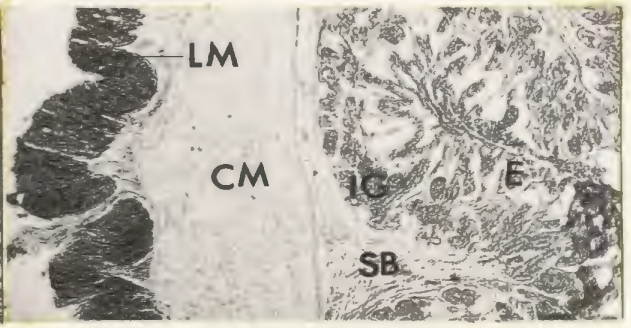
Fig. 45B. Rectum: control paraffin section for Sample 11, (X60).

Fig. 46A. Rectum: PAS-positive compounds in the striated border (ST), basement membrane (BM), goblet cells (G) of the surface epithelium and intestinal glands. (Sample 11, paraffin section, PAS technique, X250).

Fig. 46B. Rectum: control paraffin section for Sample 11, (X250).

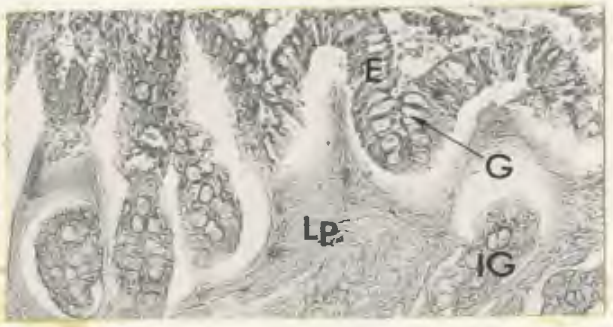


43 A

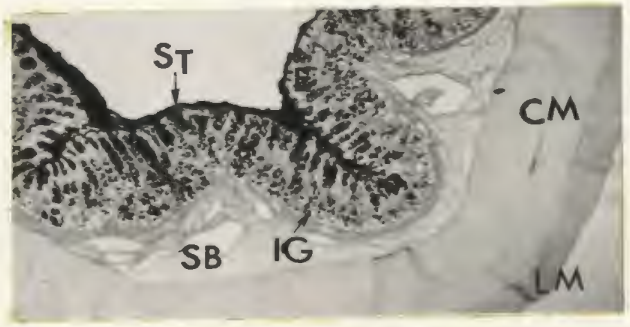


43 B

44 A

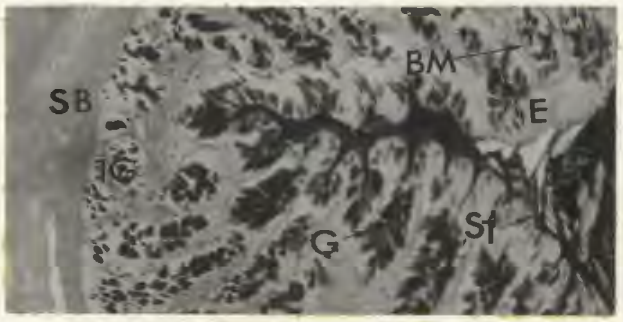


44 B

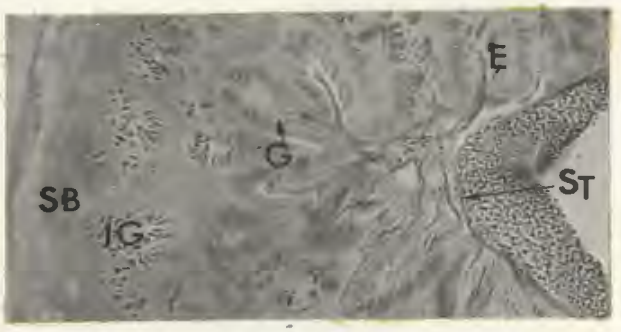


45 A

45 B



46 A



46 B

Liver. The distribution of hydrolytic enzymes and PAS-positive compounds is summarized in Table 4.

Alkaline Phosphatase. No enzyme activity could be detected within the hepatic cells. Only the wall of the bile duct exhibits an intense reaction (Figure 47B), the endothelium of arteries also shows a moderate activity. It was not certain whether the enzyme occurred in the tunica media of the arteries (see Table 4). Detectable activity of alkaline phosphatase was found within the walls of the central veins and sinusoids (Figure 47A). In the control sections no precipitate was present in the corresponding sites.

Acid Phosphatase. Moderate enzyme activity is noted within the hepatic cells of the liver, with the black precipitate randomly distributed throughout the cells (Figures 48A-B and Table 4). The columnar cells, which form the epithelial lining of the bile duct, exhibit an intense enzyme reaction (Figure 48A). The walls of blood vessels showed no activity. The control sections were negative (Figure 48B).

Esterase. In the liver esterase is confined to the columnar epithelium of the bile duct and the tunica media of arteries (Figure 49A-B and Table 4). The endothelium of arteries and the wall of the bile duct show a slight activity. The control sections were negative (Figure 49B).

Table 4. The distribution of alkaline phosphatase, acid phosphatase, esterase and PAS reactive compounds in paraffin and frozen sections of the liver.

		Liver				
	Sample	Alk (F)	Alk (P)	Acid (F)	Es (F)	PAS (P)
Hepatic	17	-	-	++	-	+
cells	18	-	-	++	-	++
Bile duct	17	-	-	+++	++	+
epithelium	18	-	-	+++	++	+
Bile duct	17	+++	++	-	+	+
wall (capsule)	18	+++	+	-	+	+
Artery	17	++	++	-	+	-
endothelium	18	++	-	-	+	-
Artery	17	+	-	-	++	-
media	18	-	-	-	++	-
Central	17	+	+	-	-	+
vein	18	+	-	-	-	+
	17	+	+	-	-	+
Sinusoid	18	+	+	-	-	+

Significance of figures employed in this Table:

(F) = frozen section
(P) = paraffin section
\- = negative
\+ = slight
++ = moderate
+++ = intense

Alk = alkaline phosphatase
Acid = acid phosphatase
Es = esterase
PAS = PAS reactive compounds

Fig. 47A. Liver: alkaline phosphatase in the endothelial cells of the central vein (CV), walls of sinusoid (S). (Sample 17, frozen section, Gomori's calcium-cobalt method, X250).

Fig. 47B. Liver: alkaline phosphatase in the endothelial cells of artery (A), capsule (C) of bile duct (BD); columnar epithelial cells (E) of bile duct. (Sample 17, frozen section, Gomori's calcium-cobalt method, X250).

Fig. 48A. Liver: acid phosphatase in the columnar epithelial cells (E) of bile duct and in the hepatic cells (HC). (Sample 18, frozen section, Gomori's lead nitrate method, X350).

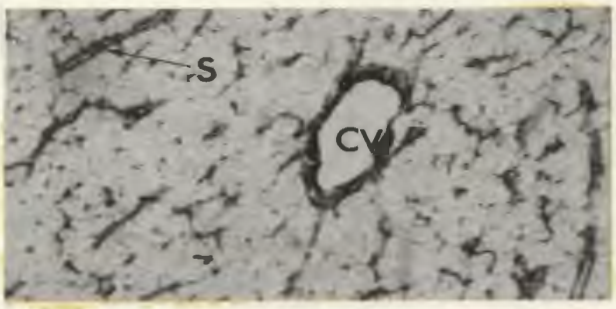
Fig. 48B. Liver: control frozen section for Sample 18, (X350).

Fig. 49A. Liver: esterase in the wall of artery (A) and the epithelial cells (E) of bile duct. (Sample 17, frozen section, α-naphthyl acetate method, X250).

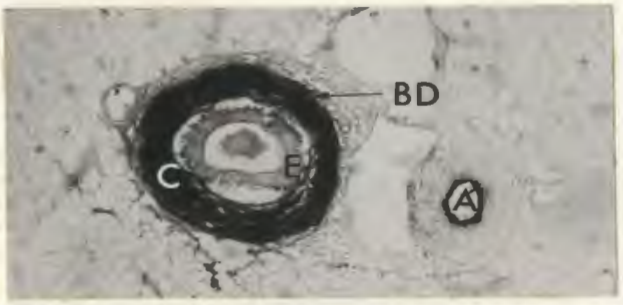
Fig. 49B. Liver: control frozen section for Sample 17, (X250).

Fig. 50A. Liver: PAS-positive compounds within the hepatic cells (HC), walls of the central vein (CV) and sinusoid (S). (Sample 7, paraffin section, PAS technique, X250).

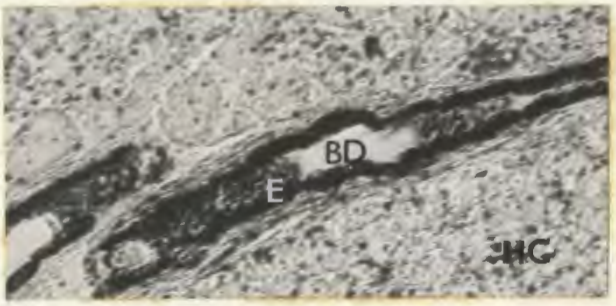
Fig. 50B. Liver: control paraffin section for Sample 7, (X250).



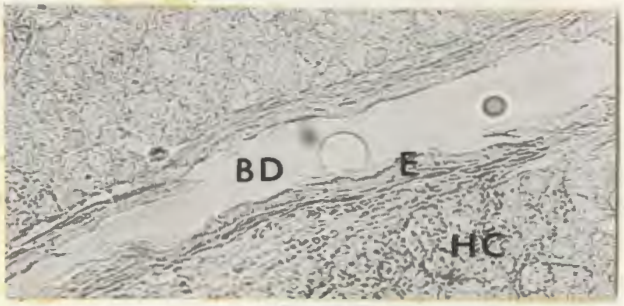
47 A



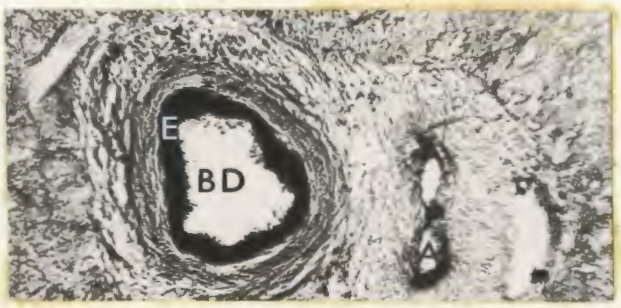
47 B



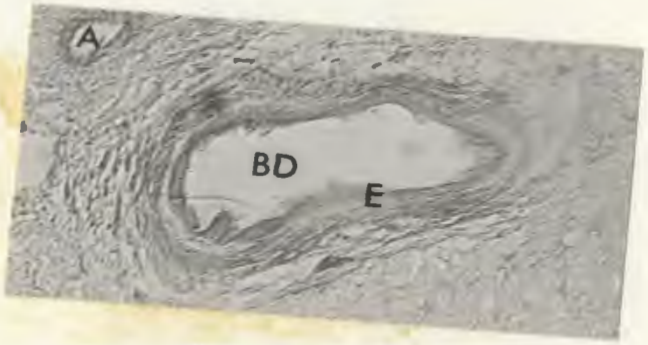
48 A



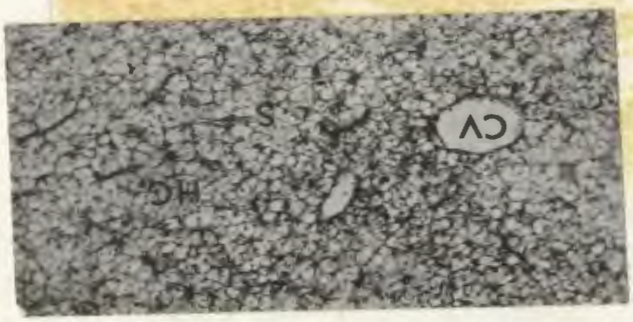
48B

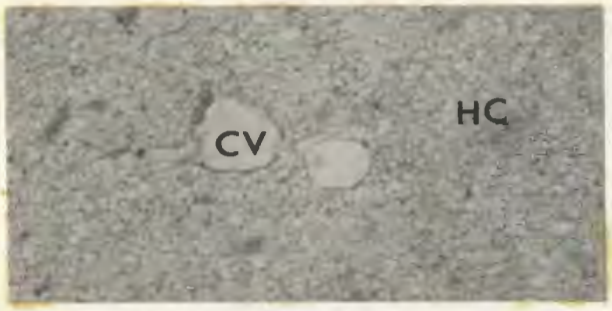


49 A



49 B





50 B

PAS Reactive Compounds. Numerous small PAS-positive particles are concentrated near the periphery of the hepatic cells, while the center of these cells, contains fewer granules (Figures 50A-B). Whether this pattern represents a fixation artefact could not be determined. Similar granules are also noted within the endothelium of the central veins and the wall of sinusoids (Table 4). The basement membrane of the bile duct shows a prominent PAS-positive reaction, while weaker staining is noted in the wall of the bile duct (Figure 15). Control sections were negative (Figure 50B).

DISCUSSION

In this thesis, alkaline phosphatase, acid phosphatase, esterase and PAS-positive substances are histochemically located in the gastrointestinal tract of cod. Differences in the intensity and distribution of these elements are shown for the various regions of this tract.

Alkaline Phosphatase.

Gomori's calcium-cobalt method is employed for the demonstration of alkaline phosphatase in this thesis. This method does not distinguish a particular phosphatase or a group of phosphatases (Newman et al. 1950); yet a significant difference in the distribution of alkaline phosphatase is found within the surface epithelium of the stomach and intestines. The surface epithelium of the stomach is negative for this enzyme while in the small intestine, rectum and caeca a considerable degree of enzyme activity is observed in the striated border and the proximal part of these cells.

Although the significance of these findings remains unexplained in this thesis, a correlation may exist in glucose absorption and the presence of phosphatase in the intestine. It is known that glucose is more effectively absorbed in the small intestine of the rat and rabbit if phosphorylated (Magee and Reid 1931, Lundgaard 1933 a, b), and that in the rat glucose absorption is about the same in the upper and middle portions and less in the lower portion of the small intestine (Chang, Zang and Li 1960). However, whether or not this

enzyme is associated with dephosphorylation of carbohydrates or other food is not resolved in literature. Deane and Dempsey (1945) showed conspicuous granules containing alkaline phosphatase in the Golgi region of epithelial cells lining the villus of the intestine in a number of species, and suggested that the enzyme is a secretion rather than a substance engaged in intracellular metabolism. Bourne and MacKinnon (1943) demonstrated glucose absorption from the rectum in guinea pigs. In cod, alkaline phosphatase is located in the striated border and the proximal portion of the epithelial cells of the caeca and rectum, and may be an indication that glucose is also absorbed in these regions.

The accumulation of alkaline phosphatase in the lamina propria of all regions of the tract, particularly in the stomach is more likely to be concerned with secretion rather than absorption of material. It was suggested by Danielli (1945) alkaline phosphatase found in connective tissue during regeneration was connected with the formation of fibrous protein, but so far nothing definite is known about the specific function of the enzyme within the lamina propria.

Alkaline phosphatase is evident in the endothelial cells of blood vessels of the submucosa throughout the tract. This was also reported by Kabat and Furth (1941), Bourne and MacKinnon (1943) for human, mouse and guinea pig. The significance of its presence in the endothelium and whether

phosphatase is secreted into the blood or absorbed by the endothelium is unknown. Alkaline phosphatase is present in the intestinal glands of cod. It is interesting to note that the Brunner's glands in the small intestine of the guinea pig do not contain this enzyme (Bourne and MacKinnon 1943).

Special attention is focused on the presence of alkaline phosphatase in the muscularis mucosae of the stomach and the muscularis externa of the whole gastrointestinal tract. It is the first time that this enzyme has been conclusively demonstrated in muscular tissue. In other vertebrates these regions were always reported to be negative (Bourne and MacKinnon 1943, Pearse 1961). Gomori (1941) observed a scarce precipitate in both smooth and striated muscle, but Pearse (1961) considered that this was due to the non-specific absorption of cobalt used in Gomori's test. The reaction for alkaline phosphatase in the muscle layers of cod is so intense and consistent throughout the tract that the argument of Pearse would not be admissible. It is doubtful that diffusion of the enzyme can account for its occurrence in the muscle layers since no sign of enzyme activity could be seen in the adjacent submucosa. The presence of the enzyme in the muscularis mucosae of the stomach and the muscularis externa is finally confirmed by the controls.

In liver, alkaline phosphatase is located in the connective tissue capsule of the bile duct, the walls of sinusoid and in

the endothelium of the central vein and arteries. The findings of phosphatase activity in hepatic tissue of other vertebrates reported in the literature are variable; no reaction was noted in the liver of guinea pig (Bourne and MacKinnon 1943), whereas in adult human, chicken and mouse only the endothelium of sinusoids and blood vessels gives a conspicuous phosphatase reaction (Kabat and Furth 1941). It is interesting to note that the endothelium in blood vessels of this organ as well in the arteries of the gastrointestinal submucosa is positive for alkaline phosphatase.

Acid Phosphatase

The physiological significance of acid phosphatase is difficult to evaluate, since little is known about its function. The Gomori's lead nitrate method employed in this investigation is a non-specific test for acid phosphatase. A number of different acid phosphatases were reported with the use of different substrates or inhibitors (Gomori 1956, Pearse 1961). In animal tissue acid phosphatases are known to be widely distributed: in prostate, spleen, liver and gastrointestinal mucosa (Wolf, Kabat and Newman 1943, Shnitka 1960, Barka 1964).

The activity of acid phosphatase in the gastrointestinal tract of the cod is demonstrated only in the surface epithelium and intestinal glands. According to Gomori (1941) acid phosphatase usually shows a diffuse distribution within cells, and is not organized in discrete granules. In cod tissues

acid phosphatase is in granular form in the cytoplasm, and the granules are concentrated at the distal portion of the surface epithelial cells. Shnitka (1960) and Barka (1963) reported such accumulations in the distal cytoplasm of the intestinal epithelium in dog, cat, rat and mouse, and Barka (1963) noted with the use of an electron microscope, that in the epithelium of the mouse small intestine the enzyme containing granules were always located in the cytoplasm above the nucleus. Gomori (1941) observed the absence of acid phosphatase from the gastric epithelium and glands in cat, dog and rat, but Wolf, Kabat and Newman (1943) reported that these locations gave positive reaction in man, guicea pig, rabbit and mouse.

Nuclear localization of acid phosphatase in the surface epithelium of cod is insignificant, but other workers have reported a positive nuclear reaction in the surface epithelium of the gastrointestinal tract in man, guinea pig, rabbit and mouse (Wolf, Kabat and Newman 1943, Gomori 1956). Gomori (1956) demonstrated that the nuclei of the surface epithelium were invariably stained by the lead nitrate method, but not by the post-coupling method, and suggested that this may be due either to a different enzyme or to diffusion artefact.

The functional implication of acid phosphatase in the cod tissue is difficult to evaluate. Barka (1963) has shown in mice that during fat absorption the concentration of this enzyme within the surface epithelium was much reduced and

that the enzyme containing granules grew smaller. In both the central and peripheral nervous systems, individual nerve cells in man, guinea pig, rabbit and mouse is one of the most consistent sites of the enzyme activity (Wolf, Kabat and Newman 1943), which may be related to the transmission of nervous impulses. However, these correlations are entirely speculatory and whether they may be applied to the occurrence of acid phosphatase in the cod gastrointestinal tract is not known.

In cod liver coarse granules of acid phosphatase are randomly distributed in the cytoplasm of hepatic cells. Similar distribution was reported by others for various vertebrates (Wolf, Kabat and Newman 1943, Gomori 1956). Gomori (1956) reported that the lining of the bile ducts was negative in most of the vertebrates (man and rabbit) he investigated, but Wolf, Kabat and Newman (1943) found that the epithelium of the bile duct in man, guinea pig, rabbit and mouse contained the enzyme. In cod, acid phosphatase is abundant in the epithelium of the bile duct.

The significance of acid phosphatase in the liver is obscure. Norberg, cited by Walker (1954), observed that liver regeneration in the rat was accompanied by a great increase of acid phosphatase, suggesting a relationship between this enzyme and protein synthesis. No hypothesis is offered to explain the occurrence of this enzyme in the liver and bile duct of cod.

Esterase

The occurrence of the non-specific esterase in tissues of the rat, rabbit, guinea pig, dog, cat, mouse and man was reported by Nachlas and Seligman (1949 a and b) and Chessick (1953). Although several classes of esterases are recognized histochemically (Pearse 1961), no specific one is favoured in this thesis where the non-specific reaction was employed. Basically a similar pattern of esterase is noted for cod and mammals (Nachlas and Seligman 1949 a, b, Chessick 1953) in the surface epithelium, gastric and intestinal glands of the gastrointestinal tract.

Chessick (1953) reported that there was no activity in the lamina propria of the villi in most mammalian species except in man. In the cod the connective tissues of the lamina propria and submucosa are slightly positive. The occurrence of esterase in the wall of arteries of the submucosa was demonstrated in all species (Nachlas and Seligman 1949 a, b, Chessick 1953) as well as in the cod.

In the rabbit, cat and man, no esterase activity could be demonstrated in the muscularis mucosae and muscularis externa of the gastrointestinal tract, but in the mouse a fairly intense diffuse reaction was noted in the cardiac and fundic stomach (Chessick 1953). In the cod the muscularis mucosae contained a considerable amount of esterase throughout the regions of the stomach. The muscularis externa is positive only in the fundic region. This pattern of distribution in

the tract of the cod resembles that of the mouse quite closely.

In the hepatic tissue of the cod the distribution of esterase is concentrated within the epithelium and the connective tissue sheath of the bile duct, and in the walls of arteries. In most mammals corresponding regions were reported to be negative (Chessick 1953). The validity of the localization of esterase in the cod tissues is supported by the control sections.

The significance of esterase within animal tissues is not yet understood, no conclusion can be drawn concerning the functions of this enzyme performed in the body (Chessick 1953).

PAS-Positive Compounds

In the cod as well as in the rat, the gastric surface epithelium reveals an intense PAS-positive reaction which in the intestine only the goblet cells are prominently stained. The striated border and basement membrane of the epithelium are strongly stained throughout the cod tract similarly to those reported in the rat and cattle (Leblond 1950, Eidinger and Ghosh 1956). The occurrence of the PAS-positive compounds within the striated border coincides with the site of alkaline phosphatase activity in the intestine, and this suggests a correlation between alkaline phosphatase and the absorption of sugar. However, the routine treatment of sections removes sugars from the tissue, and the diastase

test used here failed to demonstrate glycogen in the cod gastrointestinal tract. It has also been shown that a variety of substances, other than sugars or glycogen can be oxidized to aldehydes and react with the Schiff reagent, of which the most important group of compounds in tissues are the carbohydrate-protein complexes (Hotchkiss 1948, Leblond 1950, Eidinger and Ghosh 1956). These include the mucopolysaccharides which are probably responsible for the positive PAS reaction in the epithelium of the digestive tract, and are thought to participate in the elaboration and discharge of secretory material (Leblond 1950). The basement membrane in vertebrates is thought to be composed of reticular fibers (Ham 1965), which give a positive PAS reaction. PAS-positive granules are randomly distributed within the epithelial cells of the digestive tract; these were also demonstrated in the rat (Leblond 1950), but their significance was not explained.

The gastric lamina propria and submucosa give a weak and diffuse PAS-positive reaction, similar observation was made by Eidinger and Ghosh (1956) for the gastric submucosa of rat and cattle, however, Ham (1965) states that collagenous fibers are PAS-negative. It would seem that the staining observed in cod is due to some other material.

The cells of gastric glands give a weak and diffuse PAS reaction, and from the staining pattern only one type of cell is apparent. The variety in gastric gland cells described

in the stomachs of mammals (Leblond 1950, Ham 1965) is not apparent in the cod, Bishop and Odense (1966 a) also reported that only one type of cell is present in the gastric glands of cod. The glands in the intestine contain numerous goblet cells, but only a few in the caeca.

The gastric muscularis mucosae and the muscularis externa of the entire tract are PAS-positive. The muscle is weakly positive, as was also reported by Eidinger and Ghosh (1956) in the rat and cattle.

In the cod liver the basement membrane in the bile duct is strongly PAS-positive as is the basement membrane of the mucosa in the entire tract. The parenchymal cells show scattered granules of PAS-positive material, shown to be other than glycogen, unlike the granules found in the hepatic cells of mammals (Hotchkiss 1948, Leblond 1950, Eidinger and Ghosh 1956). This material was frequently concentrated near the walls of sinusoids and central veins in the cod.

SUMMARY

(1) The microanatomical organization of the cod gastrointestinal tract corresponds to that of other vertebrates. The intestinal wall is composed of a mucosa (surface epithelium, lamina propria, gastric or intestinal glands and muscularis mucosae) a submucosa and a muscularis externa (inner circular and outer longitudinal muscle layers). The gastric and intestinal surface epithelial cells are morphologically similar, but the epithelial lining of the intestine and caeca includes goblet cells. The muscularis mucosae is limited to the stomach. In the gastric glands only one type of cell is demonstrated while in the intestinal glands in addition to glandular cells, goblet cells are observed.

(2) Alkaline phosphatase within the gastrointestinal tract is confined to the mucosa and muscularis externa. In the stomach it is concentrated in the lamina propria, the muscularis mucosae and both the inner circular and outer longitudinal muscle layers, but is absent from the surface epithelium. In the intestine the enzyme is concentrated within the striated border of the surface epithelium and in the muscularis externa; smaller amount of alkaline phosphatase is present in the proximal region of the surface epithelium and of the intestinal glands.

(3) Acid phosphatase is demonstrated only in the distal portion of the surface epithelial cells in the form of discrete granules, and in the cells of the gastric and intestinal glands where the granules are randomly distributed within the cytoplasm.

(4) Esterase appears in several parts of the gastrointestinal tract. In the stomach the enzyme is concentrated in the distal cytoplasm of the surface epithelium, in the cells of gastric glands and in the muscularis mucosae, and a weak diffuse reaction is also noted within the lamina propria and submucosa, while the muscularis externa exhibits a positive reaction only in the fundic region. In the intestine, a similar pattern is observed in the epithelium, intestinal glands and submucosa, but all muscular layers are negative.

(5) The PAS reaction is most intense in the surface epithelium of the cod stomach and intestine, and in the inclusions of intestinal goblet cells. The striated border of the intestinal epithelial cells and the basement membrane of the gastrointestinal mucosa indicate a moderate PAS reaction. All the other regions are characterized by some PAS-positive material. Parasites, frequently noted within the gastrointestinal tract are well demonstrated by virtue of their high content of PAS material.

(6) The sites of alkaline phosphatase activity correspond to the presence of PAS-positive substances in the gastric lamina propria and in the various muscular regions of the tract. Correspondence is also noted in the surface and glandular epithelium of the intestine, but not of the stomach. Acid phosphatase and esterase, exhibit a different pattern from the above, and generally are confined to places where the other two do not occur, mainly in the distal portion of the surface epithelium and in the glands throughout the tract.

(7) In the cod liver, alkaline phosphatase is found in the connective tissue sheath of the bile duct and in the endothelium of all blood vessels. Acid phosphatase is present in the hepatic cells and concentrated in the columnar epithelium of the bile duct. Esterase is also noted in the hepatic cells, but concentrated in the epithelium of the bile duct and the media of arteries. PAS-positive compounds are distributed in the hepatic cells near the walls of sinusoids and central veins. The basement membrane of the bile duct shows a moderate PAS-positive reaction.

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APPENDIX 1

Fixatives

In histochemical work some type of fixation is usually necessary to produce accurate results (Burstone 1961), but fixation may cause partial or complete inactivation of the enzyme. In some instances fixatives may bring about morphological changes in the tissue but leave the reactive groups of the enzymes unaltered (Pearse 1961). If unfixed tissues are used in such studies diffusion of the enzyme may be severe (Chessick 1953).

In this investigation three fixatives were employed: Lison's fluid, cold acetone and Baker's formol calcium. For the study of the histological features tissues fixed in Lison's fluid and cold acetone. For the study of PAS reactive compounds the tissues were fixed in Lison's fluid. Cold acetone was recommended by Gomori (1946) for the preservation of hydrolytic enzymes to be followed by paraffin embedding. With this procedure only alkaline phosphatase could be demonstrated and no enzyme activity was noted for acid phosphatase and esterase. Holt (1959) has shown that no more than half of the initial acid phosphatase is retained in tissues after fixation, but to what extent paraffin embedding can further decrease the enzyme activity has not been determined; in this case apparently all was lost.

Lison's Fluid (Lillie 1954)

Picric acid, saturated in 95% alcohol	85 ml
Formalin, saturated	10 ml
Glacial acetic acid	5 ml

(1) Fix tissues for 24 hours in refrigerator.
(2) Wash in several changes of 70% alcohol.
(3) Dehydrate, clear, embed in paraffin.

Cold Acetone (Gomori 1946)

(1) Fix tissues in absolute acetone (2 or 3 changes), at 4° C for 24 hours.
(2) Transfer to absolute ethanol for ½ hour.
(3) To absolute ethanol/ether for ½ hour (2 changes).
(4) To 1% celloidin in ethanol/ether for 1 hour.
(5) To chloroform for ½ hour.
(6) Clear in 2 changes of benzene, ½ hour each.
(7) Infiltrate and embed in paraffin in vacuo, for 3 hours.

Baker's Formol Calcium (Lillie 1954)

Anhydrous $CaCl_2$	10 g
Concentrated formalin (40%)	100 ml
Distilled water	900 ml

(1) Fix tissues for 24 hours at 4° C.
(2) Section at -20 to -30° C at 15 μ.
(3) Incubate, wash in distilled water, mount with glycerol jelly.

Glycerol Jelly (Lillie 1954)

(1) Soak 40 g gelatin in 210 ml of distilled water.
(2) Add 250 ml glycerol and few crystal of thymol as a preservative.
(3) Heat gently and stir for 10-15 minutes.
(4) Store in refrigerator, melt just before use.

APPENDIX 2

Mallory's Triple Stain (Lillie 1954)

Staining Solutions

A. 0.5% aqueous solution of acid fuchsin

B. Aniline Blue-Orange G Staining solution

Aniline blue	0.5 g
Orange G	2.0 g
Phosphotungstic acid	1.0 g
Distilled water	100 ml

Method

(1) Bring sections from xylene through alcohols to water.
(2) Stain in 0.5% aqueous solution of acid fuchsin for 1 to 10 minutes.
(3) Without washing, pass the sections to the Aniline Blue-Orange G staining solution and allow them to stain for 20 to 60 minutes.
(4) Remove the slides from staining solution, drain off excess stain and differentiate very rapidly in 95% alcohol (2 to 3 changes).
(5) Dehydrate in absolute alcohol.
(6) Clear in xylene and mount in permount.

Results

Nuclei red; mucus, collagen fibrils and other hyaline substance deep blue; muscle and cytoplasm red to orange.

Fraenkel's Orcein Method for Elastin (Lillie 1954)

Staining Solutions

A. Orcein Stock Solution

Orcein	1.5 g
95% alcohol	120 ml
Distilled water	60 ml
Nitric acid	6 ml

B. Orth's Lithium Carmine Solution

Carmine	2.5 g
Saturated aqueous lithium carbonate	100 ml

Dissolve by boiling 15 minutes. Let cool and add 1 g thymol as a preservative.

Method

(1) Bring sections from xylene through alcohol to water.
(2) Stain nuclei with Orth's lithium carmine 2 to 5 minutes, differentiate in 1% HCl in 70% alcohol.
(3) Stain in Orcein solution for 24 hours (add stock solution about 1:1 to 3% 85% alcoholic HCl to give a dark brown colour).
(4) Stain 10 to 15 minutes in 0.25% indigocarmine in saturated aqueous picric acid solution.
(5) Rinse in 3.5% acetic acid.
(6) Dehydrate in alcohol, clear in xylene and mount in permount.

Results

Nuclei red; elastin dark brown; collagen blue green.

APPENDIX 3

Histochemical Methods

The advantages and disadvantages of using paraffin sections versus frozen sections for the histochemical localization of enzymes are summarized by Pearse (1961). The exposure of tissue to heat inevitable in paraffin embedding does inactivate enzyme activity to varying degrees. Danielli (1945) estimates that the loss of enzyme is as high as 75%. Stafford and Atkinson (1948) reported that acetone-fixed tissues following paraffin embedding suffered a reduction of acid phosphatase up to 95%, and Nachlas and Seligman (1949a) noted a decrease in esterase up to 60%. It seems that frozen sections are better material than paraffin sections for demonstration of hydrolytic enzymes, since they are not exposed to excessively high temperature.

In the course of this investigation it was found that frozen sections fixed in Baker's formol calcium were superior for the localization of the three enzymes studied. It seems that acid phosphatase and esterase were inactivated in the paraffin method, but whether the inactivation was due to fixation or paraffin embedding was not determined.

Alkaline Phosphatase

The range of optimum pH for alkaline phosphatase is pH 9.2-9.8 in vertebrates (Pearse 1961). A series of experiments was carried out to determine the optimum pH for cod tissues by exposing frozen sections from the same block to substrates range from pH 9.0-10, and was found that best result was obtained by a pH 9.6-9.7. This is higher than in mammalian tissues (Kabat and Furth 1941, Bourne and MacKinnon 1943, Deane and Dempsey 1945). In this procedure the pH of the substrate was maintained at pH 9.6-9.7 by an equal volume of a 3% sodium B-glycerophosphate and a 2% sodium diethyl barbiturate.

Gomori's Calcium-Cobalt Method (Gomori 1946)

Substrate

10 ml 3% sodium B-glycerophosphate
10 ml 2% sodium diethyl barbiturate
5 ml distilled water
20 ml 2% calcium chloride
1 ml 5% magnesium sulphate

(1) Paraffin sections were dewaxed in light petroleum and acetone, place in water and incubate at 37° C in substrate for 5½ hours. Frozen sections, cut in a cryostat at 15 µ were incubated at 37° C for 2½ hours.
(2) Washed in running water for 3-5 minutes.
(3) Sections were treated with 2% cobalt nitrate for 3-5 minutes.
(4) Rinsed in distilled water.
(5) Transferred to a 1% solution of yellow ammonium sulphide for 1-2 minutes.
(6) Paraffin sections were dehydrated in alcohol, clear in xylene and mounted in permount. Frozen sections were mounted in glycerol jelly.

Results

Sites of alkaline phosphatase activity are stained black .

Controls

Control sections were incubated in the same substrate without the sodium β-glycerophosphate.

Acid Phosphatase

The pH of the substrate is a critical factor in demonstrating the enzyme. In this study Walpole acetate buffer (Colowick and Kaplan 1955) was used to maintain the substrate at pH of 5.0.

The capriciousness of original technique used by Gomori (1941) is well known. Later Gomori (1950) improved the technique by raising the concentration of the substrate from 0.005 N to 0.014-0.05 N, and concluded that the ratio of buffer to substrate in the incubating mixture is the crucial factor, since high concentration of buffer increases the solubility of the reaction product. An artefact such as nuclear staining is impossible to avoid. However, Gomori (1950) stressed that the distribution pattern of acid phosphatase in histological sections is largely due to a specific enzymatic effect and that localization is reasonably accurate. Wolf, Kabat and Newman (1943) have shown that non-specific localized deposits of lead salts occur occasionally, and Barka and Anderson (1962) reported that a "random precipitate" type of reaction follows long incubation periods. It was noted here, that when the period of incubation exceeded 3½ hours, the localization was more diffuse. According to

Barka and Anderson (1962), this type of reaction is due to a weak acid phosphatase reaction, known to occur in most cells.

Lead Nitrate Method (Gomori 1950)

0.1 M acetate buffer pH 5	3 parts
5% lead nitrate	1 part
2% sodium β-glycerophosphate	3 parts
Distilled water	6 parts

This was filtered to remove precipitate of lead salt and diluted 1:3 with distilled water immediately before use.

(1) Frozen sections 15 μ thick were incubated at 37° C for 3-3½ hours.
(2) Rinse rapidly in distilled water.
(3) Treated with 2% acetic acid for 1 minute.
(4) Rinsed in distilled water.
(5) Treated with 1% ammonium sulphide for 1 minute.
(6) Washed in running water for 2-3 minutes.
(7) Mounted with glycerol jelly.

Results

Sites of acid phosphatase activity are indicated by black precipitates of lead sulphide.

Controls

Control sections were incubated in the same substrate without the sodium β-glycerophosphate.

Esterase

The first azo-dye method was introduced by Nachlas and Seligman (1949 a) for the demonstration of esterase, using β-naphthyl acetate as substrate. Gomori (1953) proved that the β-naphthyl acetate was unsuitable because it resulted in a readily diffusible azo-dye complex and substituted it with

α-naphthyl acetate. Since α-naphthyl acetate is insoluble in water, a small amount of acetone is added to dissolve it in the incubating medium. The substrate prepared freshly before use, was maintained at pH of 7.4 by 0.1 M phosphate buffer.

α-Naphthyl Acetate Method (Gomori 1953)

Substrate

α-naphthyl acetate in 0.25 ml acetone ------- 10 mg
0.1 M phosphate buffer, pH 7.4 ---------- 20 ml

Shaken until cloudiness disappears.

Fast B salt ---------- 50-100 mg

Mixed and filtered.

(1) Frozen sections, 15 μ in thickness were incubated for 25-30 minutes at room temperature.
(2) Washed in running water for 2-5 minutes.
(3) Mounted in glycerol jelly.

Result

Esterase activity is indicated by a brownish precipitate.

Controls

Control sections were incubated in the same substrate without the α-naphthyl acetate.

Periodic Acid-Schiff Technique (Hotchkiss 1948)

The PAS method is a well known histochemical test for the demonstration of polysaccharides, glycoproteins, glycolipids, unsaturated lipids and phospholipids (Pearse 1961). The major advantage of using periodic acid for the oxidation of the C-C bonds is that it does not oxidize the resulting aldehydes further, therefore the aldehydes can be demonstrated by Schiff reagent. In the digestive tract of cod the PAS reactive compounds are thought to be mainly mucopolysaccharides. This supposition is supported by control sections as well as by the study of Leblond (1950), who evaluated the PAS reactive compounds in the adult rat.

Reagents

A. Periodic Acid

4% periodic acid ---------- 10 ml
M/5 sodium acetate ---------- 5 ml
Distilled water ---------- 35 ml

Kept at 4° C and allowed to come to room temperature before use.

B. Schiff Reagent

Dissolve 1.0 g basic fuchsin in 200 ml of boiling water, filter, cool and add 2 g of potassium meta-bisulphite and 10 ml of 1 N HCl (8.3 ml of 12 N HCl in 100 ml of water). Let stand for 24 hours then add 5 g of activated charcoal (Norrit). Shake 1 minute and filter, and if filtrate is not colorless add a few drops of concentrated HCl. Keep in refrigerator until use.

C. Iodide Thiosulphate Solution

Potassium iodide ---------- 1.0 g
Sodium thiosulphate ---------- 1.0 g
Distilled water ---------- 20 ml
While stirring add 30 ml of 95% alcohol and 0.5 ml 2N HCl.

D. Sulphite Solution

2 ml 10% potassium metabisulphite
0.5 ml concentrated HCl
50 ml distilled water

E. Fast Green FCF

0.02% of Fast Green FCF solution in distilled water.

(1) Bring sections to water.
(2) To periodic acid at room temperature for 5 minutes.
(3) Rinse in distilled water.
(4) Transfer to iodide thiosulphate solution for 5 minutes.
(5) Rinse in distilled water.
(6) Stain in Schiff reagent for 15 minutes.
(7) Two changes of sulphite solution.
(8) Wash under tap for 3-5 minutes.
(9) Counter-stain in 0.02% Fast Green for 30 seconds.
(10) Wash in 95% alcohol for 30 seconds to differentiate Fast Green.
(11) Dehydrate, clear in xylene and mount in permount.

For Nuclear Stain

(8a) Rinse in distilled water.
(8b) Stain in Harris-Hematoxylin for 1 minute.
(8c) Wash under tap for 5 minutes.

Results

Glycogen, mucin, hyaluronic acid, reticulin, colloid droplets, basement membranes, amyloid infiltration and other elements may show a positive reaction -- rose to purplish red.

Controls

(A) Acetylation

Control sections were acetylated with a mixture of pyridine and acetic anhydride at a ratio of 24:16 for 24 hours at room temperature prior to the PAS procedure.

Lillie (1954) reports that acetylation for 24 hours blocks a subsequent periodic acid reaction, since NH_2

and OH groups become esterified. This result was obtained in all sections from the cod gastro-intestinal tract and gives a basis for the confirmation of the validity of the Hotchkiss test.

(B) Diastase Digestion

Sections were also subjected to a 1% diastase solution at room temperature for 5-6 hours before staining. The enzyme removes any glycogen that might be present.