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### A STUDY OF THE PARASITES OF LIPARIS ATLANTICUS (JORDAN AND EVERMANN) WITH EMPHASIS ON THE HISTOCHEMICAL MORPHOLOGY OF SPATHEBOTHRIUM SIMPLEX, LINTON, 1922 (CESTODA)

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University of New Hampshire, Ph.D., 1970  
Zoology

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JORDAN AND EVERMANN WITH EMPHASIS ON THE HISTO-  
CHEMICAL MORPHOLOGY OF SPATHEBOTHRIUM SIMPLEX  
LINTON, 1922 (CESTODA)

BY

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A.B., Colgate University, 1963

M.S., Adelphi University, 1966

A THESIS

Submitted to the University of New Hampshire  
In Partial Fulfillment of  
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Doctor of Philosophy

Graduate School  
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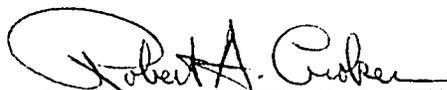
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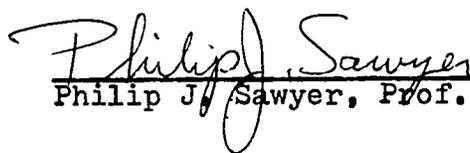
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Arthur E. Teeri, Prof. of Biochemistry

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## SECTION I

### INTRODUCTION

Adult members of the cestode order Spathebothridea are mainly parasitic in ancient, and probably primitive, groups of fishes (Wardle and McLeod, 1952). Spathebothrium simplex belongs to this order and was first described by Linton (1922) from Liparis liparis at Woods Hole. From the moment of its description, the taxonomic status of this cestode has been a moot point. In his original description (1922), Linton recognized that the cirrus, uterine, and vaginal apertures alternated irregularly with respect to the dorsal and ventral surfaces of the worm, as did the reproductive organs. For this reason, Linton did not include this worm in the order Pseudophyllidea (cestode parasites of fish and fish eating birds and mammals), members of which are characterized by having the uterine pore on the ventral surface. Linton made no attempt to place S. simplex in any definite scheme. However, Poche (1926) and Fuhrmann (1931) felt that S. simplex should be included in the family Cyathocephalidae Nybelin, 1922, which was at that time in the Pseudophyllidea. Yamaguti (1934) found S. simplex in Crystallias matshushima from Toyama Bay, Japan, and agreed with Poche and Fuhrmann in including it in the Cyathocephalidae, but created a new subfamily, Spathebothriinae, to contain it. Hart and Guberlet (1936) found S. simplex in

Liparis fuscensis from Puget Sound on the west coast of North America. These workers believed that the lack of a scolex in the adult worm was sufficient reason to establish a new superfamily, Spathebothrioidea, to contain this tapeworm. Hyman (1951) felt that Hart and Guberlet's creation of this superfamily in order to contain S. simplex was on inadequate grounds, and felt that the cestode should be retained in the family Cyathocephalidae of the Pseudophyllidea. In 1952 Wardle and McLeod removed both the Cyathocephalidae and the Diplocotylidae from the Pseudophyllidea, and established a new family, Spathebothriidae, and a new order, Spathebothridea, to contain these three families. Spathebothrium simplex Linton, 1922, is the only species of the only genus in the Spathebothriidae. The basic morphology and taxonomic position of S. simplex was described by Wardle and McLeod (1952) in their description of the family Spathebothriidae:

Of the order Spathebothridea. Small forms with a bluntly tapering anterior end and a sharply tapering posterior end, and with the anterior end completely undifferentiated. Longitudinal parenchymal muscles lacking. Genital apertures alternating irregularly from one flat surface to the other. Vaginal aperture behind the cirrus aperture. Uterine aperture at the same level as the vaginal aperture but lateral to it. No utero-vaginal depression. Reproductive organs serially repeated up to thirty-six sets. Testes arranged in two lateral bands extending through the whole body. Ovary rosettiiform, with a central opening. Uterus a much-convoluted tube. Eggs spindle-shaped, operculated, 40 by 20 microns. Life cycle unknown. Type genus and only known genus, Spathebothrium Linton, 1922, with one species, simplex Linton, 1922, from the teleostean fish Liparis liparis in North American Atlantic water.

The objectives of this investigation were primarily to study some aspects of the histochemical morphology of Spathebothrium simplex Linton, 1922, which is parasitic in

the local seasnail, Liparis atlanticus (Jordan and Evermann), and secondarily to make a survey of the parasites of L. atlanticus with observations on histopathology, host-parasite, and parasite-parasite interactions. No previous histochemical studies have been performed on spathebothriid cestodes, and knowledge of the parasites of tide pool fishes of the New England coast is very incomplete.

## SECTION II

PREVIOUS HISTOLOGICAL AND CYTOLOGICAL OBSERVATIONS ON  
S. SIMPLEX

Linton (1922) noted that the cuticle (now recognized to be a tegument) consisted of two layers, an outer made up of short rod-like structures and an inner structureless layer. The outer layer constituted about two-thirds the thickness of the cuticle. The cuticle averaged ten microns in thickness. Hart and Guberlet (1936) found the cuticle to measure five microns in thickness. The remainder of the worm can be divided into cortical and medullary regions. The cortical or outer region is cellular, measures about forty microns in thickness, and has the vitellaria and external excretory (osmoregulatory) canal impressed on its medial surface (Hart and Guberlet, 1936). This region is called the subtegument in current terminology. The medullary region contains all the remaining organs, and spaces not filled by organs are filled with loose parenchyma.

Linton (1922) and Yamaguti (1934) noted the lack of a definite layer of inner longitudinal muscle fibers in S. simplex. These muscle fibers are a characteristic feature of the majority of other cestodes and divide the parenchyma into cortical and medullary regions (Wardle and McLeod, 1952). Linton (1922) saw no indication of a circular muscle layer, but Yamaguti (1934) observed a small number of dorso-ventral and transverse fibers. The former extend from cortex to

cortex or from cortex to internal organs, while the latter are found only in anterior and posterior regions where there are no organs.

The spherical testes lie in lateral fields on each side of the strobila and are located medially with respect to the vitellaria (Yamaguti, 1934). A testis is as long as 110 microns in its long axis; they tend to decrease in size near the posterior end of the worm. Testes are first found anterior to the first set of reproductive organs, and do not extend posterior to the uterine duct of the most posterior set of reproductive organs (Hart and Guberlet, 1936).

Combined vasa efferentia form a short, thin-walled, convoluted vas deferens of large diameter at the level of the cirrus pouch. The vas deferens constricts abruptly upon entering the cirrus pouch and continues as an internal seminal vesicle (Hart and Guberlet, 1936). The cirrus pouch is short but with relatively thick muscular walls (Linton, 1922). Yamaguti (1934) noted that the cirrus pouch was surrounded by radially arranged prostatic cells and that the cytoplasm of these cells occasionally contained strongly eosinophilic granules.

Vitellaria are ovoid, 50 to 60 microns in longest diameter, and are usually located lateral to the testes. Occasionally they are located medial to the testes (Hart and Guberlet, 1936). In mature worms the vitellaria extend from slightly posterior to the cirrus of the first set of reproductive organs to posterior to the last testes. Hart and Guberlet (1936) described the vitelline cells as generally

spherical in shape and about 17 microns in diameter. The cytoplasm of mature vitelline cells has a granular appearance. They further observed a small non-cellular region in the center of each vitelline follicle and noted that the common vitelline duct first appeared on the surficial side of the ovary anterior to the center of the ovary.

The ovary is a rosette-shaped organ with an opening through the center, and may lie on the same side of the worm as the cirrus aperture or on the opposite side (Hart and Guberlet, 1936). It is situated nearer the cirrus of the following reproductive unit than to the cirrus of its own unit (Yamaguti, 1934). The ovarian lobes extend laterally to the testes and occasionally extend lateral to the testes. Ova average 15 microns in diameter and their nuclei appear granular due to scattered chromatin (Hart and Guberlet, 1936).

Distally the vagina is a small duct with a heavy-walled sphincter at the vaginal aperture. The vagina passes posteriorly from the aperture until it approaches the ovary (Hart and Guberlet, 1936). Proximally the vagina enlarges and continues as a seminal receptacle. This structure nears the ovary, constricts, and forms a seminal canal (Yamaguti, 1934). The seminal canal is then joined by the oviduct (Hart and Guberlet, 1936). There is a small sphincter, the oocapt, located around the region where the ovary releases ova into the oviduct (Yamaguti, 1934). The union of the oviduct and the seminal canal forms the relatively thick-walled uterine duct, which is immediately joined by the common vitelline

duct. These ducts are surrounded by large, closely packed cells, possibly the "shell-gland" (Hart and Guberlet, 1936). The "shell-gland" is currently referred to as Mehlis' gland. After passing through these gland cells the uterus becomes greatly convoluted, and gradually changes from a relatively thick-walled to a thin-walled structure as it approaches the uterine aperture. The diameter of the uterus increases as it nears the aperture (Hart and Guberlet, 1936).

Hart and Guberlet (1936) described the eggs as being spindle-shaped, measuring 20 by 40 microns. Yamaguti (1934) described the eggs as being elongate, oval, and somewhat thick-shelled. They measure 39 to 45 microns by 24-28 microns with an operculum located near the middle of the egg shell.

Hart and Guberlet (1936) described the excretory (currently referred to as the osmoregulatory system) system as being composed of two lateral canals on each side of the strobila. The inner canal lies median to the testes, while the outer one passes between the testes and the vitellaria. Transverse connections between median canals occur at the level of each cirrus. Connections between the two canals on the same side occur without relation to the reproductive organs. The lateral canals exhibit irregularly occurring accessory lateral openings to the outside. In the anterior and posterior ends of the worm the canals branch and anastomose with one another in no discernible pattern.

Yamaguti (1934) observed calcareous corpuscles in the anterior end of the cestode only. Hart and Guberlet (1936)

made no mention of calcareous corpuscles in the anterior end of the worm, but did observe them in the posterior excretory canals and posterior to the last set of reproductive organs.

## SECTION III

LIPARIS ATLANTICUS (SEASNAIL)

Much of the material discussed below concerning the ecology of L. atlanticus is taken from the University of New Hampshire doctoral dissertation of Robert Detwyler (1963) entitled Some Aspects of the Biology of the Seasnail, Liparis atlanticus Jordan and Evermann.

Other Names: New England seasnail, dusky seaslug, sea-snail, snail-fish.

Description: The seasnail, L. atlanticus (Jordan and Evermann), belongs to the family Cyclopteridae, order Perciformes. Probably the best general account of L. atlanticus is that of Bigelow and Schroeder (1953). The fish is tadpole-shaped and soft-bodied. The snout is broad and rounded. The belly is soft and flat. There is an abrupt sidewise flattening of the body close behind the vent. A prominent sucking disc is located on the ventral surface between the pectoral fins and is used for attachment. There are 28 to 32 fin rays in the dorsal fin. The dorsal fin rays in the male are longer than in the female. The anal fin possesses 23-27 rays, and originates under, or a little behind the posterior region of the dorsal fin. There are ten to twelve rays in the caudal fin. The pectoral fins are large and fan-like and extend under the throat. The number of pectoral fin rays varies

from 28 to 30. The pectoral fins are notched, with the lower lobe containing seven rays. These rays usually extend beyond the fin membrane, giving them a fringed appearance. The teeth are tri-lobed and are arranged in eight or nine oblique rows in each half of the jaw. The skin is scaleless and smooth except at spawning time when breeding tubercles appear on the male snout. The lateral line is reduced to a series of pores on the head. Coloration is, in a general way, correlated to environment. In the natural environment the fish are black, gray, or dark brown. In the laboratory aquaria the fish take on an olive, reddish-brown, or light tan color. Fins are frequently barred with white, blue, or pink, and the body often shows blotches of these colors. The maximum size attained is about one hundred millimeters.

Range: Bigelow and Schroeder (1953) cite the general range of L. atlanticus as rocky shores along the North American coast from northeastern Newfoundland, the northern part of the Gulf of St. Lawrence and the grand Banks to southern New England. L. atlanticus is rarely found south of Cape Cod, but it has been collected at Woods Hole and along the Connecticut and New Jersey coasts.

Habitat: Seasnails are found adhering to the undersides of rocks and clinging to vegetation in littoral tide pools and in the sublittoral. The species moves inshore during the fall and winter to spawn, but is rarely found inshore after late May. How far offshore the fish migrates is not known.

Temperature probably has the greatest effect on migration and habitat selection. When the water temperature rises above 12° C, the fish are rarely found in the tide pools, and are not found again in numbers until the fall when the temperature falls below 12° C. Breeding occurs in the tide pools throughout later winter and early spring.

Food Habits: Bigelow and Schroeder (1953) presumed that seasnails feed chiefly on small crustaceans and shellfish.

Detwyler (1963) examined the contents of the stomachs of 48 seasnails. He found that the amphipod Gammarus locusta (now recognized as G. oceanicus) and the isopod Jaera marina comprised 55% of the diet of L. atlanticus. The remainder of the diet consisted of polychaete remains (possibly Harmothoe imbricata) and plant material (believed to be Chondrus crispus), and unidentifiable crustacean remains.

## SECTION IV

## MATERIALS AND METHODS

One-hundred-twenty-eight seasnails (L. atlanticus) were collected during the period December, 1967 to May, 1969 at Odiorne's Point, Rye, New Hampshire, and Rye Ledges, New Hampshire. Collection of fish took place during the periods of lowest tides each month, when weather conditions permitted.

The fish were exposed by turning over rocks in tide pools under which the fish were hiding. Seasnails were captured by using small dip nets or by hand. When the fish were captured, they were placed in a plastic pail, transported alive to the marine cold room (13-15° C) of the Zoology Department, and placed in an aquarium until they were examined.

Some fish were sacrificed and examined immediately upon return to the laboratory, while others were held in the aquarium for several days to allow time for removal of undigested food from the intestinal tract. Before each fish was sacrificed, its sex and standard length was determined. Standard length is here defined as the distance from the tip of the snout to the base of the caudal fin. All fish were killed by severing the spinal column at the base of the skull. The operculum was removed from one side of the fish and the gills were scraped with a scalpel to obtain mucus and gill tissue. The scrapings were then placed on a microscopic

slide with a drop of saline. A coverslip was applied to this and the slide was examined microscopically for the presence of parasites on the gills. Ciliate parasites observed on these gill scrapings were either fixed in alcohol-formalin-acetic acid (AFA) and stained with chrome alum-gallocyanin (Barka and Anderson, 1963), or were fixed and stained with the nigrosin-HgCl<sub>2</sub>-formalin technique (Borror, 1968).

A ventro-lateral incision was made from the operculum to the anus exposing the internal organs of the fish. Direct cardiac puncture with a sterile pipette was performed on a few fish in order to obtain blood for making blood smears. These smears were then fixed in 100% methyl alcohol, allowed to air dry, and were stained by the Giemsa method.

The entire viscera was then placed in Ringer's solution in a Syracuse watch glass. In some instances the entire viscera was then fixed. In other fish, the intestine was freed from the rest of the viscera and fixed by itself. For the majority of fish the intestine was carefully removed from the viscera, opened up, and examined under a binocular dissecting microscope. The number and position of the parasites in each intestine was recorded. A small amount of feces and mucosal scrapings were placed on a microscopic slide with a drop of saline. A coverslip was applied, and this slide was examined for the presence of microscopic intestinal parasites, particularly intestinal Protozoa.

Whole mounts of trematodes and cestodes were made by fixing the worms in AFA and staining them with either chrome

alum-galloycyanin (using chromotrope 2R as a counterstain), Harris' hematoxylin, or Grenacher's borax carmine. These specimens were then examined to identify the helminths and to study their morphology.

Acanthocephalans were removed from the intestine, rinsed in Ringer's solution, and placed in distilled water in a Syracuse watch glass to allow the proboscis to become completely everted. Identifications were made by Dr. Wilbur L. Bullock.

Nematode parasites were removed from the host and fixed in Travassos' fixative. Temporary mounts of these parasites were made by adding a few drops of glycerin to the vial containing the fixative and the specimens. This procedure was continued until the specimens were placed in pure glycerin, after which they were cleared, examined microscopically, and identified.

All tissues (both parasite and host) used in the histological and histochemical aspects of this study were fixed in a variety of either hot or ice cold fixatives. In general, tissues fixed in cold fixatives produced a more accurate histological "picture", since hot fixatives tended to cause some tissue distortion. The fixatives employed were: Bouin's, alcohol-formalin-acetic acid, neutral buffered formalin, Zenker's, Helly's, Gendre's, formol-calcium, cold acetone, and 85% ethyl alcohol. Appropriate washing was used after each fixative. Each tissue was then dehydrated and embedded in "paraplast" in a paraffin oven at 58° C. Serial sections, seven microns in thickness, were cut on a rotary microtome,

mounted on slides with or without albumin adhesive (depending on the staining technique to be used), and stained by various techniques. Frozen sections were cut on a cryostat at 12 microns.

For histological study the following techniques were employed:

Ehrlich's hematoxylin and eosin (other counter-stains used were chromotrope 2R, orange G, and fast green)  
 Gomori-Wheatley trichrome  
 Mallory's phosphotungstic acid hematoxylin  
 Basic fuchsin and picro-indigo carmine  
 Mallory's rapid one step stain (Humason, 1962)  
 Cameron and Steele simplified aldehyde-fuchsin (Cameron and Steele, 1959)  
 Lillie's buffered azure-eosinate (pH 4.1 and 6.0) (Lillie, 1948)

The techniques employed for the histochemical portion of this investigation were chosen in order to determine the presence of: 1) Glycogen, 2) Protein-carbohydrate complexes, 3) Nucleic acids, 4) Proteins, 5) Alkaline glycerophosphatase, 6) Calcium, 7) Lipid. The majority of histochemical methods used were from Barka and Anderson (1963).

1) Glycogen: Paraffin sections were stained by the periodic acid-Schiff technique (PAS) (Lillie, 1948). Control sections were digested in filtered, human saliva for one hour at 37° C. In an attempt to further identify the PAS positive material, control slides were treated in several ways. One group of control slides was treated with the PAS stain without prior oxidation with periodic acid to determine the presence of free aldehyde groups. A second group of control slides was exposed to a blocking technique for 1-2 glycol groups (acetylation).

A PAS positive substance, which after acetylation becomes negative, possesses 1-2 glycol groups. This reaction is reversible by treatment with potassium hydroxide. Another group of control sections was treated with a mixture of methyl alcohol and chloroform at 60° C for three hours to determine the presence of glycolipid. The Himes and Moriber triple stain (Humason, 1962) was utilized to determine the presence of general polysaccharide.

2) Protein-carbohydrate complexes: In addition to the PAS technique, the procedures listed below were used to determine the presence of protein-carbohydrate complexes.

Alcian blue, colloidal iron, and Astra blue for acid mucopolysaccharides.

Alcoholic and aqueous toluidine blue for acid mucopolysaccharides.

Alcian blue and PAS combined, and colloidal iron and PAS combined for differentiation between acid mucopolysaccharides and mucoproteins (Mowry, 1963).

3. Nucleic acids: For the determination of deoxyribonucleic acid (DNA) the Feulgen nuclear reaction (Di Stefano, 1952), methyl green-pyronin Y, chrome alum-gallocyanin, and Himes and Moriber triple stains were utilized. Ribonucleic acid (RNA) was stained for with the methyl green-pyronin Y, chrome alum-gallocyanin, and buffered azure-eosinate techniques. Control slides were digested with ribonuclease for one hour at 37° C.

4. Proteins: Paraffin sections were subjected to the brom-phenol blue technique (Mazia et al, 1953) with and without the presence of mercuric salts (HgCl<sub>2</sub>) to determine the presence of basic protein and "total" protein. The Himes

and Moriber triple stain was also used to observe "total" protein distribution.

5) Alkaline Glycerophosphatase: Alkaline glycerophosphatase distribution was determined by the Gomori technique as modified by Bullock (1953, 1958).

6) Calcium: The distribution of calcium was examined by the use of the alizarine red S technique (Humason, 1962).

7) Lipid: Lillie's supersaturated oil red O technique was used on frozen sections to study the distribution of general lipids.

Details of procedure and formulary of stains used will be found in the appendix.

Sections were photographed on Panatomic-X or Kodachrome II film with a Zeiss Ikon Contaflex super BC 35 millimeter camera mounted on a Bausch and Lomb trinocular compound microscope.

Since the life cycle of S. simplex is unknown, an attempt was made to work out the life cycle of this cestode. Tapeworms were teased apart in sea water in petri dishes to liberate eggs. These were placed in the marine cold room (13-15° C) in nine inch finger bowls. Each finger bowl also contained two or three dozen amphipods (the majority of which were Gammarus oceanicus, but probably some Marinogammarus spp. were also present). Amphipods were used for life cycle studies because they have been shown to serve as intermediate hosts for other spathebothriid cestodes. For 42 days the amphipods were allowed to feed on the tapeworm fragments

and eggs in various stages of development. During this period, and at the end of it, amphipods were observed under a dissecting microscope to check for the presence of larval stages. Several hundred amphipods were utilized and examined in this portion of the investigation.

## SECTION V

## RESULTS

The general morphology of S. simplex is shown in figures 1-3.

1. Collection Data- S. simplex was the most frequently encountered helminth parasite in the seasnail. Only nine of 132 seasnails were not parasitized by this cestode, and two of these hosts had been held for over one week in an aquarium prior to examination. This is ample time for a host to rid itself of many of its parasites. The maximum number of parasites recorded was 13 tapeworms in one host. The worms were always found in the anterior region of the intestine, the majority of worms with their anterior ends intruding deeply into the pyloric ceca (Fig. 4). Worms were often entwined around each other, and were difficult to separate without tearing.

All the cestodes collected in late September 1968 were either immature or just maturing (Fig. 1). The hosts captured during this season were the smallest caught throughout the year, ranging from 20 to 46 millimeters in standard length. For the remainder of the year, the fishes were generally quite a bit larger, and very few immature worms were found. The longest cestodes came from hosts that were captured in March and April.

## 2. Histochemical Observations

a) Tegument- The entire tegument of S. simplex was PAS positive after salivary digestion and lipid extraction (Fig. 5-6). Substances that are PAS positive after salivary digestion and after treatment with lipid solvents are glyco- or mucoproteins. The outer edge of the tegument (made up of short rod-like structures) does not stain as positively with the PAS technique as does the inner region. The basement membrane underlying the tegument is also PAS positive. The staining of the tegument with this technique varied with the fixative used. Gendre's fixative tended to produce a lighter staining, while tissues fixed in neutral buffered formalin stained heavily. Because exposure of similar sections to a mixture of hot methanol and chloroform did not decrease the intensity of the PAS reaction, the PAS positive material was not a glycolipid. Acetylation prior to exposure to the PAS technique resulted in the loss of all positive staining, while if this were followed by a de-acetylation all PAS positive staining returned (Fig. 7-8). Sections subjected to the PAS procedure without prior oxidation with periodic acid were all PAS negative (Fig. 9). The above two controls show that the PAS positive reaction in the tegument and basement membrane was due to the presence of 1-2 glycols and not due to the presence of free aldehyde groupings. The tegument stained yellowish-red with the Himes and Moriber triple stain, indicating the presence of mucoprotein and/or mucopolysaccharide.

Acid mucopolysaccharides do not stain with the PAS technique, but do stain positively with Alcian blue, colloidal iron, and Astra blue. They also exhibit metachromasia with toluidine blue. The outer edge of the tegument in the anterior region of the worm stained lightly with Astra blue. The entire tegument stained very lightly with the Alcian blue and colloidal iron methods (Fig. 10). The teguments of Podocotyle reflexa and Prosorhynchus sp., other helminths in the same host intestine, stained intensely with all three of the above stains. The tegument did exhibit slight metachromasia when stained with aqueous toluidine blue and viewed under water. However, if the sections were dehydrated through the alcohols, this metachromasia was lost.

The entire tegument of the worm gave a strongly positive reaction for alkaline glycerophosphatase at a pH of 9.2. The enzyme activity appeared heaviest in the anterior tegument, in front of the first set of reproductive organs, and diminished slightly in the posterior regions (Fig. 11-12).

The brom-phenol blue technique is considered to be specific for basic proteins. In the presence of  $\text{HgCl}_2$  this stain will combine with other proteins to give a total protein picture. Some basic protein is present in the tegument, as light staining of this structure did occur with brom-phenol blue. In the presence of  $\text{HgCl}_2$  the tegument stained intensely, indicating the presence of much proteinaceous material.

No histochemical evidence of lipids, nucleic acids, calcium, or glycogen was observed with the technique used.

b) Subtegument- Small granules of PAS positive material, i.e. glycogen, that were labile to digestion with saliva, were found scattered throughout the entire length of the subtegument. The majority of granules were intra-cellular. These glycogen deposits in the subtegument were somewhat lighter in the middle and posterior regions of the worm.

The cells of the subtegument contained relatively large amounts of RNA as evidenced by their positive staining with the chrome alum-gallocyanin and pyronin Y techniques. This positive staining was labile to ribonuclease digestion. These cells exhibited cytoplasmic basophilia when stained with azure-eosinate at a pH of 4.1. This basophilia was also labile to ribonuclease digestion. DNA content of the subtegumental cells appeared slight, as only light positive staining occurred in the nuclei of these cells with the Feulgen, chrome alum-gallocyanin, Himes and Moriber triple stain, and methyl green and pyronin Y reactions.

The subtegumental cells exhibited slight metachromasia when stained with aqueous toluidine blue. This metachromatic staining reaction was labile to alcoholic dehydration, eliminating the possibility of the staining reaction being due to sulfated acid mucopolysaccharides since the latter exhibit alcohol resistant metachromasia.

Intense positive staining occurred in these cells when stained with brom-phenol blue in the presence of  $\text{HgCl}_2$ , indicating the presence of protein. A negligible staining reaction was given by these cells when this stain was used

without  $\text{HgCl}_2$ . The cells of the subtegument stained bright yellow with the Himes and Moriber triple stain.

In the anterior region of the cestode, some light alkaline glycerophosphatase activity was observed in the cells of the subtegument. The remainder of the subtegument did not react positively. The staining reaction in the anterior subtegument was heavier in worms that were fixed in situ in the host intestine than in worms that had been removed from the intestine, rinsed in Ringer's solution, and then fixed.

Small lipid deposits were found in the cells of the anterior subtegument. A few small granules that reacted positively to the oil red O technique were apparent in the anterior subtegument of the worm.

c) Parenchyma- Heavy glycogen deposits, labile to salivary digestion, are located in and outside the cells of the medullary parenchyma anterior to the first set of reproductive organs. These deposits extend nearly to the most anterior region of the holdfast (Fig. 13-14). The remainder of the medullary parenchyma (from the first reproductive organs to the posterior of the worm) contained the next largest concentration of glycogen found in the cestode.

The cytoplasmic processes of the cells of the medullary parenchyma were positive after all the previously mentioned controls and stained reddish-yellow with Himes and Moriber stain. These regions stained lightly with Alcian blue and colloidal iron, and exhibited metachromasia with

alcoholic toluidine blue. The cells exhibited metachromasia with aqueous toluidine blue when viewed under water. Metachromatic staining was lost after alcoholic dehydration. The cells stained purplish with aldehyde fuchsin.

The cytoplasm of the parenchymal cells anterior to the first set of reproductive organs contained RNA, while the parenchyma of the remainder of the worm had very little. DNA content of the parenchymal cells was very slight. Light positive staining of the parenchymal cells did occur with mercuric brom-phenol blue. Numerous small lipid droplets were observed in some of the cells of the parenchyma.

d) Male Reproductive System- Mature spermatozoa in the testes, seminal vesicle, vas deferens, and seminal receptacle contained relatively heavy glycogen deposits. Immature spermatozoa in the testes contained no glycogen. The inner lining of the cirrus, cirrus pouch, seminal vesicle, and the epithelial lining of the prostatic portion of the male reproductive system were all PAS positive after the various controls. The prostatic epithelium gave a particularly strong reaction (Fig. 15). All these regions stained reddish-yellow with the Himes and Moriber triple stain. The large pyriform cells seen in the medullary region on the sides and base of the cirrus pouch were PAS negative and stained yellow with the Himes and Moriber triple stain (Fig. 16).

Large amounts of DNA were found in the mature spermatozoa. RNA content was greater in the developing spermatozoa than in the mature spermatozoa. The large pyriform prostatic

cells bordering the cirrus pouch contained large amounts of RNA as shown by ribonuclease labile cytoplasmic basophilia when stained with azure-eosinate at pH 4.1. No positive reaction for basic protein was noted in the male reproductive system, but the tissues of this system, especially the pyri-form prostatic cells, showed some affinity for mercuric brom-phenol blue, indicating some cellular protein content.

Alkaline glycerophosphatase was present along the outer edges of the cirrus pouch. However, this apparent reaction may be merely the result of diffusion from the adjacent tegument. No cellular lipid was observed.

e) Female Reproductive System- Glycogen was not observed in the vitellaria or the ovaries, although the strands of parenchyma surrounding these regions contained glycogen. Minute amounts of glycogen were observed in the developing embryos. Fine granules were also observed in the uterine wall.

The egg shell was strongly PAS positive after all controls used, as were the uterine and vaginal walls, wall of the seminal receptacle, and the colloidal material around the oncosphere. The above first four regions stained reddish-yellow with the Himes and Moriber triple stain, while the colloidal material around the oncosphere stained more red than yellow (Fig. 17). With this stain, the most mature egg shells (those highest up in the uterus) stained yellow, while those less mature (lower in the uterus) stained reddish-yellow. With the PAS and Himes and Moriber techniques the cells of

Mehlis' gland produced a staining reaction comparable to that of the egg shell, uterine and vaginal walls, and wall of the seminal receptacle (Fig. 18).

The colloidal area surrounding the oncosphere stained heavily with Alcian blue, Astra blue, and colloidal iron, indicating the acid mucopolysaccharide nature of this material. All other parts of this system stained negatively with these stains.

Large amounts of DNA were found in the nuclei of the developing embryos and in the nuclei of the vitelline cells. Appreciable amounts of RNA were observed in the cytoplasm of the vitelline cells and developing embryos (Fig. 19-20). The cytoplasm of the cells of Mehlis' gland also showed considerable RNA.

The largest concentration of basic protein in S. simplex appeared as globules in the cytoplasm of the vitelline cells. Basic protein was found also in the eggs of the lower uterus, but eggs high up in the uterus lost their affinity for brom-phenol blue and appeared almost colorless. Tissues fixed with neutral buffered formalin produced the most precise intra-cellular localization of basic protein in the vitelline cells. The vitelline cells stained a bright yellow with the Himes and Moriber triple stain (Fig. 17). The egg shells and vitellaria stained intensely with mercuric brom-phenol blue. All of the worm's tissues showed some affinity for mercuric brom-phenol blue.

The only region of the female reproductive system that reacted positively to the alkaline glycerophosphatase technique at a pH of 9.2 was the outer edge of the vaginal aperture. Again, tegument overlying this area was probably the cause of this reaction.

The largest concentration of lipid in the cestode was found between the egg shell and the developing oncosphere (Fig. 21). Large lipid droplets were almost always observed in this region.

f) Calcareous Corpuscles- The largest concentration of calcareous corpuscles was found in the parenchyma anterior to the first set of reproductive organs. They were, however, observed in fewer numbers in the parenchyma throughout the entire length of S. simplex. None were observed in the osmoregulatory canals. The calcareous corpuscles reacted negatively to the PAS technique, but stained heavily with the Alcian blue, Astra blue, and colloidal iron techniques. No nucleic acids appeared to be present in these structures. The calcareous corpuscles gave a strong positive reaction with the alkaline glycerophosphatase technique, even in control sections (Fig. 11-12). However, no evidence of actual phosphatase activity was observed as both experimental and control sections gave comparable staining reactions. This reaction was undoubtedly due to the presence of calcium in these structures, since they stained positively for the presence of calcium when the alizarine red S technique was used.

g) Osmoregulatory System- The osmoregulatory canals did not stain with colloidal iron, Astra blue, Alcian blue, or alkaline glycerophosphatase techniques. Very light staining of the walls of the canals occurred with mercuric bromphenol blue. No lipid was observed in osmoregulatory canals. However, some of the sections investigated by the oil red O technique were torn and some leakage of lipid from the canals into the surrounding tissues may have occurred.

3. Histological Observations- The literature descriptions of the eggs of S. simplex do not mention the presence of filaments. Eggs teased out of the uterus, and sometimes observed in the uterus, as well as eggs passed in the feces of L. atlanticus, displayed filaments radiating out from a small, raised area at one end of the egg shell. These filaments measure 13 to 17 microns in length, and are more easily observed when the eggs are stained with 0.001% thionin than when the eggs are unstained (Fig. 22).

I know of no description of the morphology of the nervous system of S. simplex. Although I did not attempt a detailed study of this particular system, some observations were made. Paired ganglia, connected by cross commissures, are located in the anterior fourth of the holdfast. Two longitudinal nerves arise from this structure, one on each side of the worm (Fig. 2). Also, there appear to be nerves arising from the ganglia that pass anteriorly. The number of these was not determined.

4. Histopathology- Since the holdfast of S. simplex is unarmed and undifferentiated, little tissue damage is done to the host, especially in light infections. However in heavy infections the worm mass can drastically flatten the host intestinal mucosa (Fig. 23-24). In heavy infections the PAS positive brush border of the intestine appeared to have been worn away. Due to this abrasion the distribution of alkaline glycerophosphatase normally present in the brush border was seriously diminished or completely lacking in heavy infections. This histopathology would tend to impair host intestinal absorption.

5. Life Cycle- The life cycle of S. simplex is unknown. The elucidation of the life cycle was attempted during this investigation. As described earlier, tapeworms were teased apart in sea water in petri dishes to liberate eggs. These were then placed in the marine cold room (13-15°C) and were observed daily for 35 days. Some embryos did appear to develop as indicated by the presence of oncospheres with six hooks in some eggs. However, no procercooids were observed.

Attempts to infect possible intermediate hosts were unsuccessful. Amphipods (mostly Gammarus oceanicus, but probably some Marinogammarus spp.) were placed in nine inch diameter finger bowls with sea water in the marine cold room, and pieces of cestodes and embryos at various stages of development were added. The amphipods were allowed to feed on these eggs and tissues in the hope that a more advanced larval stage would develop in the amphipods. No cestode larval

stages were observed after an examination of hundreds of these amphipods. However, encysted metacercariae were observed in a few of the amphipods. Undoubtedly these infections occurred prior to the collection of the amphipods in the field. No attempt was made to identify these metacercariae.

Other Parasites observed in Liparis atlanticus

The parasite fauna of L. atlanticus will be discussed according to taxonomic groupings. Also included in the results will be observations on histopathology, if any, and location of the parasite in the host. The majority of the parasites in this study were found in the intestine.

Protozoa: Examination of Giemsa stained blood smears for haematozoa from 17 fish yielded negative results. For this reason this procedure was discontinued and full attention was given to the examination of the gills and intestinal tract.

1. Eimeria sp.- Every fish examined was infected with sporozoans of the genus Eimeria. The heaviest concentration of parasites was usually found to occur in the mucosal layer of the rectum. In very heavy infections parasites were observed also in the submucosa of the rectum (Fig. 25). Parasites were seen throughout the entire length of the intestine, even as far anterior as the mucosa of the pyloric ceca. This parasite appeared to cause the most serious histopathological damage to the host of any parasite studied, sometimes invading and destroying the majority of the intestinal mucosa in any given section.

2. Myxosporidia- Spores of an unidentified species of myxosporidian were seen in the feces of one fish examined in October, 1968. A heavy infection of morphologically

similar spores was observed in the gall bladder of one fish examined in May, 1969. However, not all fish were examined specifically for the presence or absence of Myxosporidia. Therefore the incidence of infection may have been higher than was recorded. No apparent histopathology was associated with the parasite.

3. An unidentified species of trichodinid ciliate was observed in varying degrees of infection on the gill filaments of 38 of 68 hosts examined for this parasite (Fig. 26-27). Whether all these infections were acquired in the ocean or after the fish were introduced into aquaria in the marine cold room is not known. After each collection of fish was examined, the aquarium housing the fish was washed with soap and water. However, while the fish were alive the parasite could have rapidly spread from infected to non-infected fish, as no intermediate host is required for the completion of the life cycle. Whether members of this genus are considered true parasites or ecto-commensals is debatable. No lesions were observed on the gill filaments that could have been attributed to the presence of this organism. However, heavy infections covering much of the gill surface might impair respiration.

#### Trematoda:

1. Prosorhynchus (crucibulum?)- A species of the gasterostome genus Prosorhynchus, possibly P. crucibulum, was found in 15 of 128 hosts examined. The majority of worms

were sexually mature. The parasite was always found as far forward in the pyloric ceca as was possible for it to be. This made removal of specimens somewhat difficult. There were usually no more than three (many times only two or one) parasites found in the host. An exception to this was one host that was collected in January, 1968. Twenty-seven specimens of this parasite were removed from this host. The tegument of the parasite is spinous, with the spines being more abundant anteriorly. These spines caused severe abrasion of the pyloric mucosa. In one cecum observed, all the tissues had been worn away except the serosa.

2. Podocotyle reflexa and P. atomon- Members of the genus Podocotyle, probably P. reflexa for the most part, were the second most frequently encountered parasite of this survey. There may have been a few P. atomon observed as well. Only 11 of 128 L. atlanticus examined were not parasitized by this trematode. Podocotyle spp. were found throughout the entire length of the intestine, but were only found in the anterior regions of the intestine when there were very few S. simplex in that region. When the incidence of infection of this trematode was high, the number of S. simplex present was reduced, and vice versa. When the incidence of S. simplex was high, the species of Podocotyle were located in the mid- and posterior intestine, or even in the rectum. Histopathological damage caused by this parasite was limited mainly to the grasping of small amounts of the intestinal mucosa (Fig. 28). In heavy infections, the worms tended to become pressed

against the mucosa, and caused the normally columnar-shaped epithelium to assume a cuboidal or even squamous shape.

3. Metacercariae- Encysted trematode metacercariae were observed on the gills of 17 of 68 hosts examined for the presence of this parasite. The incidence of infection was highest in those hosts captured in the late fall and early winter. The number of parasites present per gill scraping varied from one to five or six. Metacercariae were located generally at the base of the gill filaments adjacent to the gill arches. No attempt was made to identify these metacercariae.

Numerous metacercariae were found encysted in the musculature of the body wall in one L. atlanticus that had been decalcified, embedded, serially sectioned, and stained (Fig. 29). Whether these metacercariae were the same species as the above is not known, as again, no attempt was made to identify these parasites.

#### Acanthocephala!

1. Echinorhynchus gadi- Twenty-seven of 128 hosts examined were found infected with the acanthocephalan parasite E. gadi. All specimens were immature. The parasite was always found with its proboscis embedded in the intestinal wall. When moderate to heavy infection of S. simplex was present, E. gadi was attached to the wall of the mid- or posterior intestine. One specimen was found as far posterior as the rectum. However, three E. gadi were found attached to the

wall of the anterior intestine, and one of these had invaded the pyloric ceca, in hosts that harbored no more than four S. simplex. Histopathology was restricted to the immediate area of the intestinal wall that was penetrated by the proboscis of the parasite (Fig. 30). The proboscis rarely extended deeper than the lamina propria of the intestinal wall. Whenever the proboscis penetrated complete cellular destruction occurred. There was very little, if any, evidence of generalized tissue reaction to the presence of this parasite.

#### Nematoda:

1. Thynnascaris sp.- Twelve of 128 hosts examined were infected with a nematode parasite tentatively identified as belonging to the genus Thynnascaris. The specimens were larval stages and were entwined in the mesenteries in the coelom of the host. In only one host were two individuals present. In all other infections only one individual was observed.

Examination of microscopic sections of the stomach of L. atlanticus revealed the presence of encysted nematode larvae in three stomach walls (Fig. 31). The parasite was always encapsulated by host connective tissue. These parasites were not identified.

#### Arthropoda:

No parasitic arthropods were found on L. atlanticus during this investigation.

## SECTION VI

## DISCUSSION

To my knowledge there have been no previous histochemical investigations on spathebothridean cestodes. The majority of previous cestode histochemical studies were on members of the order Cyclophyllidea (parasites of birds and mammals). A few histochemical studies were reported on members of the order Pseudophyllidea (parasites of fish and fish eating birds and mammals).

1. Histochemical Observations

a) Tegument- The entire tegument of S. simplex stains uniformly with the PAS technique after salivary digestion and lipid extraction. The outer edge stains a little less intensely than the inner region. On the basis of the criteria set forth by Barka and Anderson (1963), this staining reaction indicates that the tegument consists of a protein-carbohydrate complex, probably a mucoprotein. The yellowish red staining of this structure with the Himes and Moriber triple stain lends support to this belief. The very positive staining of the tegument with mercuric brom-phenol blue, and its less intense staining with aqueous brom-phenol blue, illustrated the proteinaceous nature of the tegument, some of this protein being basic. Ohman-James (1968) found that in the scolex and neck regions of plerocercoid and adult Diphyllobothrium dendriticum (Pseudophyllidea) PAS positive, diastase labile

material was located in the tegument. No histochemical techniques were performed on the remainder of the strobila of D. dendriticum. In both S. simplex and D. dendriticum this PAS staining reaction in the tegument was eliminated by acetylation and restored by de-acetylation, indicating that the original positive staining was due to 1-2 glycol groups (Culling, 1963).

The distribution of glycolipids in D. dendriticum (Ohman-James, 1968) was not investigated, and none were observed in S. simplex. Bogitsh (1963) noted a similar lack of glycolipid in the cyclophyllidean cestode Hymenolepis microstoma. Bogitsh (1963) also observed the PAS positive, diastase labile reaction in the tegument of H. microstoma, and showed that this reaction was due to the presence of 1-2 glycols and not due to the presence of free aldehyde groupings. However, this reaction in the tegument varied depending on the region investigated. The tegument of the scolex and neck regions were lightly PAS positive, while the reaction increased in the remainder of the immature segments and persisted for the remainder of the length of the strobila. This differential staining of the tegument was not noted in S. simplex. The tegument of D. dendriticum stained positively with the acrolein-Schiff technique, showing protein to be present (Ohman-James, 1968). Staining similar to that observed in S. simplex was noted in the tegument of H. microstoma (Bogitsh, 1963) when the brom-phenol blue technique was utilized. However, there was only a very slight reaction for

basic protein in the tegument of H. microstoma. The tegument of S. simplex stained somewhat more intensely, but still lightly.

In both D. dendriticum (Ohman-James, 1968) and H. microstoma (Bogitsh, 1963) the outer edge of the tegument stained intensely with colloidal iron and Alcian blue. This strong reaction was not observed in the tegument of S. simplex. In S. simplex only the anterior region of the tegument stained lightly with Astra blue. However the entire outer edge of the tegument stained lightly with both colloidal iron and Alcian blue. The tegument of S. simplex did stain metachromatically with aqueous toluidine blue when viewed under water. This metachromasia was lost if the sections were dehydrated in alcohol. All the above staining reactions are specific for acid mucopolysaccharides (Barka and Anderson, 1963). Thus there appeared to be a small amount of acid mucopolysaccharide in the tegument of S. simplex, but not as much as was found in D. dendriticum and H. microstoma. This staining reaction in S. simplex probably is not due to the presence of sulphated acid mucopolysaccharides, as according to Barka and Anderson (1963) these compounds exhibit alcohol resistant metachromasia. Monné (1959) demonstrated acid mucopolysaccharides in the teguments of several tapeworms and suggested that these substances act as host enzyme inhibitors. However it should be noted that all the cestodes he studied were cyclophyllideans. Lee (1966) also feels that tapeworms are protected against host digestive enzymes by a

layer of mucopolysaccharide in the tegument. Apparently further protection would be afforded the parasite by the resistant mucoprotein also found in the tegument.

The distribution of alkaline phosphatase has been investigated in several cestodes. The tegument of the plerocercoid of D. dendriticum (Ohman-James, 1968) exhibited a reaction for non-specific phosphatase, the most intense reaction being in the scolex and outer edge of the bothria. The reaction in the adult was more intense and occurred along the entire length of the tegument of the parasite. Alkaline phosphatase was present in the outer zone of the tegument of Anoplocephala perfoliata (Cyclophyllidea), but only on the outer face of the proglottids, that were stacked like paper cups so that only this outer face was fully exposed. The coarse microvilli at the posterior margin of each proglottis showed intense enzymatic activity (Lee and Tatchell, 1964). Bogitsh (1963) found alkaline phosphatase present in the entire tegument (except the regions overlying the scolex) of H. microstoma. Erasmus (1957a, 1957b) found the distribution of alkaline phosphatase in the teguments of Taenia pisiformis and Moniezia expansa (both cyclophyllidean cestodes) to be similar to that found by Bogitsh (1963) in H. microstoma. Comparable results were observed in Moniezia expansa (Howells and Erasmus, 1969). Takahashi (1959) has demonstrated alkaline phosphatase in the tegument of the plerocercoid of Diphyllobothrium mansonii (Pseudophyllidea). Arme (1966) found alkaline phosphatase along the entire tegument of both

plerocercoid and adult Ligula intestinalis (Pseudophyllidea). In the present study alkaline glycerophosphatase has been demonstrated along the entire tegument of S. simplex. Enzyme activity appeared heaviest in the anterior regions of the tegument, and diminished slightly in the posterior regions of the tegument.

The function of the tegumental phosphatases is not clear. Bullock (1949, 1958) believes that the cuticular phosphatases of the Acanthocephala are concerned with the absorption of nutrients or possibly with the elimination of fatty acid waste products. Erasmus (1957a, 1957b) suggested that cestode tegumental phosphatases are involved in the release of phosphate ions which are associated either with the phosphorylated passage of substances through the tegument, or possibly with carbohydrate metabolism. Phifer (1960), however, has shown that ammonium molybdate, which inhibits the phosphatases of Hymenolepis diminuta (Cyclophyllidea) in macerated tissues of the cestode, does not inhibit the active transport of glucose when this cestode is incubated in a medium containing ammonium molybdate and  $^{14}\text{C}$  glucose. This would indicate that in this particular cestode, the phosphatases are not involved directly in the active transport of glucose. Lee (1966) felt that in the above instance, it is possible that the ammonium molybdate was unable to reach the phosphatases of the living worm, and thus failed to inhibit the enzymes. Lee further suggested that phosphatases are probably present in the tegument of all cestodes, but that

the distribution of these enzymes varies along the length of the strobila and in the tegument of individual proglottids from one species to another. He believed that this may reflect the mechanisms involved in the uptake of nutrients by different species of cestodes and/or the areas of the worm which are involved in this function. Therefore Lee and Tatchell (1964) postulated that in Anoplocephala perfoliata the part of the proglottis which is involved in the uptake of nutrients is that which is not overlapped by the proglottis in front of it, since they found that the maximum enzyme activity occurs in this region of the tegument.

The localization of alkaline glycerophosphatase in the tegument of S. simplex would lend further support to the above theories as all substances moving in or out of the worm must cross the tegument. Also, the areas of heaviest enzyme distribution (the anterior region of the tegument) are usually located in the ceca of the host. Most host intestinal absorption occurs in this region, and the cestode, in competition with the host for metabolites, would benefit by such an enzyme. Sometimes the entire worm is found in a cecum, and thus the distribution of the enzyme along the entire tegument would be beneficial to the cestode. Even when not in a cecum, the worm is always found in the anterior regions of the intestine, again a region of active host intestinal absorption, where the parasite must compete with the host for nutrients.

Moderate amounts of lipid were observed in the teguments of H. diminuta, H. nana, Hydatigera taeniaeformis and

Dipylidium caninum (Waitz and Schardein, 1964). No lipid was observed in the tegument of S. simplex. The tegument of D. dendriticum was also devoid of lipid (Ohman-James, 1968).

No glycogen was found by any of the above workers in the teguments of any of the cestodes that they investigated. A similar lack of glycogen in the tegument of S. simplex was noted in this investigation.

b) Subtegument- Small granules of glycogen were found scattered throughout the entire length of the subtegument of S. simplex. These deposits were lighter in the middle and posterior regions of the worm. Ohman-James (1968) did not observe similar glycogen deposits in the subteguments of both plerocercoid and adult D. dendriticum, but she studied only the scolex and neck regions. Takahashi (1959) demonstrated heavy glycogen deposits in the subtegumental cells of Diphyllobothrium mansoni. The subtegument of Hydatigera taeniaeformis contained heavy glycogen concentrations (Waitz, 1963). Bogitsh (1963) demonstrated small amounts of PAS positive, diastase labile material, i.e. glycogen, in the subtegumental cells of the scolex and neck regions of H. diminuta. In contrast to S. simplex, glycogen deposits increased in the subtegumental cells in the middle and posterior regions of H. diminuta. Hedrick and Daugherty (1957) also observed small glycogen deposits in the subtegument of H. diminuta and Raillietina cesticillus (Cyclophyllidea).

The very light metachromasia of the cells of the subtegument of S. simplex was observed also in D. dendriticum

(Ohman-James, 1968). This reaction is probably due to the presence of non-sulphated acid mucopolysaccharides since the metachromasia is labile to alcoholic dehydration. Bogitsh (1963) did not observe this in H. diminuta.

The subtegumental cells of S. simplex contain relatively large amounts of RNA as evidenced by their various staining reactions. This reaction can be explained physiologically by the fact that the cestode probably undergoes a fairly rapid rate of growth. Heavy RNA concentrations do not occur in the subtegumental cells of the neck region in S. simplex or in D. dendriticum (Ohman-James, 1968). Bogitsh (1963) had reported heavy concentrations in H. microstoma.

The DNA content of the subtegumental cells of S. simplex was slight, all activity being restricted to the nuclei of the cells. Similar results were observed in all other investigations reviewed by the author.

The staining of these cells with mercuric brom-phenol blue, and their bright yellow staining with the Himes and Moriber triple stain indicated their protein nature. This would be expected as it has been previously shown that their RNA content is high, indicating active protein synthesis. However this protein does not appear to be basic in nature, as the cells gave a negligible staining reaction when stained with brom-phenol blue without  $HgCl_2$ . A comparable staining reaction was noted in the subtegumental cells of H. microstoma (Bogitsh, 1963). However the subtegumental cells of the neck region of both adult and plerocercoid D. dendriticum

stained positively with the ninhydrin-Schiff reaction, indicating the basic nature of the protein present (Ohman-James, 1968).

Light alkaline glycerophosphatase activity was observed in the subtegumental cells of the anterior region of the worm. This reaction was more intense in worms that were fixed in situ in the host intestine than in worms that had been removed from the intestine prior to fixation. Certain Acanthocephala also give a positive staining reaction for alkaline glycerophosphatase when fixed in situ in the host intestine, and a negative reaction when they are removed from the host intestine prior to fixation (Bullock, personal communication). Ohman-James (1968) observed a strong reaction for non-specific phosphatase in the subtegument of D. dendriticum plerocercoids. The reaction in the adult was even stronger in the subtegument and occurred along the entire length of the parasite. Arme (1966) noted alkaline phosphatase activity in the subtegument of plerocercoid and adult Ligula intestinalis. Alkaline phosphatase activity was strong in the subtegumental cells of H. microstoma (Bogitsh, 1963). Erasmus (1957a, 1957b) found results similar to those of Bogitsh in Moniezia expansa and Taenia pisiformis.

The very slight staining reaction of the subtegumental cells of S. simplex may be due partially to the differences between the histochemical procedures used in my studies and in previous investigations. Probably the function of the

subtegumental phosphatases would be similar to their hypothesized tegumental function, namely transport of nutrients to the interior of the worm.

Small lipid droplets were found in the subtegument of the anterior regions of the S. simplex. The subtegument of the neck of plerocercoids of D. dendriticum contained no lipid (Ohman-James, 1968). The subtegument of H. diminuta was devoid of lipid, while some was present in the subtegument of R. cesticillus (Hedrick, 1958).

c) Parenchyma- Most of the glycogen in S. simplex was concentrated in the medullary parenchyma throughout the length of the strobila. The heaviest deposits were found in the region anterior to the first set of reproductive organs, and extended to very near the anterior end of the holdfast. Glycogen distribution in the parenchyma of S. simplex is essentially the same as that reported in H. diminuta and R. cesticillus (Hedrick and Daugherty, 1957). Heavy glycogen deposits were demonstrated in the parenchyma of D. mansoni (Takahashi, 1959). Similar deposits were observed in the parenchyma of H. taeniaeformis (Waitz, 1963), and D. dendriticum (Ohman-James, 1968). In H. microstoma (Bogitsh, 1963), the parenchyma of the scolex was devoid of glycogen, the remainder of the parenchyma of the strobila being rich in glycogen.

According to Cheng (1964) most of the stored food in cestodes is in the form of glycogen. Certainly this appears to be a valid statement concerning S. simplex. Because the

amount of glycogen is greatest in the anterior regions of the worm, one can assume there is a difference in the metabolic rate along the length of the strobila (Cheng, 1964). However in S. simplex, this difference in the amount of glycogen in the parenchyma is not great. Therefore it would appear that the greatest amount of metabolic activity occurs just anterior to the first set of reproductive organs (a region that is comparable to the neck region of other cestodes), with the metabolic rate diminishing gradually posteriorly.

The cytoplasmic processes of the cells of the medullary parenchyma exhibit staining reactions that indicate the presence of both mucoproteins and mucopolysaccharides. Since the metachromatic staining of these cells was lost after alcoholic dehydration, one can assume the absence of sulphated acid mucopolysaccharides (Barka and Anderson, 1963). Ohman-James (1968) observed similar staining of the parenchymal cells in D. dendriticum. Bogitsh (1963) did not observe the positive PAS staining reaction of the cells of the parenchyma after the various controls, but did observe acid mucopolysaccharide deposits as small granules in the parenchyma of the neck. The purplish staining of the parenchyma of S. simplex with aldehyde-fuchsin showed the elastic nature of these cells (Culling, 1963). According to Culling (1963) this stain may be specific for sulphated mucopolysaccharides. However the staining reaction of the parenchyma in S. simplex does not support this belief.

The slight content of RNA in the parenchyma of the

anterior regions of S. simplex is similar to the findings of Ohman-James (1968) in D. dendriticum. Only light RNA concentrations were found in the neck regions of the parenchyma of both plerocercoid and adult D. dendriticum. Bogitsh (1963) however, observed much RNA in the parenchymal regions of the neck and immature proglottids of the strobila of H. microstoma. This RNA almost completely disappeared in the mature proglottids. This same phenomenon was noted in S. simplex. This would appear to be plausible as there is no neck region as such in S. simplex and most of the sets of reproductive organs reach maturity at the same time.

DNA content of all parenchymal cells studied was slight, a finding that was similar to that of all other investigators on various tapeworms.

No alkaline phosphatase activity was noted in the parenchyma of S. simplex. The parenchyma of D. dendriticum did exhibit light enzyme activity (Ohman-James, 1968).

The parenchyma of D. dendriticum stained lightly for the presence of protein, but negatively for the presence of basic protein (Ohman-James, 1968). Comparatively little protein was present in the parenchyma of H. microstoma, the cells being completely devoid of basic protein (Bogitsh, 1963). Comparable staining results were obtained in S. simplex.

Numerous small lipid droplets were observed in some of the cells of the parenchyma of S. simplex. Hedrick (1958) observed much larger concentrations of lipid droplets in the parenchyma of H. diminuta and R. cesticillus. King and

Lumsden (1969) have shown autoradiographically that much of the linoleic acid absorbed by H. diminuta is located initially in the parenchyma. The possible role of this lipid will be discussed further in the section dealing with the female reproductive system.

d) Male Reproductive System- Heavy glycogen deposits were observed in mature spermatozoa in the testes, seminal vesicle, vas deferens, and seminal receptacle of S. simplex. Immature spermatozoa contained no glycogen. In immature proglottids of H. microstoma the developing testes contained no glycogen (Bogitsh, 1963), while in mature proglottids glycogen was found abundantly in the sperm in both the seminal vesicle and seminal receptacle. Similar results were observed in H. diminuta and R. cesticillus (Hedrick and Daugherty, 1957). It is assumed that this glycogen is being used as an energy source by the spermatozoa.

The inner lining of the cirrus, of the cirrus pouch, and of the seminal vesicle in S. simplex were all PAS positive after the various controls. The epithelial lining of the prostatic portion of the male reproductive system stains especially strongly. These regions all stain reddish-yellow with the Himes and Moriber triple stain, further substantiating the presence of mucoprotein in these regions. Bogitsh (1963) noted similar PAS positive staining of the lining of the cirrus pouch, cirrus, and seminal vesicles of H. microstoma. However, he did not observe PAS positive staining of

the epithelial lining of the prostatic portion of the male reproductive system as noted in S. simplex. The staining of the prostatic epithelium with this technique suggests that these cells may be glandular in nature. Possibly, they may produce a substance that is secreted into the terminal portions of the male reproductive system.

The large pyriform cells on the sides and base of the cirrus pouch stained negatively with the PAS technique, and yellow with the Himes and Moriber triple stain, and contained large amounts of RNA. Burt and Sandeman (1969) postulated a glandular function for comparable cells observed in Bothriomonus sturionis (Spathebothridea), but did not investigate them histochemically. The histochemical staining reaction of these cells in S. simplex supports Burt's and Sandeman's postulated glandular function for these cells. The product of these cells would probably be released into the male reproductive system, but their exact function is speculative. These cells did show some affinity for mercuric brom-phenol blue, indicating the proteinaceous nature of their contents. No basic protein was observed in any cells of the male system, but some tissues did show some affinity for mercuric brom-phenol blue.

Large amounts of DNA were found in the mature spermatozoa of S. simplex. The same is true of the spermatozoa in H. microstoma (Bogitsh, 1963). In S. simplex, RNA content was greater in the developing spermatozoa than in the mature spermatozoa. Again, this finding agrees with those of Bogitsh (1963) in H. microstoma.

The only alkaline glycerophosphatase activity in the male reproductive system of S. simplex was observed along the outer edges of the cirrus pouch. This reaction may be a result of diffusion from the adjacent tegument. Alkaline phosphatase was noted in the genital anlagen (both male and female) in the plerocercoid of Ligula intestinalis, and in most parts of the adult reproductive system, especially the cirrus, of the same species (Arme, 1966).

e) Female Reproductive System- No glycogen was observed in the vitellaria or ovaries of S. simplex. Glycogen was observed in the parenchyma around these regions. Small amounts of this carbohydrate were observed in the developing embryos and in the uterine wall. The mature oncosphere of H. microstoma contained little glycogen (Bogitsh, 1963), while those of H. diminuta and R. cesticillus (Hedrick and Daugherty, 1957) contained significant and trace amounts respectively. These researchers also observed glycogen granules in the vitellaria. The vitellaria of S. simplex contained no glycogen.

Because of their staining reactions with the PAS technique after use of the various controls, the egg shell, uterine and vaginal walls, wall of the seminal receptacle, colloidal area around the oncosphere, and the cells of Mehlis' gland all contained a carbohydrate-protein complex, probably a mucoprotein. The staining reaction of these areas with the Himes and Moriber triple stain substantiated this premise. The cells of Mehlis' gland and the region around the oncosphere

of H. microstoma gave similar PAS positive reactions (Bogitsh, 1963). Bogitsh made no mention of the staining reaction of the uterine, vaginal, or seminal receptacle walls. There was however a positive staining reaction of the colloidal material around the oncosphere with Alcian blue. This finding of acid mucopolysaccharide also was seen in the colloidal material around the oncosphere of S. simplex. Johri (1957) noted similar staining of the colloidal material around the oncosphere of Multiceps smythi, along with the finding of mucoprotein in the same area.

The exact function of Mehlis' gland is not known. Cheng (1964) suggested the following possible functions: 1) the gland secretes a fluid that enhances the hardening and tanning of the egg shell, 2) the secretions of the gland cause a release of the shell globules from the vitelline cells, 3) the secretion activates the spermatozoa which are then passed down the ootype, 4) the secretion may form a thin membrane around the cells forming the egg, and the shell globules then build up from within this membrane, or 5) the secretion lubricates the uterus, facilitating passage of the eggs. The cells of Mehlis' gland in S. simplex contained significant amounts of RNA, indicating that above protein synthesis was occurring in the cells of the gland. Hanumantha-Rao (1960) found similar staining of the cells of Mehlis' gland in the pseudophyllidean cestode Penetrocephalus ganapatii when the PAS and Himes and Moriber triple stains were employed. He further attempted to analyze these cells histochemically,

material that may be a lecithin-like phospholipid. These compounds may aid in the release of egg shell precursors from the vitelline cells. After the egg shell is completed, the lecithin remaining in the embryo would account for the PAS positive reaction. Although this appears plausible, no histochemical tests for phospholipid were carried out on S. simplex.

Burt and Sandeman (1969) described the seminal receptacle of Bothrimonus sturionis as having a lining of gland cells. In S. simplex no gland cells were distinguished in the walls of the seminal receptacle, but the staining reaction of the wall would indicate a possible glandular function.

RNA concentration in the vitelline cells of S. simplex is due to the very active protein synthesis occurring in these cells. This is supported by the very intense yellow staining of these cells with the Himes and Moriber triple stain. The nature of the protein being synthesized in these cells is definitely basic, since bright blue cytoplasmic globules appeared in these cells when stained with brom-phenol blue without HgCl<sub>2</sub>. Much DNA was observed in the nuclei of the vitelline cells.

Egg shell formation in S. simplex appears to follow the pattern shown by Schistocephalus solidus (Pseudophyllidea) and the caryophyllaeid cestodes Caryophyllaeus laticeps and C. fennica (Mackiewicz, 1968). The vitelline cells of these cestodes gave staining reactions with brom-phenol blue comparable to those seen in S. simplex. Egg shell material has its

origin in the globules of the vitelline cells. These globules are composed of basic protein as evidenced by their affinities for brom-phenol blue. The loss of coloration to the eggs when stained with brom-phenol blue (without mercuric salts) in the upper uterus is due to the loss of  $\text{-NH}_2$  groups during the hardening and tanning of the protein. This loss of coloration was noted in S. simplex. Furthermore, the eggs in the lower uterus of S. simplex stain reddish-yellow, while those high up in the uterus stain bright yellow. This also indicates the proteinaceous nature of the egg shell, the mature shell containing negligible amounts of basic protein. The tanning of the egg shell is caused by the action of the enzyme polyphenol oxidase on the phenols and the proteins found within the egg shell producing materials (Smyth, 1956).

The concentrations of both DNA and RNA were high in the developing embryos, indicating a high rate of mitosis and protein synthesis.

Heavy lipid deposits were observed in the eggs of S. simplex. These were located between the oncosphere and the egg shell. This lipid could possibly be metabolized in the place of glycogen. Johri (1957) observed large lipid droplets in the eggs of M. smythi. Hedrick (1958) found large lipid deposits in the eggs of H. diminuta, but only diffuse amounts in the eggs of R. cesticillus. It has been shown autoradiographically that much of the linoleic acid absorbed by H. diminuta is located initially in the parenchyma, and much of this is eventually found in the developing eggs (King and Lumsden, 1969).

The only region of the female reproductive system that exhibited alkaline glycerophosphatase activity was the outer edge of the vaginal aperture, and this reaction may have been due to the overlying tegument and its strong positive reaction for this enzyme. Alkaline phosphatase was observed in the genital anlagen in plerocercoids and in most parts of the reproductive system and egg shell of adult Ligula intestinalis (Arme, 1966).

f) Calcareous Corpuscles- Ohman-James (1968) found that in both plerocercoid and adult D. dendriticum the calcareous corpuscles gave positive PAS reactions after diastase digestion, and stained positively for the presence of acid mucopolysaccharides. In S. simplex the calcareous corpuscles did not stain with the PAS technique, but did stain positively with the various stains used to detect acid mucopolysaccharides. In both D. dendriticum (Ohman-James, 1968) and in S. simplex the calcareous corpuscles were the only structures that gave a positive histochemical test for calcium. Calcareous corpuscles of Taenia saginata (Cyclophyllidea) have been shown to contain calcium, protein, and acid mucopolysaccharide as part of their inorganic and organic moiety (Chowdhury, et al, 1962). In S. simplex the calcareous corpuscles gave a strong positive reaction for alkaline glycerophosphatase in both experimental and control sections. However, no evidence of actual phosphatase activity was observed as both experimental and control sections gave staining reactions. This reaction was due undoubtedly to the presence of calcium in these

corpuscles, as evidenced by their positive staining with alizarine red S.

g) Osmoregulatory System- The walls of the osmoregulatory canals of H. microstoma (Bogitsh, 1963) exhibited alkaline phosphatase activity. Erasmus (1957a, 1957b) observed similar results in both T. pisiformis and M. expansa. Enzymatic activity was noted also in the walls of the osmoregulatory canals of both plerocercoid and adult L. intestinalis (Arme, 1966). In both adult and plerocercoid D. dendriticum no alkaline phosphatase activity was observed in the walls of the osmoregulatory canals (Ohman-James, 1968). No alkaline glycerophosphatase activity was noted in the osmoregulatory system of S. simplex. Ohman-James (1968) observed that the walls of the excretory canals in adult and plerocercoid D. dendriticum gave a positive reaction to the PAS technique after the various controls. No such reaction was observed in S. simplex.

2. Nervous System- Although a detailed study of the nervous system of S. simplex was not undertaken, paired ganglia connected by cross commissures were observed in the anterior fourth of the holdfast. Two longitudinal nerves arise from the structure, and there also appear to be anterior nerves arising from the ganglia. This very brief observation seems to agree, in general, with the detailed observations of Rees (1958, 1969) on the nervous system of three pseudophyllidean cestodes, Bothriocephalus scorpii, Clestobothrium crassiceps, and Acompscephalum tortum.

No alkaline phosphatase activity was observed in the nervous system of S. simplex. The main nerve cords and peripheral nerves of D. dendriticum plerocercoid and adult showed alkaline phosphatase activity (Ohman-James, 1968). Arme (1966) noted activity of this enzyme in the main longitudinal nerve cords in plerocercoid and adult L. intestinalis. Neurosecretory cells in the rostellum of H. diminuta were demonstrated by paraldehyde-fuchsin technique of Cameron and Steele (Davey and Breckenridge, 1967). This technique did not reveal similar cells in the holdfast of S. simplex.

3. Morphology of the Egg of S. simplex - As mentioned previously, the descriptions of the eggs of S. simplex make no mention of the presence of filaments on the egg. In this investigation, such filaments were observed radiating out from a small raised area at one end of the egg shell. Burt and Sandeman (1969) noted similar filaments radiating out from the eggs of Bothrimonus sturionis, and postulated a possible adhesive function for the filaments. This would also be a plausible explanation for the function of the filaments on the eggs of S. simplex. Since the eggs are released with the feces into the tide pools, and since the tide pools are subject to severe wind, wave, and tidal action, it would appear to benefit the life cycle of the parasite if the eggs were able to adhere to some structure in the tide pool rather than drift as part of the plankton. In this way, there would be a greater concentration of eggs in a smaller area, thus increasing the possibility of infecting the intermediate host(s).

4. Histopathology Associated with *S. simplex* Infection- The major amount of histopathology associated with cestode infections is related, usually directly, to the morphology of the scolex of the particular worm involved. Thus the histopathology caused the host by a cestode with an ornate and well armed scolex such as *Acanthobothrium coronatum* (Tetraphyllidea) (Rees and Williams, 1965), is much more severe than that caused by the cestodes with unarmed scolices such as the pseudophyllideans *Parabothrium gadi-pollachi* and *Abothrium gadi* (Williams, 1960). Little tissue damage is done to the host by the undifferentiated holdfast of *S. simplex* in light infections. However the intestinal mucosa becomes markedly flattened in heavy infections. The number of goblet cells present in heavy infections appears to be about the same as the number present in light infections. Rees (1969) observed the histopathological effects that were caused by the pseudophyllidean cestode *Acomsocephalum tortum*. The holdfast of this cestode is completely undifferentiated also. Rees (1969) noted the disappearance of goblet cells from the host intestinal mucosa in heavy infections, and believed that the disappearance of goblet cells in the host intestine is due to the discharge of contents of exposed goblet cells as a protection for the mucosa. Bullock (personal communication) has found that acanthocephalans tend to stimulate mucous cell production, the goblet cells increased in number in areas of irritation. In heavy *S. simplex* infections the columnar cells of the host intestine sometimes assumed an almost

squamous shape. A. tortum only caused the columnar cells to show a slight diminution in height (Rees, 1969). The brush border of the intestine of L. atlanticus was entirely worn away in heavy infections. This would undoubtedly impair host intestinal absorption. Similar abrasion of the brush border and flattening of the columnar cells of the bile duct of mice infected with H. microstoma was observed by Bogitsh (1966). In L. atlanticus no vacuolization of the cytoplasm of the mucosal cells in areas of contact with S. simplex occurred. This vacuolization was observed in the epithelial lining of the bile duct of mice infected with H. microstoma (Bogitsh, 1966).

### Analysis of Collection and Life Cycle Data

S. simplex was the most commonly encountered helminth parasite in this investigation. The greatest incidence of infection observed was 13 tapeworms in one host. Detwyler (1963) also found S. simplex to be the most frequently found parasite in an examination of 30 L. atlanticus from the northeast New England coast. One of these fish contained 36 tapeworms, and cestodes in all fish were found most commonly in the anterior intestine with their anterior ends protruding into the pyloric ceca. The same was true in this investigation. Zhukov (1960) found S. simplex in Liparis sp. while studying the parasitic worms of the fishes of the Sea of Japan and South Kuril shallow waters.

Interestingly, all cestodes collected in late September 1968 were either immature or just maturing, while the longest (and therefore containing the greatest number of sets of genitalia), worms were removed from hosts that were captured in late March and April. Detwyler (1963) observed that seasnails did not move inshore until late September and early October when the ocean temperature had decreased. His observation was supported during this investigation. Seasnails are believed to move offshore in the warmer summer months (Detwyler, 1963). Since no fish were available during the summer, no observations were made on the parasite fauna of L. atlanticus during this period. However, it is tempting to postulate that infection of L. atlanticus with S. simplex occurs when the fish move inshore to the tide pools in early

fall. The finding of only immature and just maturing cestodes in the fish collected at this time would tend to support this theory.

Detwyler (1963) recorded that crustaceans made up nearly 75% of the total diet of L. atlanticus, and that the amphipod Gammarus locusta (now recognized to be G. oceanicus) was the most frequently encountered crustacean in the examined stomachs.

Other spathebothriid cestodes are known to utilize amphipod intermediate hosts. Awachie (1966) found Gammarus pulex to serve as intermediate host for Cyathocephalus truncatus in a stream of Wales. Stark (1965) recorded the fact that Gammarus zaddachi served as an intermediate host for Diplocotyle sp. in a British estuary. Neotonic plerocercoids of Diplocotyle sp. were reported from Marinogammarus finmarchicus and M. pirloti from Scotland (Sandeman, 1962), while Burt and Sandeman (1969) observed that Bothrimonus sturionis utilized Gammarus oceanicus as an intermediate host in Newfoundland.

The attempt to elucidate the life cycle of S. simplex utilizing gammarids (mostly G. oceanicus) as possible intermediate hosts produced only negative results. No larval cestodes were observed in the gammarids after they were allowed to feed on the eggs of S. simplex. However, many factors that could have had an effect on the infection of the amphipods and/or development of the eggs such as light intensity, oxygen concentration, and water movement, were not taken into consideration

during this aspect of the investigation. Mueller (1966) has found that these factors as well as others must be considered in the laboratory propagation of Spirometra mansonoides (Pseudophyllidea).

Discussion of the Parasites Found in *L. atlanticus*

Protozoa:

1. Haematozoa- In an extensive investigation of the blood of 1142 marine fish, representing 68 different species, Laird and Bullock (1969) observed intraleucocytic haemogregarines in a blood smear from one *L. atlanticus* captured at Kent Island, New Brunswick, Canada. These parasites, identified as *Haemogregarina* sp., bore some superficial resemblance to the sporozoan parasite *Atoxoplasma*. The erythrocytes of a second seasnail, also captured at Kent Island, New Brunswick, were parasitized by an organism, probably viral in nature, and caused a disease designated as piscine erythrocytic necrosis (PEN) (Laird and Bullock, 1969).

In this present investigation, examination of Giemsa stained blood smears from 17 *L. atlanticus* produced only negative results.

2. *Eimeria* sp.- The incidence of this parasite in *L. atlanticus* during this investigation was 100%. The genus *Eimeria* is a large one, being present in all vertebrate classes. Levine (1962) estimated that there might be 3,500 different species of the genus in mammals, and perhaps 34,000 in chordates. Bullock (personal communication) has found seasnails from Kent Island, New Brunswick, to be parasitized by this coccidian. The genus does parasitize other marine fish. *Eimeria gadi* has been reported from cod in Soviet waters, and *E. sardinae* has been reported from certain clupeid

fishes, mainly sardines, also in Soviet waters (Dogiel, et al., 1961). E. clupearum is parasitic in herring and mackerel, and E. brevoortiana is parasitic in menhaden. With the possibility of 34,000 species occurring, the species reported in this investigation may very well be undescribed.

3. Myxosporidia- Members of the order Myxosporidia are parasites of lower vertebrates, especially fishes (Cheng, 1964). Spores of an unidentified species of myxosporidian were observed in the feces of one fish, and in the gall bladder of a second fish examined during this investigation. Unfortunately not all L. atlanticus in this study were examined specifically for the presence of myxosporidians. I know of no report of myxosporidians from L. atlanticus. However, numerous myxosporidians have been reported from marine fishes. Noble (1939) has described the myxosporidians Leptotheca compressa, L. sphaerula, Sphaeromyxa gibbonsia, S. ovula and Trilospora californica from tide pool fishes of Santa Barbara, California.

4. The incidence of an unidentified species of trichodinid on the gills of 68 seasnails examined was quite high, about 56%. Detwyler (1963) recorded the presence of this parasite on the gills of one of the seasnails that he examined, but did not know if the infection was acquired before or after the host was introduced into the aquarium. This may have occurred with some of the infected fish in this examination. Bullock (personal communication) has also recorded the

presence of this ciliate on the gills of L. atlanticus. To date, there have been more than 90 species of the genus Trichodina described from the gills and skin of marine and freshwater fishes (Lom and Hoffman, 1964). This parasite can be fatal to fish, especially in hatcheries and aquaria, where heavy infections can rapidly build up. Fishes with small opercular openings are especially susceptible to the pathological effects of the parasite, mainly on the covering of the gill surface with subsequent impairment of respiration. Whether the parasite observed on the gills of the seasnails is a new species or not, was not determined.

#### Trematoda:

1. Proisorhynchus (crucibulum?)- The three most commonly reported species of Proisorhynchus in marine fishes are P. aculeatus, P. crucibulum and P. squamatus. All three species are quite similar morphologically, the main differentiation between species being one of relative size. P. crucibulum is the largest measuring 2-6 mm. (Dawes, 1946). The specimens collected from L. atlanticus during this investigation conform best to the diagnostic characteristics given for P. crucibulum by Dawes (1946). Dawes (1946) believed that there are reasons for supposing that both P. aculeatus and P. squamatus are identical with P. crucibulum but does not state these reasons. Yamaguti (1958) retained the above three species as separate species, and cited Liparis spp. as being a definitive host for P. squamatus in Sweden. Chubrick (1952) stated that cercariae of P. squamatus are found in

Mytilus edulis, and the metacercariae are found in Liparis liparis.

Almost all of the specimens collected during this investigation were sexually mature with eggs in the uterus. The morphology of the worms appeared to agree with that given by both Dawes (1946) and Yamaguti (1958) for mature, adult worms. None were encysted; rather all were found free in the pyloric ceca. If the three species are synonymous, then it is possible that Mytilus edulis, also found in tide pools along with L. atlanticus, could serve as an intermediate host for P. crucibulum. At any rate, I accept the taxonomy and description of Dawes (1946) and believe that the species collected during this investigation was P. crucibulum.

To my knowledge this is the first report of P. crucibulum from L. atlanticus from the Atlantic coast of the U.S.A. Zhukov (1960) reported the parasite from Liparis spp. from the Sea of Japan and South Kuril shallow waters. Linton (1940) reported this trematode from the conger eel, Conger conger, at Woods Hole.

2. Podocotyle reflexa and P. atomon- There are 17 species of the genus Podocotyle (sensu stricto) parasitic in marine fishes of the colder waters of the Pacific and Atlantic oceans or connecting cold waters (Pritchard, 1966). The incidence of infection of L. atlanticus with species of the genus Podocotyle in this investigation was over 90%. The majority of specimens were identified as P. reflexa and were

sexually mature. This identification is based on the fact that most of these trematodes possessed a cylindrical shaped body, and their esophagus was about as long as the pharynx. These characteristics are attributed to P. reflexa by Dawes (1946). However, a few specimens were examined that did appear to agree with the characteristics attributed to P. atomon, specifically the esophagus being nearly twice as long as the pharynx. According to Dawes (1946) P. atomon is a very variable species, and artifacts produced by inappropriate methods of fixation may modify specimens so that they answer the description of other species in the genus. This is an important concept to consider, especially when the differences among species is primarily based on relative length and shape, characteristics that can change with the degree of distention and contraction of the organism at the time of fixation.

P. atomon is a commonly found parasite of tide pool fishes in British and American waters. Rees (1945) has reported P. reflexa from the five-bearded rockling, Onos mustelus, a tide pool fish, at Aberystwyth. Zhukov (1960) reported both P. atomon and P. reflexa from Liparis spp. in the Sea of Japan and South Kuril waters. P. reflexa has also been reported from Cyclopterus lumpus (a fish closely related to L. atlanticus) from Sweden (Yamaguti, 1958).

Cercariae of P. atomon are found in Littorina rudis (= L. saxatilis) and metacercariae occur in Gammarus spp. and other amphipods (Yamaguti, 1958). During this investigation

metacercarial cysts were observed in Gammarus oceanicus, but no attempt was made to identify them. These may well have been species of Podocotyle.

Detwyler (1963) found no species of Podocotyle in L. atlanticus. However, he did report a trematode tentatively identified as a possible species of the genus Pycnadenoides. This parasite was the second most frequently encountered in his survey. Although I have not examined any of Dr. Detwyler's specimens, I would tend to question this identification as no members of the genus Pycnadenoides were found during this present, more extensive, parasite investigation.

Both species of Podocotyle appeared to have a reciprocating relationship with S. simplex in regards to both degree of incidence and location in the host intestine. Detwyler (1963) observed the same phenomenon with S. simplex and Pycnadenoides in L. atlanticus.

3. Metacercariae- No attempt was made to identify the encysted metacercariae on either the gills or in the musculature of L. atlanticus. Cryptocotyle lingua (a trematode parasite of fish-eating birds and marine mammals) uses snails of the genus Littorina as one intermediate host. This genus of snail is certainly prevalent in tide pools along the New Hampshire coast, but whether the metacercariae in L. atlanticus were C. lingua or not is not known. Also, whether L. atlanticus is a normal or an abnormal intermediate host for these metacercariae is not known.

### Acanthocephala:

1. Echinorhynchus gadi- E. gadi is the most common acanthocephalan parasite of marine fish in northern water. It is circumpolar in distribution, and is most common in gadid fishes. Detwyler (1963) reported this parasite from L. atlanticus as has Bullock (personal communication). Linton (1933) found E. gadi in 54 species of marine teleost at Woods Hole, the highest incidence occurring in Pollachius virens, Gadus morhua, and Pseudopleuronectes americanus. Yamaguti (1963) lists Gammarus locusta (= G. oceanicus in the western Atlantic) as one of the intermediate hosts used by this parasite. This amphipod has been shown to constitute a major part of the diet of L. atlanticus, and this is undoubtedly the source of infection for L. atlanticus. However, none of the E. gadi examined were mature, and therefore L. atlanticus may not be a normal definitive host for this acanthocephalan. E. gadi also appeared to have a reciprocal relationship with S. simplex regarding number of parasites present and location in the host intestine. Histopathology due to this parasite was similar to that found in the white sucker intestine infected with the acanthocephalan Neoechinorhynchus cristatus by Chaicharn and Bullock (1967). That is, only destruction of the epithelium and the underlying lamina propria of the infected intestine occurred.

### Nematoda:

1. Thynnascaris sp.- Twelve of 128 hosts examined were parasitized by a nematode parasite belonging to the

genus Thynnascaris. The identification of this parasite was made by Dr. Robin M. Overstreet of the Gulf Coast Research Laboratory, Ocean Springs, Mississippi. Prior to his identification, I had keyed the specimens out as belonging to the genus Contracaecum. Members of this genus are parasites of fishes, birds, and piscivorous mammals (Yamaguti, 1961). Dr. Overstreet (personal communication) stated the following:

All the specimens that you sent me are a species of Thynnascaris Dollfus, 1933 (= Contracaecum Railliet and Henry, 1912, pro parte). Both of the diverticula can be clearly seen, and the excretory pore is located near the level of the nerve ring. All specimens were larval stages, and the definitive host is fish.

For a review of the taxonomy of the genera Contracaecum and Thynnascaris the reader is referred to Hartwich (1957).

Large nematodes were found in the coelom and ovaries of one of the seasnails examined by Detwyler (1963). These were probably the same genus as was found in this present investigation. Kahl (1936) reported Contracaecum clavatum from Liparis sp. at Woods Hole. Overstreet (1968) reported two species of larval Contracaecum in the marine fish Synodus foetens.

No attempt was made to identify the encysted nematode larvae seen in microscopic sections of the stomachs of three L. atlanticus.

#### Arthropoda:

No parasitic arthropods were found during this investigation. Detwyler (1963) did find one specimen of the

parasitic copepod Lernaeocera branchialis in his investigation, and felt that its occurrence on L. atlanticus may have been accidental.

## SECTION VII

## SUMMARY

The histochemical morphology of the spathebothriid cestode Spathebothrium simplex, parasitic in the marine teleost Liparis atlanticus, has been investigated.

1. The tegument is composed primarily of mucoprotein, while the outer edge of this structure contains acid mucopolysaccharide. Alkaline glycerophosphatase is present in the tegument along the entire length of the parasite, enzyme activity being heaviest in the anterior regions of the cestode. Some basic protein is present in the tegument.
2. Small glycogen deposits are found in the cells of the subtegument of S. simplex. Considerable amounts of RNA are present in the cytoplasm of the cells of this region, while only small amounts of DNA are found in their nuclei. These cells contain much protein. Light alkaline glycerophosphatase activity is present in the cells of the subtegument in the anterior region of the worm.
3. The medullary parenchyma contains heavy glycogen deposits especially in the anterior region of the worm. The cells of this region contain some acid mucopolysaccharide. Trace amounts of RNA, DNA, and lipid are present in the parenchymal cells.

4. Mature spermatozoa contain relatively heavy glycogen deposits. Immature spermatozoa contain none. The cells lining much of the male reproductive system contain mucoprotein. The prostatic cells are rich in RNA and protein. Large amounts of DNA are present in mature spermatozoa. RNA content is greater in immature than mature spermatozoa.
5. Minute amounts of glycogen are present in the developing embryos and uterine wall. The egg shell, uterine and vaginal walls, cells of Mehlis' gland, and the wall of the seminal receptacle all contain mucoprotein. The colloidal material around the oncosphere contains both mucoprotein and mucopolysaccharide. Nuclei of developing embryos are rich in DNA. The cytoplasm of vitelline cells and Mehlis' gland contain much RNA. The cytoplasm of the vitelline cells is rich in basic protein. Newly formed egg shells contain basic protein, while more mature egg shells show only trace amounts. The largest concentration of lipid in S. simplex is located between the egg shell and developing oncosphere.
6. Calcareous corpuscles contain much calcium and some acid mucopolysaccharide.
7. The eggs of S. simplex display filaments radiating out from a raised area at one end of the egg shell. These filaments may serve an adhesive function. This is the

first description of such filaments on the eggs of S. simplex. Observations on the morphology of the nervous system of S. simplex were made.

8. Little histopathological damage is done to the host by this cestode except in heavy infections where the intestinal mucosa is markedly flattened.
9. Attempts to work out the life cycle of S. simplex using gammarid amphipods as possible intermediate hosts were unsuccessful.

A study of the parasites of L. atlanticus showed S. simplex to be the most frequently encountered helminth parasite in this host. Other parasites found in L. atlanticus were:

1. An unidentified species of the sporozoan genus Eimeria was present in every fish examined. In heavy infections this parasite caused severe histopathological damage to the host intestinal mucosa.
2. Spores of an unidentified species of myxosporidian were observed in the feces of one fish and in the gall bladder of a second. Not all fish were examined for the presence of this parasite.
3. An unidentified species of trichodinid ciliate was found on the gills of 38 of 68 hosts examined for this parasite.

4. A gasterostome, tentatively identified as Proisorhynchus crucibulum, parasitized 15 of 128 hosts examined. This parasite was always found in the pyloric ceca of the host, and abraded the intestinal mucosa.
5. The trematode Podocotyle reflexa was the second most frequently encountered helminth parasite in this study. A few P. atomon were also found. These parasites had a reciprocal relationship with S. simplex in respect to number and location of parasite. Histopathology caused by these trematodes was limited to grasping of small bits of host intestinal mucosa except in heavy infections where flattening of the mucosa occurred.
6. Unidentified trematode metacercariae were observed on the gills and in the musculature of the body wall of L. atlanticus.
7. Immature specimens of the acanthocephalan Echinorhynchus gadi were found in 27 of 128 hosts examined. Histopathology caused by this parasite was limited to the immediate area of penetration of the intestinal mucosa by the proboscis of the parasite.
8. Twelve sea snails were parasitized by larval states of a species of the nematode genus Thynnascaris. This parasite was found in the mesenteries and coelom of the host.
9. Unidentified encysted larval nematodes were seen in microscopic sections of the stomach wall of L. atlanticus.

10. No haematozoa or arthropods were observed in or on L.  
atlanticus.

SECTION VIII

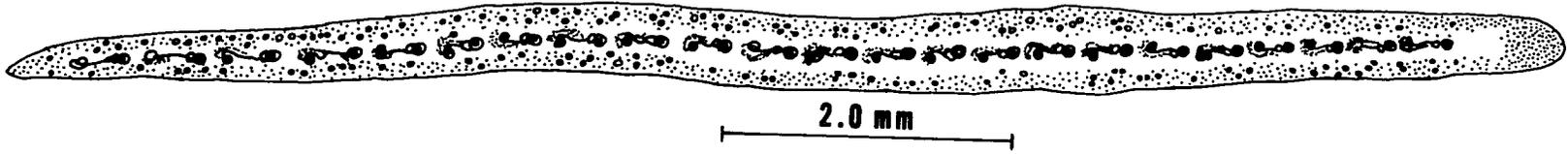
FIGURES

Figure 1 Immature S. simplex showing developing genitalia

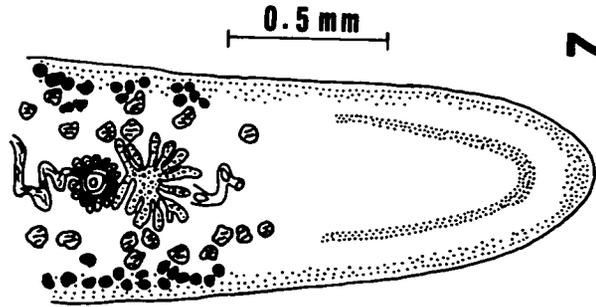
Figure 2 Undifferentiated holdfast of mature S. simplex. Note two longitudinal nerves connected by a cross commissure

Figure 3 Reproductive organs of mature S. simplex

1



2



3

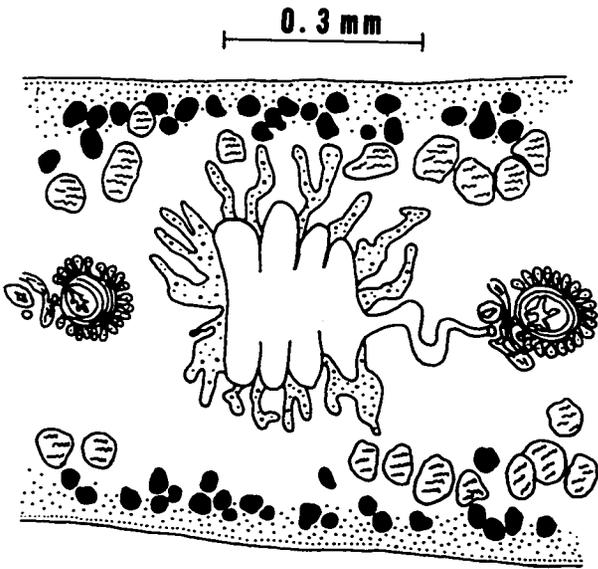


Figure 4 Section through pyloric ceca of L. atlanticus  
showing S. simplex in the ceca. Bouin's,  
Gomori-Wheatley trichrome. X 200



Figure 4

Figure 5 PAS positive staining in tegument and parenchyma of S. simplex after salivary digestion. NBF, PAS-hematoxylin. X 200

Figure 6 Section of S. simplex similar to fig. 5 after lipid extraction. Note retention of PAS positive staining in tegument, parenchyma, and egg shells. NBF, PAS-hematoxylin. X 200



Figure 5

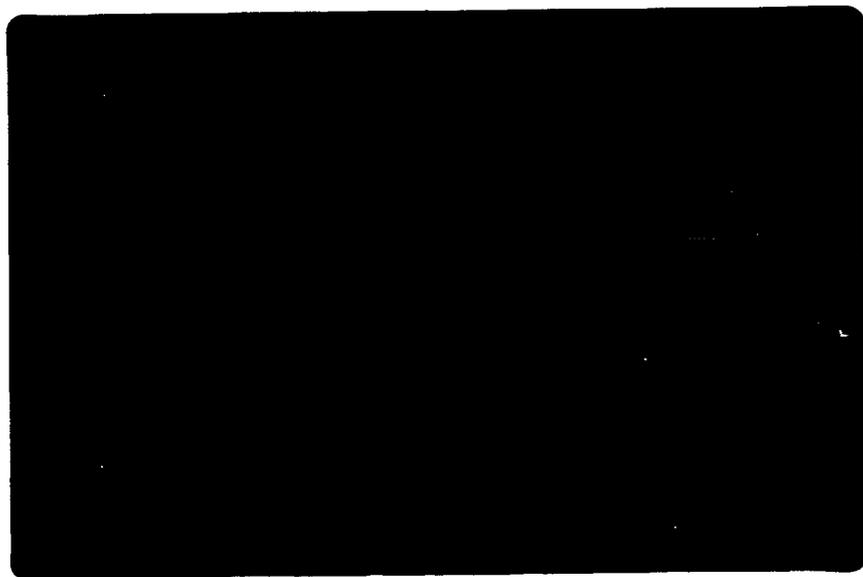


Figure 6

Figure 7 Section of S. simplex comparable to fig. 6 after acetylation. Note lack of any PAS positive staining. The dark staining material is the nuclear counterstain. NBF, PAS-hematoxylin. X 200

Figure 8 Section comparable to fig. 7 after de-acetylation. Note return of PAS positive staining. NBF, PAS-hematoxylin. X 200

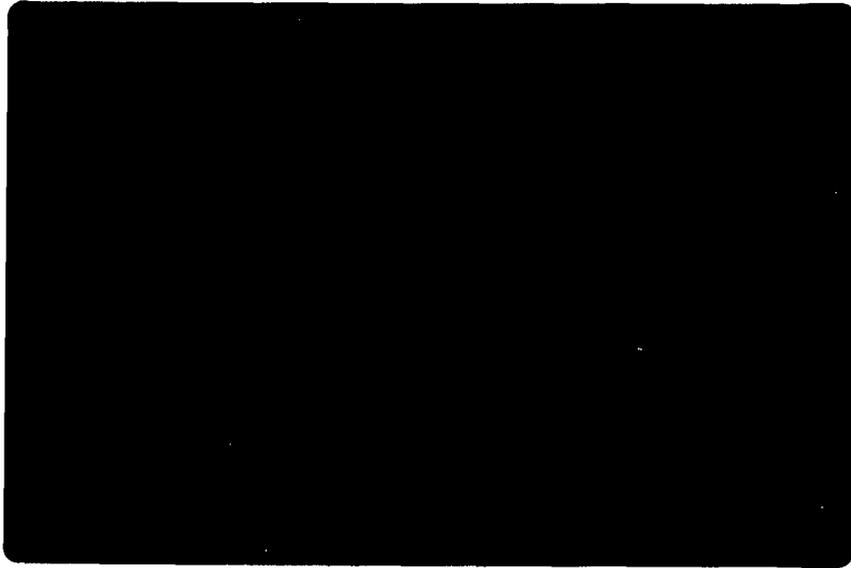


Figure 7

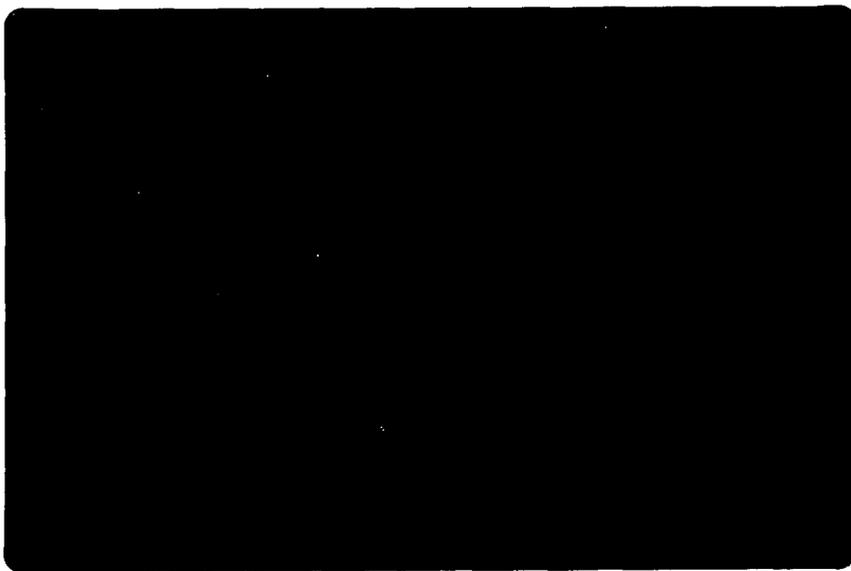


Figure 8

Figure 9 Section of S. simplex exposed to the PAS technique without prior oxidation with periodic acid. Note lack of any PAS positive staining. NBF, PAS-hematoxylin. X 200

Figure 10 Section through the intestine of L. atlanticus showing the positive staining of the tegument of S. simplex for acid mucopolysaccharides (blue). NBF, Alcian blue-neutral red. X 200

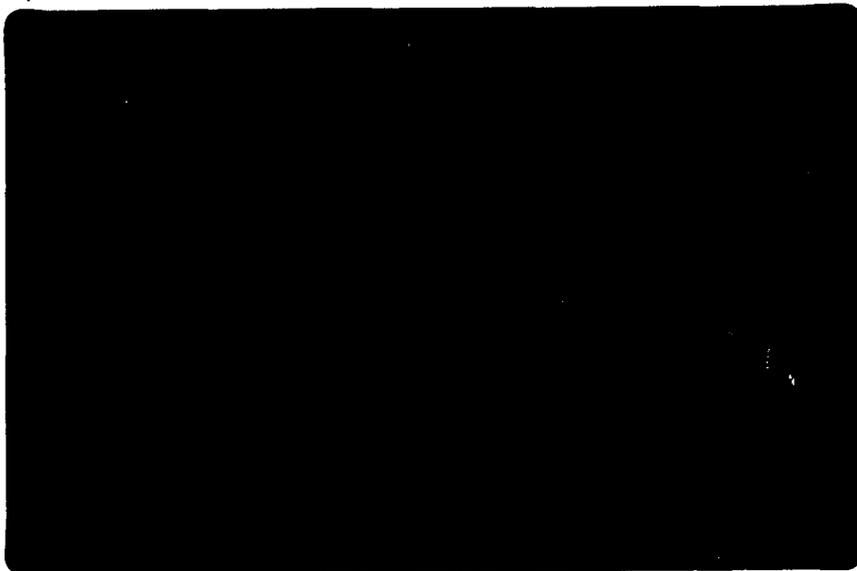


Figure 9

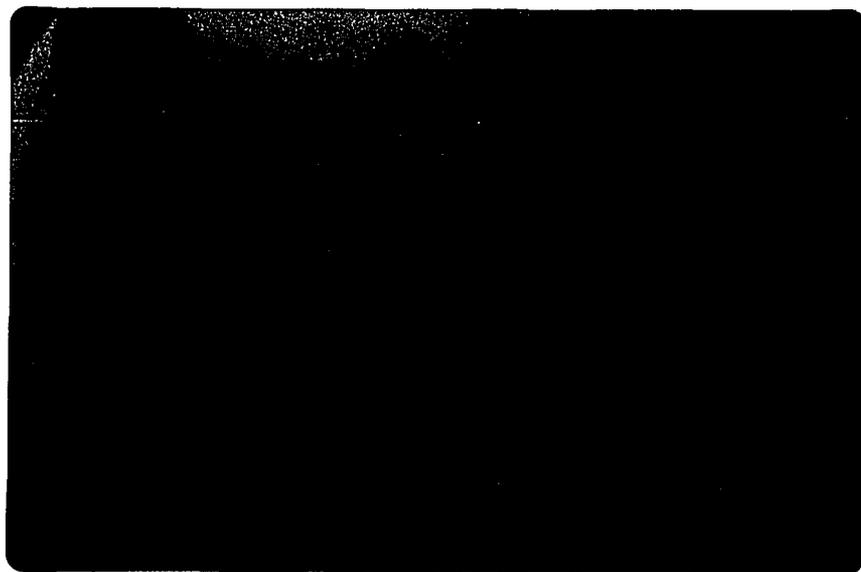


Figure 10

Figure 11 Section through the anterior intestine of L.  
atlanticus. Note strong alkaline glycerophosphatase activity in the tegument of the parasite. Also note the light enzyme activity in the subtegument of the anterior region (smaller section of cestode) and the light positive reaction in the brush border and lamina propria of the host intestine. X 200

Figure 12 Section comparable to fig. 11, control. Note lack of all enzyme activity and false positive reaction in the calcareous corpuscles. X 200



Figure 11



Figure 12

Figure 13 Anterior end of S. simplex showing heavy glycogen deposits in medullary parenchyma anterior to first set of reproductive organs. Gendre's, PAS-hematoxylin. X 200

Figure 14 Section comparable to fig. 13 after salivary digestion. Note absence of glycogen deposits in medullary parenchyma. Gendre's, PAS-hematoxylin. X 200



Figure 13

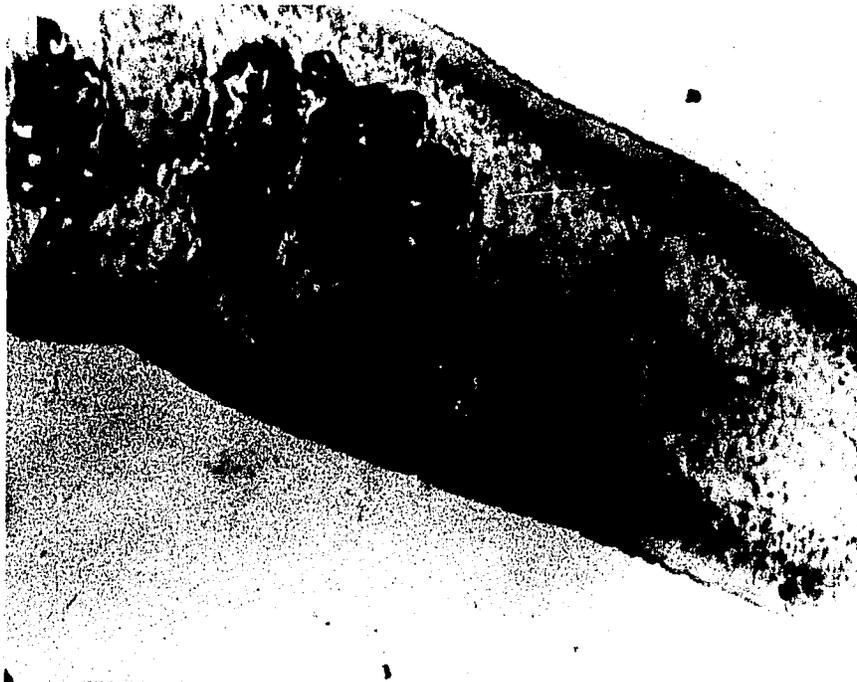


Figure 14

Figure 15 Strong PAS positive reaction in the prostatic epithelium of S. simplex after salivary digestion. Gendre's, PAS-hematoxylin. X 860

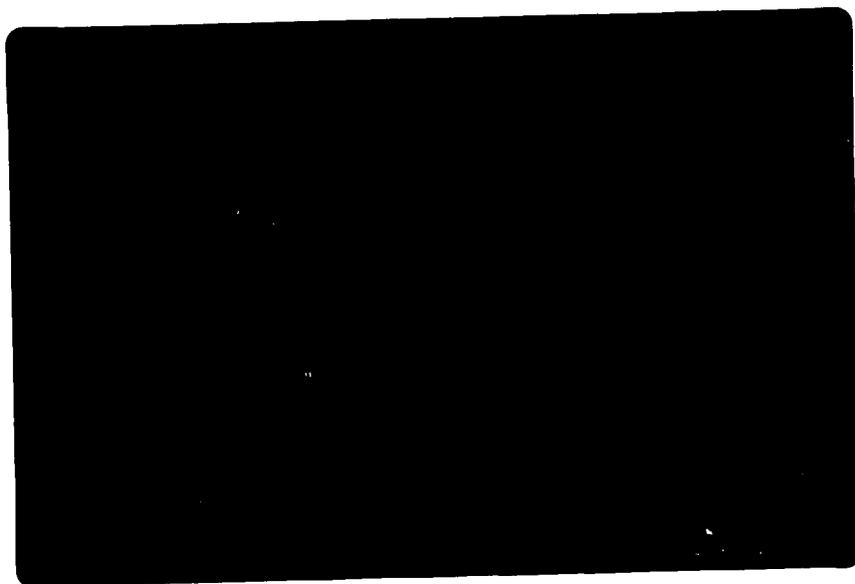


Figure 15

Figure 16 Prostatic cells surrounding cirrus pouch of S. simplex. Bouin's, Gomori-Wheatley trichrome. X 860



Figure 16

Figure 17 Section of S. simplex showing bright yellow staining reaction of vitellaria indicating the presence of protein, and reddish staining of vaginal wall and seminal receptacle wall indicating the presence of carbohydrate-protein complexes. Zenker's, Himes and Moriber triple stain. X 860

Figure 18 PAS positive staining reaction in the cells of Mehlis' gland in S. simplex after salivary digestion. NBF, PAS-hematoxylin. X 860

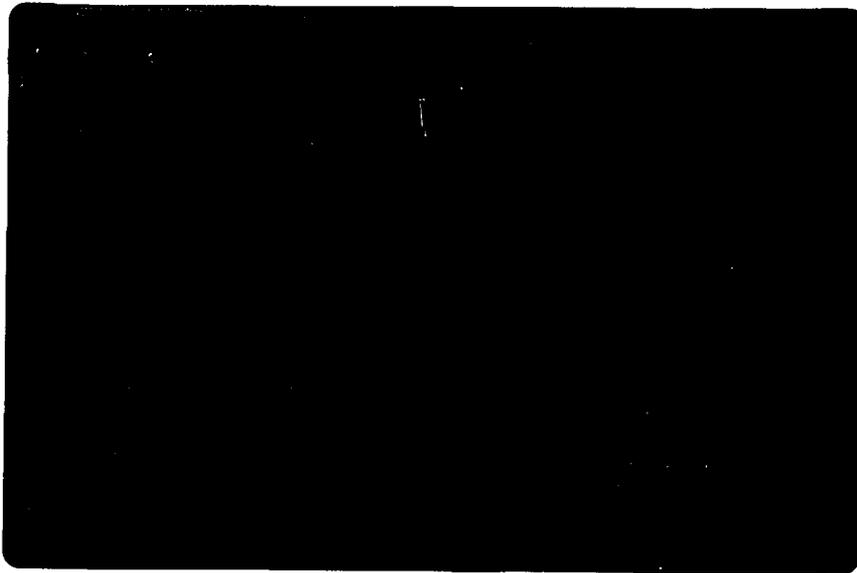


Figure 17

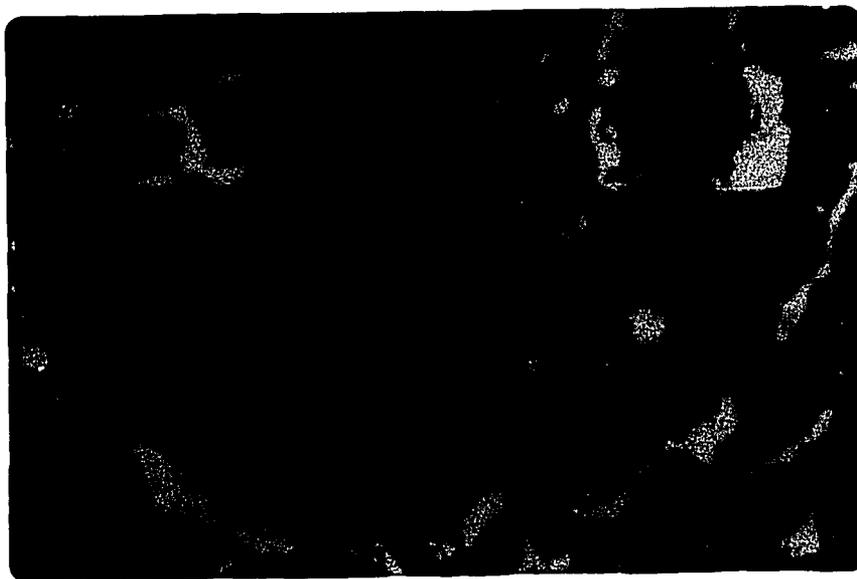


Figure 18

Figure 19 RNA content in the vitelline follicles of S.  
simplex. NBF, chrome alum-galloycyanin. X 860

Figure 20 Section comparable to fig. 19 after RNase  
digestion. Note much lighter staining of the  
vitelline follicles. NBF, chrome alum-  
galloycyanin. X 860



Figure 19



Figure 20

Figure 21 Section through the uterus of S. simplex  
showing lipid droplets (red) located between  
the egg shell and the developing oncosphere.  
Frozen section, oil red O-hematoxylin. X 860

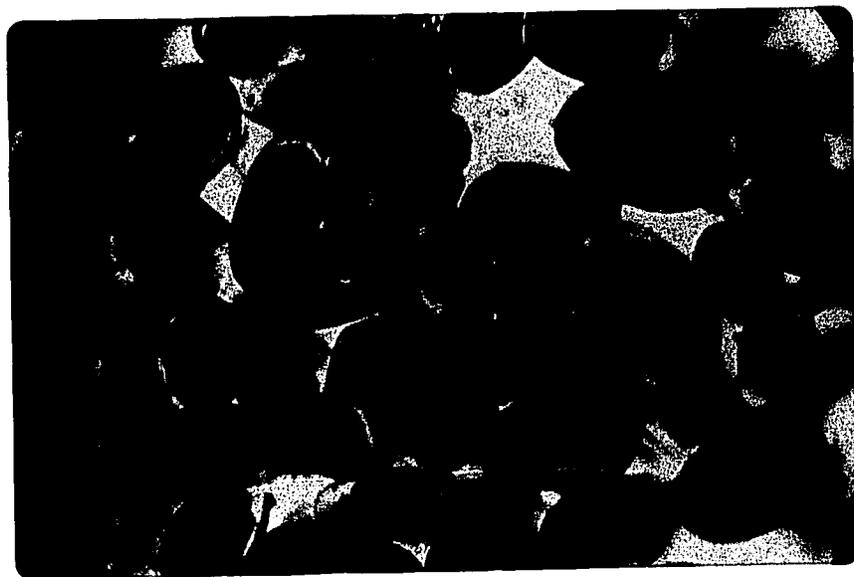


Figure 21

Figure 22 Eggs of S. simplex showing filaments radiating out from raised knob at one end of the egg.  
0.001% thionin X 1940

Figure 23 Section through the intestine of L. atlanticus showing heavy infection with S. simplex and resulting flattening of the intestinal mucosa.  
Bouin's aldehyde-fuchsin. X 70



Figure 22



Figure 23

Figure 24 Higher magnification of fig. 23. Note compression of the intestinal mucosa immediately to the left of the parasite. Bouin's, aldehyde-fuchsin. X 860

Figure 25 Section through the rectum of L. atlanticus showing heavy infection of Eimeria sp. in the mucosa and submucosa. Note the two sections of an E. gadi on the right and section of P. reflexa in the middle of the photograph. Zenker's, Mallory's phosphotungstic acid hematoxylin. X 200



Figure 24



Figure 25

Figure 26 Trichodinid ciliates on gill filament of  
L. atlanticus. AFA, chrome alum-gallo-  
cyanin chromotrope 2R. X 200

Figure 27 Aboral view of a trichodinid ciliate  
showing cilia and skeletal ring.  
nigrosin-HgCl<sub>2</sub>-formalin. X 970

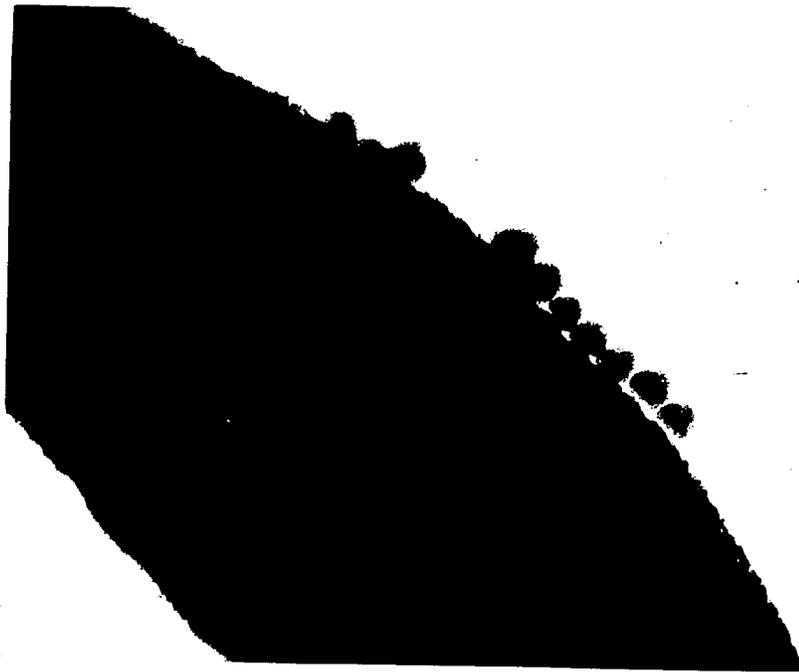


Figure 26



Figure 27

Figure 28 Section through the intestine of L. atlanticus showing the oral sucker of P. reflexa grasping the host mucosa. Note presence of E. gadi in upper right corner. Zenker's, Mallory's phosphotungstic acid hematoxylin. X 200

Figure 29 Encysted metacercaria in the musculature of L. atlanticus. Bouin's, PAS-hematoxylin. X 200



Figure 28



Figure 29

Figure 30 Section through the intestine of L. atlanticus showing the proboscis of E. gadi penetrating the intestinal mucosa. Note tissue destruction at the point of penetration. Zenker's, Mallory's phosphotungstic acid hematoxylin. X 200

Figure 31 Section through the stomach wall of L. atlanticus showing an encysted larval nematode. NBF, aldehyde-fuchsin. X 860



Figure 30

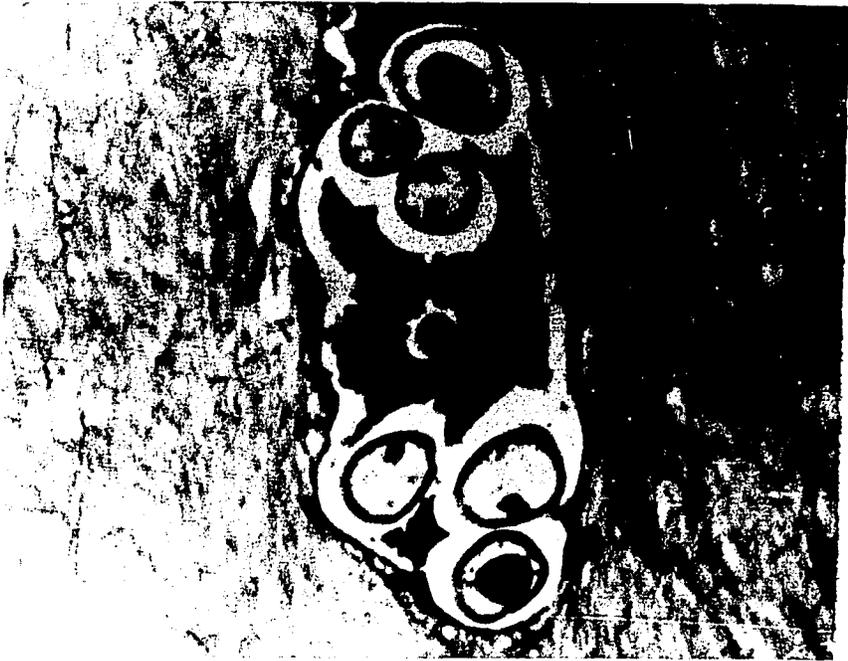


Figure 31

## SECTION IX

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## SECTION X

## APPENDIX

## FORMULARY OF STAINS AND PROCEDURES

Ringer's Solution (Humason, 1962)

Sodium chloride . . . . .	0.65 gm.
Potassium chloride. . . . .	0.042 gm.
Calcium chloride. . . . .	0.025 gm.
Distilled water . . . . .	100.0 ml.

Bouin's Fixative (Humason, 1962)

Picric acid, saturated aqueous. . . . .	75.0 ml.
Formalin. . . . .	25.0 ml.
Glacial acetic acid . . . . .	5.0 ml.

Gendre's Fluid (Humason, 1962)

95% ethyl alcohol saturated with picric acid. . . . .	80.0 ml.
Formalin. . . . .	15.0 ml.
Glacial acetic acid . . . . .	5.0 ml.

Formol - Calcium (Culling, 1963)

Formalin. . . . .	10.0 ml.
Calcium chloride. . . . .	1.0 gm.
Distilled water . . . . .	90.0 ml.

Neutral Buffered Formalin (Barka and Anderson, 1963)

40% formaldehyde solution . . . . .	100.0 ml.
Distilled water . . . . .	900.0 ml.
Acid sodium phosphate, monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) . . . . .	4.0 gm.
Anhydrous <sup>2</sup> disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ). . . . .	6.5 gm.

Helly's Fixative (Humason, 1962)

Potassium dichromate. . . . .	2.5 gm.
Mercuric chloride . . . . .	4.0-5.0 gm.
Distilled water . . . . .	100.0 ml.
Formalin (add just before using). . . . .	5.0 ml.

Zenker's Fixative (Humason, 1962)

Potassium dichromate. . . . .	2.5 gm.
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Mercuric Chloride . . . . .	4.0-5.0 gm.
Distilled water . . . . .	100.0 ml.
Glacial acetic acid . . . . .	5.0 ml.

Alcohol-Formalin-Acetic Acid (AFA) (Bullock, personal communication)

Formalin. . . . .	5.0 ml.
Glacial acetic acid . . . . .	5.0 ml.
Glycerin. . . . .	10.0 ml.
95% ethyl alcohol . . . . .	24.0 ml.
Distilled water . . . . .	46.0 ml.

Travassos' Fixative (Bullock, personal communication)

0.85% saline. . . . .	92.0 ml.
Formalin. . . . .	5.0 ml.
Glacial acetic acid . . . . .	3.0 ml.
Glycerin. . . . .	5.0 ml.

Giemsa Method For Blood Smears (Bullock, personal communication)

Giemsa Stock Solution:

Giemsa Powder. . . . .	1.0 gm.
Glycerin . . . . .	66.0 ml.
Methyl alcohol . . . . .	66.0 ml.

Work Giemsa powder into glycerin and place in 60° C incubator for two hours. Add methyl alcohol and stopper tightly.

0.2M Phosphate Buffer, pH 6.5. (Humason, 1962)

Giemsa Working Solution (make fresh after every ten slides):

Giemsa stock. . . . .	1.0 ml.
Phosphate buffer. . . . .	2.0 ml.
Distilled water . . . . .	47.0 ml.

Procedure:

1. Thin blood smear is made and air dried.
2. Fix smears in absolute methanol for 5-10 minutes.
3. Place slides in Giemsa working solution for 1-2 hours.
4. Rinse off excess stain in buffered, distilled water (47.0 ml. distilled water, and 2.0 ml. phosphate buffer pH 6.5).
5. Let slides air dry.

Nigrosin-HgCl<sub>2</sub>-Formalin (Borrer, 1968)

## Solution A:

HgCl<sub>2</sub>, sat. aq., 10.0 ml; glacial acetic acid, 2.0 ml; formalin, 2.0 ml; t-butanol, 10.0 ml.

## Solution B:

Formalin, 20.0 ml; nigrosin, water soluble, 4.0 gm; distilled water, 100.0 ml.

## Working Solution:

To 12 volumes of A add one volume of B.

## Procedure:

Place a drop of concentrated suspension of organisms on a clean slide, and pipette onto it from a height of 2-3 cm. a drop of solution C. After a few seconds, wash the culture fluid to the end of the slide by additional drops of solution C. Practically all of the specimens will be fixed, stained, and attached to the slide, and after about 15 seconds the preparation may be dehydrated in alcohol, cleared, and covered.

Chrome Alum-Gallocyanin (Barka and Anderson, 1963)

Dissolve 5.0 gm of chrome alum (chromium potassium sulfate) in 100.0 ml. of distilled water. Add 0.15 gm. of gallocyanin and boil the solution for 5 minutes. Cool, filter, and adjust to a final volume of 100.0 ml. with distilled water. This solution can be used for one month.

## For staining sections:

1. Bring sections from xylene to water, using iodine if necessary.
2. Stain 24-48 hours in chrome alum-gallocyanin.
3. Wash in tap water and rinse in distilled water.
4. Dehydrate thru 70% and 85% ethyl alcohol.
5. Counterstain about 1 minute in 0.1% chromotrope 2R in 95% ethyl alcohol.
6. Complete dehydration with 2 minutes in 95% ethyl alcohol and 3 minutes in absolute ethyl alcohol.
7. Clear in xylene and mount.

## For staining helminth whole mounts:

1. Bring helminths down to water from storage solution (usually 70% ethanol).
2. Stain 24-48 hours in chrome alum-gallocyanin.
3. Wash in several changes of distilled water.

4. Dehydrate through the alcohols. Counterstaining in 0.001% chromotrope 2R in 100% ethyl alcohol usually accentuates muscle in contrast to nuclei and glands (which stain with gallo-cyanin). Staining time varies from 20 minutes to an hour or more. For short times dehydrate in 100% ethyl alcohol for a while first so that you can transfer from the counterstain into the clearing agent.
5. Clear in clearing agent best suited for parasite and mount.

Harris' Hematoxylin (Humason, 1962)

Dissolve 1.0 gm. hematoxylin in 10.0 ml. absolute ethyl alcohol. Dissolve 20.0 gm. potassium or ammonium alum,  $\text{Al}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$  or  $\text{Al}_2(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ , in 200.0 ml. distilled water and boil. Add hematoxylin and boil  $\frac{1}{2}$  minute. Add 0.5 gm. mercuric oxide. Cool rapidly. Add a few drops of glacial acetic acid. Do not keep longer than one or two months.

Procedure:

1. Bring helminths from storage solution to water.
2. Stain helminths in Harris' stock diluted 10:1 with distilled water overnight.
3. Rinse in tap water, upgrade through 35%, 50%, and 70% ethyl alcohol.
4. Destain in acid alcohol (1-2 ml. conc. HCl in 98-99 ml. 70% ethyl alcohol) until specimens are light pink.
5. Place helminths in alkaline alcohol (small amount of sodium bicarbonate in a petri dish with 70% ethyl alcohol) until specimens turn light blue.
6. Dehydrate, clear, and mount.

Grenacher's Borax - Carmine (Meyer and Penner, 1962)

Carmine. . . . .	3.0 gm.
Borax. . . . .	4.0 gm.
Distilled water. . . . .	100.0 ml.
70% alcohol (preferably methyl). . . . .	100.0 ml.

Add the carmine and the borax to the water and boil until the carmine is dissolved (one half hour or more), or better, allow the mixture to stand for two or three days, with an occasional stirring, until this occurs, and then add the alcohol. Allow the solution to stand for a few days and then filter.

**Procedure:**

1. Transfer helminths from 70% ethyl alcohol into undiluted Grenacher's borax-carmin, and stain for approximately 12 hours (overnight).
2. Add conc. HCl drop by drop to dish containing helminths and stain, agitating the dish until the carmine is precipitated out as a brick red flocculent mass. Allow the dish to stand for 6-8 hours, or preferably overnight.
3. Place specimens in 1% acid-alcohol and destain until they are light pink. This usually takes several hours, depending on the size of the specimens.
4. Place specimens in 85% ethyl alcohol for at least one hour and change the alcohol at least three times during this time period.
5. Complete dehydration, clear, and mount.

**Ehrlich's Hematoxylin (Humason, 1962)**

Hematoxylin . . . . .	2.0 gm.
Ammonium alum, $Al_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$ . . . . .	3.0 gm.
Alcohol, absolute ethyl or methyl . . . . .	100.0 ml.
Glycerin. . . . .	100.0 ml.
Distilled water . . . . .	100.0 ml.

Let mixture ripen in the light for 6-8 weeks.

**Hematoxylin and Eosin (H and E)**

**Procedure:** Various fixatives.

1. Bring sections from xylene to water, using iodine if necessary.
2. Stain section in Ehrlich's hematoxylin 1:9 in distilled water for 30 minutes.
3. Dehydrate thru 85% ethyl alcohol.
4. Counterstain sections in 0.05% eosin in 95% ethyl alcohol for 15-30 seconds.
5. Complete dehydration, clear, and mount.

**Gomori-Wheatley Trichrome (Bullock, personal communication)**

Chromotrope 2R. . . . .	0.6 gm.
Fast green. . . . .	0.3 gm.
Phosphotungstic acid. . . . .	0.7 gm.
Glacial acetic acid . . . . .	1.0 ml.
Distilled water . . . . .	100.0 ml.

**Procedure:** Good after Bouin's, Gendre's, and neutral buffered formalin. Fair after Helly's. Poor after Zenker's.

1. Bring sections from xylene to water, using iodine if necessary.
2. Stain in trichrome 8-15 minutes.
3. Rinse sections in 1% acetic acid in 90% ethyl alcohol until excess stain no longer flows from slide.
4. Dip slide twice in absolute ethyl alcohol.
5. Dehydrate in a second change of absolute ethyl alcohol for 30 seconds.
6. Clear in xylene and mount.

Mallory's Phosphotungstic Acid Hematoxylin (Culling, 1963)

Hematoxylin. . . . .	1.0 gm.
Phosphotungstic acid . . . . .	20.0 gm.
Distilled water. . . . .	1000.0 ml.

This ripens in 6-8 weeks.

Procedure: Zenker's fixed material.

1. Bring sections to 95% ethyl alcohol, using iodine in the 95% ethyl alcohol.
2. Place sections in a 0.5% sodium thiosulfate solution for 5 minutes.
3. Wash in tap water.
4. Place sections in 0.25% potassium permanganate for 5 minutes.
5. Wash sections in tap water and rinse in distilled water.
6. Place sections in 5% oxalic acid for 5 minutes.
7. Rinse sections in distilled water, and wash them in running water for 5 minutes.
8. Stain sections in phosphotungstic acid hematoxylin for 12-24 hours (overnight).
9. Dehydrate sections rapidly in 95% and absolute ethanol or acetone.
10. Clear with 50:50 mixture of the dehydrating agent and xylene, followed by two changes of xylene.
11. Mount sections.

Basic Fuchsin and Picro-Indigo Carmine (Shumway, 1926)

Procedure: Any general fixative, preferably containing formalin.

1. Bring sections from xylene to water, using iodine if necessary.
2. Place sections in a saturated aqueous solution of basic fuchsin for 20 minutes.
3. Without rinsing, place sections in a solution composed of one part saturated aqueous indigo carmine and 5 parts of saturated aqueous picric acid for 5 minutes.

4. Transfer sections to 70% ethyl alcohol and decolorize for a few seconds only until sections appear light pink.
5. Dehydrate rapidly in 95% ethyl alcohol and absolute ethyl alcohol for a few seconds until sections appear blue or green.
6. Clear and mount.

Mallory's Rapid One-Step Stain (Humason, 1962)

Distilled water . . . . . 200.0 ml.  
 Dissolve each of the below before adding next stain  
 Phosphotungstic acid. . . . . 1.0 gm.  
 Orange G. . . . . 2.0 gm.  
 Aniline blue. . . . . 1.0 gm.  
 Acid fuchsin. . . . . 3.0 gm.

Keeps for several months.

Procedure: Any general fixative.

1. Bring sections from xylene to water, using iodine if necessary.
2. Stain in above staining solution for 5 minutes.
3. Wash in running water 3-5 seconds.
4. Dehydrate rapidly thru 95 and 100% ethyl alcohol.
5. Clear and mount.

Aldehyde-Fuchsin (Gabe's method, Cameron and Steele, 1959)

To 200.0 ml. of boiling distilled water add 1.0 gm. of basic fuchsin. Boil one minute, cool, and filter. Add 2.0 ml. conc. HCl and 2.0 ml. paraldehyde. Leave stoppered at room temperature. Withdraw a drop daily and place on filter paper. When the solution has lost its red color (about 3 or 4 days), filter and discard the filtrate. Dry precipitate on filter paper and scrape it carefully into a small bottle for storage. Yield should be about 1.9 gm.

To make staining solution dissolve 0.25 gm. of dry stain in 50.0 ml. of 70% ethyl alcohol. This solution is stable for six months.

Halmi's Counterstain (Cameron and Steele, 1959)

Fast green. . . . . 0.2 gm.  
 Orange G. . . . . 1.0 gm.  
 Chromotrope 2R. . . . . 0.5 gm.  
 Phosphotungstic acid. . . . . 0.5 gm.

Dissolve in 100.0 ml. of distilled water and add 1.0 ml. of glacial acetic acid.

Procedure: After Bouin's or neutral buffered formalin.  
Process one slide at a time.

1. Bring sections from xylene to water.
2. Oxidize sections for one minute in Gomori's fluid; 0.15%  $\text{KMnO}_4$  in 50.0 ml. of 0.2%  $\text{H}_2\text{SO}_4$ . Make up fresh every 2 or 3 hours.
3. Rinse sections in 2.5% sodium bisulfite for a few seconds. Make this solution up fresh daily.
4. Rinse in distilled water.
5. Place sections in 35% ethyl alcohol for 30-60 seconds.
6. Place sections in 70% ethyl alcohol for 30-60 seconds.
7. Stain sections in aldehyde-fuchsin for 2-15 minutes (10 minutes is good for routine).
8. Quickly remove slide from stain, wipe back of slide with tissue, rinse in 95% ethyl alcohol, and transfer to fresh 95% ethyl alcohol until no more stain comes out of sections (about 2 to 5 minutes).
9. Place sections in 70% alcohol for 30-60 seconds.
10. Place sections in 35% alcohol for 30-60 seconds.
11. Counterstain in Halmi's for 20-30 seconds.
12. Wipe back of slide and differentiate in 0.2% acetic acid in 95% ethyl alcohol for 2-3 minutes.
13. Place sections in 95% alcohol for 30 seconds.
14. Place sections in absolute ethyl alcohol for 2 minutes.
15. Clear in xylene and mount.

Buffered Azure - Eosinate (Lillie, 1948)

Azure-eosinate stock:

Azure-eosinate stain. . . . .	1.0 gm.
Glycerin. . . . .	50.0 ml.
100% methyl alcohol . . . . .	50.0 ml.

Buffered azure-eosinate:

Stock azure-eosinate . . . . .	0.5 ml.
Acetone. . . . .	5.0 ml.
Buffer (see below) . . . . .	2.0 ml.
Distilled water. . . . .	32.5 ml.

Milliliters of Buffer for Various pH Levels:

M/10 Citric Acid	M/5 Disodium Phosphate	pH
1.6	0.4	3.05
1.2	0.8	4.1
1.0	1.0	4.95
0.8	1.2	6.0

**Procedure:** Various fixatives, but not those containing picric acid such as Bouin's.

1. Bring sections from xylene to water, using iodine if necessary.
2. Stain in buffered azure-eosinate solution for one hour.
3. Rinse briefly in distilled water.
4. Dehydrate in 3 changes of acetone, 30-60 seconds per change.
5. Clear in acetone-xylene (equal parts for 2 minutes).
6. Clear in xylene and mount.

Ribonuclease Digestion (Pearse, 1961)

1. Bring sections to distilled water using iodine if necessary.
2. Incubate sections for one hour at 37° C in buffered enzyme solution (described below), using simultaneously digested controls in solvent without enzyme.  
 Solvent: 8.0 gm. sodium chloride, 0.28 gm. anhydrous disodium phosphate, and 1.97 gm. sodium acid phosphate monohydrate. Make up to one liter with distilled water. Solvent is now at a pH of 6.0 and acts as a buffer.  
 Working solution: Add 5.0 mg. of ribonuclease to 50.0 ml. of buffer.
3. Stain sections by whatever method desired.

Periodic-Acid Schiff (PAS) (Lillie, 1948)

**Schiff Reagent:** Dissolve 1.0 gm. of basic fuchsin in 100.0 ml. of boiling water. Filter at 50-60° C. Add 2.0 gm. sodium bisulfite and 20.0 ml. N HCl. Stopper tightly and store in dark overnight. The next day add 300.0 mg. activated charcoal. Shake one minute and filter. Repeat until solution is water white. Store in refrigerator.

**Procedure:** Various fixatives, preferably non aqueous or only slightly aqueous, such as Gendre's, if glycogen is to be preserved and stained.

1. Bring sections from xylene to water, using iodine if necessary.
2. Hydrolize sections 7-8 minutes in 0.5% aqueous periodic acid.
3. Rinse sections for 5 minutes in running water.
4. Place sections in Schiff reagent for 15 minutes.
5. Place sections in 3 changes of freshly prepared dilute sulfurous acid (10.0 ml. of 10% sodium

bisulfite solution, 10.0 ml. N HCl, 180.0 ml. distilled water). At least 2 minutes each change.

6. Place sections in running water for one minute.
7. Place sections in Ehrlich's hematoxylin (1:9 in distilled water) for 30 minutes.
8. Place sections in running water for 20-30 minutes.
9. Dehydrate, clear and mount.

#### Salivary Digestion:

1. Bring sections from xylene to water, using iodine if necessary.
2. Place sections in saliva that has been strained through cheesecloth for one hour at 37° C.
3. Wash sections in running water for 5 minutes.
4. Proceed with the PAS technique beginning with periodic acid hydrolysis.

#### Acetylation:

1. Bring sections from xylene to water, using iodine if necessary.
2. Place sections for 6 hours at 58° C in a 2:3 mixture of acetic anhydride:pyridine.
3. Rinse sections in absolute ethanol for 2 minutes and briefly in distilled water.
4. The acetylated sections are then subjected to the PAS reaction together with the non-acetylated controls beginning with periodic acid hydrolysis.

#### Himes and Moriber Triple Stain (Humason, 1962)

##### Azure A-Schiff reagent:

Azure A. . . . . 0.5 gm.  
Bleach solution. . . . . 100.0 ml.

##### Bleach:

Potassium or sodium metabisulfate, 10% aqueous 5.0 ml.  
N HCl . . . . . 0.5 ml.  
Distilled water . . . . . 90.0 ml.

##### Basic Fuchsin-Schiff reagent:

Stock: Naphthol yellow S. . . . . 1.0 gm.  
1% aqueous acetic acid. . . . . 100.0 ml.

Working solution: Stock solution . . . . . 2.0 ml.  
1% aqueous acetic acid. 100.0 ml.

**Procedure: Any general fixative.**

1. Bring sections from xylene to water, using iodine if necessary.
2. Hydrolyze sections in N HCl at 60° C for 12 minutes, no longer.
3. Rinse sections in distilled water.
4. Treat sections with Azure A-Schiff for 5 minutes.
5. Rinse sections in distilled water.
6. Place sections in 2 changes of bleach, 2 minutes each change.
7. Rinse sections in distilled water.
8. Oxidize sections in 0.5% aqueous periodic acid for 2 minutes.
9. Rinse sections in distilled water.
10. Treat sections with basic fuchsin-Schiff for 2 minutes.
11. Rinse sections in distilled water.
12. Place sections in 2 changes of bleach, 2 minutes each change.
13. Rinse sections in distilled water.
14. Stain sections in naphthol yellow S for 2 minutes.
15. Rinse sections briefly in distilled water.
16. Dehydrate sections in tertiary or isopropyl alcohol, 2 changes.
17. Clear in xylene and mount.

Alcian Blue (Barka and Anderson, 1963)

**Procedure:** Fix tissues in neutral buffered formalin or Carnoy's fixative.

1. Bring sections from xylene to water.
2. Stain sections in 0.1-0.2% Alcian blue in 0.01 N HCl for 10 minutes.
3. Rinse sections briefly in 3 changes of distilled water.
4. Rinse sections briefly in 0.01 N HCl.
5. Rinse sections briefly in distilled water.
6. Use a red nuclear counterstain such as safranin.
7. Dehydrate, clear in xylene, and mount.

Colloidal Iron (Barka and Anderson, 1963)

**Stock solution:** To 750.0 ml. of boiling distilled water add 12.0 ml. of a 32% ferric chloride solution with constant stirring. This is stable for months.

**Working solution:** Dilute the stock solution with glacial acetic acid in the proportion of 10:1. This solution can be used for about one week.

Procedure: Fix tissues in neutral buffered formalin or Carnoy's.

1. Bring sections from xylene to water.
2. Stain sections in colloidal iron-acetic acid solution for 10 minutes.
3. Wash sections in 2 changes of 5% acetic acid, 5 minutes to each change.
4. Place sections for 10 minutes into the following freshly prepared solution: 30.0 ml. of 2% potassium ferrocyanide and 60.0 ml. of 1% HCl.
5. Rinse sections in distilled water.
6. Use a red nuclear counterstain such as safranin.
7. Dehydrate, clear in xylene, and mount.

Astra Blue (Barka and Anderson, 1963)

Procedure: Fix tissues in neutral buffered formalin or Carnoy's.

1. Bring sections from xylene to water.
2. Stain sections with 1% Astra blue in 1% acetic acid for 5-10 minutes.
3. Rinse sections in 1% acetic acid and in distilled water.
4. Use a red nuclear counterstain such as safranin.
5. Dehydrate, clear in xylene, and mount.

Toluidine Blue (Aqueous) (Pearse, 1961)

Procedure: Fix tissues in neutral buffered formalin or Carnoy's.

1. Bring sections from xylene to water.
2. Stain sections in 0.5% aqueous toluidine blue for 4-6 hours.
3. Rinse sections in distilled water.
4. For histochemical purposes examine immediately under water.
5. Mount in glycerine jelly.

Toluidine Blue (Alcoholic) (Pearse, 1961)

Procedure: Fix tissues in neutral buffered formalin or Carnoy's.

1. Bring sections from xylene to water.
2. Stain sections in 0.1% toluidine blue in 30% ethyl alcohol for 5-20 minutes.
3. Rinse sections in 95% ethyl alcohol.
4. Dehydrate sections in absolute ethanol.
5. Clear in xylene and mount.

Colloidal Iron-Periodic Acid-Schiff (Mowry, 1963)

Procedure: Any general fixative, neutral buffered formalin preferred.

1. Bring sections from xylene to water, using iodine if necessary.
2. Place sections for 2 hours in working solution of colloidal iron (see colloidal iron technique).
3. Rinse sections in 3 changes of 30% acetic acid, 10 minutes each change. (Bring control sections to water, without exposure to colloidal iron.)
4. Wash sections in running water for 5 minutes; rinse briefly in distilled water.
5. Treat sections for 20 minutes in freshly prepared mixture of equal volumes of 2% HCl and 2% potassium ferrocyanide.
6. Wash sections for 5 minutes in running water; rinse briefly in distilled water.
7. Oxidize sections for 10 minutes in 0.5% aqueous periodic acid.
8. Wash sections in running water for 5 minutes; rinse in distilled water.
9. Treat sections in Schiff's reagent for 10 minutes (see PAS technique).
10. Rinse sections in 3 changes of dilute sulfurous acid for 2 minutes each (see PAS technique).
11. Wash sections for 5 minutes in tap water and briefly in distilled water.
12. Stain in Harris' hematoxylin for 5 minutes.
13. Wash sections in tap water for a few seconds.
14. Dip sections for 10-20 seconds in acid alcohol.
15. Wash sections for 3 minutes but no longer in running tap water.
16. Dehydrate, clear in xylene, and mount.

Alcian Blue-Periodic Acid Schiff (Mowry, 1963)

Procedure: Any general fixative, neutral buffered formalin preferred.

1. Bring sections from xylene to water, using iodine if necessary.
2. Rinse sections in 3% acetic acid for 3 minutes.
3. Stain sections for 2 hours in Alcian blue solution (see Alcian blue technique).
4. Dip sections in running water and rinse for 3-5 minutes in 3% acetic acid.
5. Rinse sections for 3 minutes in running water and briefly in distilled water.
6. Oxidize sections for 10 minutes in 0.5% aqueous periodic acid.
7. Wash sections in running water for 5 minutes; rinse in distilled water.

8. Treat sections in Schiff's reagent for 10 minutes (see PAS technique).
9. Rinse sections in 3 changes of dilute sulfurous acid for 2 minutes (see PAS technique).
10. Wash sections for 5 minutes in running water and briefly in distilled water.
11. Stain sections for 5 minutes in Harris' hematoxylin.
12. Wash sections in tap water for a few seconds.
13. Dip sections for 20-30 seconds in acid alcohol.
14. Wash sections for 3 minutes in running tap water.
15. Dehydrate, clear in xylene, and mount.

#### Feulgen Reaction (DiStefano, 1952)

Procedure: Any general fixative, but not after Bouin's.

1. Bring sections from xylene to water, using iodine if necessary.
2. Place sections in 10% perchloric acid for 12-24 hours at room temperature.
3. Drain slides and put them in Schiff's reagent (see PAS technique) for 20-30 minutes.
4. Wash sections in 3 two minute baths of dilute sulfurous acid (see PAS technique).
5. Wash sections in running water for 10 minutes.
6. Dehydrate, counterstaining in 0.01% fast green or 0.1% orange G in 95% ethyl alcohol.
7. Clear in xylene and mount.

#### Methyl Green-Pyronin Y (Barka and Anderson, 1963)

Procedure: Fix tissues in Bouin's, Helly's or Carnoy's.

1. Bring sections from xylene to water, using iodine if necessary.
2. Stain sections for 15 minutes at room temperature with the following staining solution: dissolve 0.5 gm. methyl green in 100.0 ml. of 0.1 M acetate buffer, pH 4.4, and add 0.2 gm. pyronin Y. Before this solution is made up, any methyl violet that may be a contaminate in the methyl green should be extracted with chloroform using a separatory funnel and the buffer (in which the stain is dissolved).
3. Wash sections for 3 minutes in a mixture of one part absolute ethanol to 3 parts tertiary butanol.
4. Wash sections in 2 changes of tertiary butyl alcohol for 2 and 3 minutes.
5. Clear in 2 changes of xylene, 10 minutes each, and mount.

Mercuric Brom-phenol Blue (Mazia et al., 1953)

## Solution:

Mercuric chloride. . . . . 10.0 gm.  
 Brom-phenol blue . . . . . 01.0 gm.  
 95% ethyl alcohol. . . . . 100.0 ml.

Procedure: Fix tissues in Bouin's, Carnoy's or neutral buffered formalin.

1. Bring sections from xylene to water.
2. Treat sections with brom-phenol blue solution for 15 minutes.
3. Wash sections for 20 minutes in 0.5% acetic acid.
4. Wash sections in water or buffer at a pH of 6.0-7.0 for 3 minutes.
5. Dehydrate, clear in xylene and mount.

For the specific staining of basic protein the procedure is modified as follows:

1. After bringing sections from xylene to water, stain them in 0.1% aqueous brom-phenol blue for 10 minutes.
2. Wash sections in 0.5% acetic acid for 20 minutes.
3. Place sections in water or buffer at a pH of 6.0-7.0 for 3 minutes.
4. Dehydrate, clear in xylene, and mount.

Gomori's Alkaline Phosphatase (modified by Bullock, 1953, 1958)

Procedure: Fix tissues in cold acetone or 85% ethyl alcohol for 24 hours in refrigerator. Immediately embed the tissues, and section within 24 hours of embedding.

1. Bring sections from xylene to water.
2. Place sections in the substrate mixture for 30 minutes at 37° C.
3. Place the sections in 2%  $\text{Ca}(\text{NO}_3)_2$  for 3 minutes.
4. Place the sections in 2% cobalt chloride for 10 minutes.
5. Wash the sections briefly in distilled water.
6. Place the sections in 2% yellow ammonium sulfide solution for 15 minutes. Make up fresh every 2 hours.
7. Place the sections in 2 changes of 35% ethyl alcohol, 5 minutes each change.
8. Place sections in distilled water for 5 minutes.
9. Counterstain sections in carmalum for 15 minutes.
10. Place sections in distilled water for 5 minutes.
11. Dehydrate, clear in xylene, and mount.

Substrate mixtures for alkaline phosphatase

## Controls:

2% Ca(NO <sub>3</sub> ) <sub>2</sub> . . . . .	.20.0 ml.
1% sodium barbitol. . . . .	.10.0 ml.
Distilled water . . . . .	.50.0 ml.

## Experimentals:

2% Ca(NO <sub>3</sub> ) <sub>2</sub> . . . . .	.20.0 ml.
1% sodium barbitol. . . . .	.10.0 ml.
Distilled water . . . . .	.50.0 ml.
2% sodium glycerophosphate. . . . .	.20.0 ml.

Alizarine Red S (Humason, 1962)

Procedure: Fixative should preferably be one containing 80% ethyl alcohol for maximum preservation of calcium.

1. Bring sections from xylene to water.
2. Stain sections for 3-5 minutes in a 1% aqueous solution of alizarine red S.
3. Rinse sections in distilled water.
4. Stain sections for one minute in a 0.1% aqueous solution of toluidine blue.
5. Rinse sections in distilled water.
6. Dehydrate, clear in xylene, and mount.

Oil Red O (Lillie, 1948)

## Solution:

The stain is prepared from a filtered stock solution of isopropyl alcohol saturated with oil red O. Dilute 6.0 ml. of the stock solution with 4.0 ml. of distilled water; let stand for 10 minutes and filter it onto the section.

## Procedure:

1. Stain frozen sections in oil red O solution for 2-30 minutes (15 minutes for routine study).
2. Wash sections in tap water.
3. Stain nuclei with hematoxylin.
4. Rinse in distilled water and mount in glycerin jelly.