THE IMPORTANCE OF PHASIC ACTIVITY IN A STUDY ON THE EFFECTS OF TWO DIGENETIC TREMATODES ON THE DIGESTIVE GLAND CELLS OF LITTORINA SAXATILIS (OLIVI)

By

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TABLE OF CONTENTS

		PAGE
ABSTRACT		i
ACKNOWLEDGEMENTS		ii
CHAPTER		
I	BY WAY OF AN INTRODUCTION	1
II	THE HOST: THE DIGESTIVE GLAND CELLS OF UNINFECTED *LITTORINA SAXATILIS*	5
	1. Introduction	5
	2. Materials and Methods	10
	3. Observations	12
	a. Light Microscopy	12
	b. Electron Microscopy	15
	4. Discussion	25
III	THE PARASITES: AN ULTRASTRUCTURAL STUDY OF THE TEGUMENTS OF *CRYPTOCOTYLE LINGUA* AND *MICROPHALLUS SIMILIS* SPOROCYSTS	52
	1. Introduction	52
	2. Materials and Methods	57
	3. Observations	60
	a. General	60
	b. Tegument	60
	4. Discussion	66
IV	THE INFLUENCES OF *CRYPTOCOTYLE LINGUA* AND *MICROPHALLUS SIMILIS* ON THE DIGESTIVE GLAND CELLS OF *LITTORINA SAXATILIS*	84
	1. Introduction	84
	2. Materials and Methods	88
	3. Observations	98
	a. General Pathology	98
	b. Histochemistry of Infected Digestive Gland Cells	106
	c. Ultrastructure of the Infected Digestive Gland Cells	117
	4. Discussion	124

(Contd.)

PAGE

REFERENCES 171

APPENDIX I HISTOLOGICAL, HISTOCHEMICAL AND ULTRASTRUCTURAL TECHNIQUES

ABSTRACT

The digestive gland epithelium of Littorina saxatilis is composed of digestive and secretory cells. Digestive activity in both types of cells has been shown to take place in a consistently rhythmic pattern. Such phasic activity is more pronounced in digestive cells where three phases of digestion are recognized. The digestive activity is seen to be closely correlated with and dependent on the periodicity of the tide.

An ultrastructural study on the digenetic trematodes, Cryptocotyle lingua and Microphallus similis, revealed that the body walls of the larval stages are similar in many respects. In both instances, endocytotic activity appears to be the primary method of food acquisition.

Observations on the effects of trematode larvae on the digestive gland cells, suggest that M. similis sporocysts destroy more cells than do C. lingua rediae. While the nature of cell deterioration as induced by either parasite was found to be similar, the damage appeared to occur more rapidly in digestive cells affected by M. similis sporocysts. A prerequisite to the parasitological study presented in this dissertation, was a thorough understanding of the morphological and histochemical features of the uninfected digestive gland cells for each of the phases of digestion.

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CHAPTER I

BY WAY OF INTRODUCTION

Gastropods are of interest to parasitologists because they act as obligatory intermediate hosts for digenetic trematode larvae. Good reviews of current knowledge on the effects of these parasitic larvae on the snail host are to be found in Cheng and Snyder (1962a), Fretter and Graham (1962), Wright (1966), Cheng (1967), Robson and Williams (1971a, 1971b), and Erasmus (1972).

The pathologies induced by the trematode larvae range from minimum alterations to gross physiological and behavioral changes, castration and death. The most common sites for physical and metabolic modifications are the gonads and the food-laden digestive gland. The role of the latter during digestion, allows it to serve as a high source of energy, thereby providing a suitable location in which most digenetic larvae can undergo major development. The larvae usually occur in the visceral haemocoel, immediately adjacent to the tubules of this gland.

The overall influence of parasites on the digestive gland varies, depending on such factors as parasite size, mobility, toxicity and host reaction. An indication of such variation is obtained from papers by Kendall and Ollerenshaw (1963) and Davis and Farley (1973) who reported minimum deterioration in the digestive efficiency of infected snails. In contrast, Rees (1936) and James (1965)

demonstrated that not only is there interference with natural absorption of food, but that in certain instances, the gut becomes totally obliterated.

A prerequisite for the study of parasitic effects on the digestive gland is a thorough understanding of the anatomy and physiology of this structure. Merdsoy (1971), in an ultrastructural examination of the intertidal prosobranch, Littorina littorea, revealed the existence of phasic activity for the digestive cells of this gland. While he was unable to correlate any specific phase of the digestive cycle with an imposed tidal rhythm of feeding as had been done previously for the bivalve Lasaea rubra (Morton, 1956), the relevance of his work to the study of trematode-gastropod relationships at once become apparent. It seemed very likely that what some authors termed "parasitic effects" of the digestive cells, represented at least in some instances, the state of the cell in one of its characteristic phases of the digestive cycle. Preliminary work by Davis (1972) suggested that a feeding and digestion cycle which could be correlated with tide periodicity, might prevail in the intertidal prosobranch Littorina saxatilis. If this proved to be the case, then the specific phases of digestion could be isolated by collecting the periwinkles at different times over the tide cycle, and the parasitic effects could be studied for each of the digestion phases. In this way, the confusion that

exists between "parasitic effects" and rhythmic activity can be resolved.

Davis (1972) suggested that the most common parasites of L. saxatilis, Cryptocotyle lingua and Microphallus similis, have differing effects on their host. The former, characterized by a redial stage, enjoys a harmonious relationship with the gastropod. It causes no alteration in digestive efficiency and increases its host's growth rate and longevity. This condition contrasts to that of snails infected by M. similis sporocysts which show a decrease in longevity. This dissertation will examine the relative influences of the two parasites on the digestive gland cells of L. saxatilis at the light and electron microscope levels, in an effort to better understand their differing effects. The description is preceded by an in-depth morphological analysis of the rhythmic activity displayed by non-parasitized digestive gland cells. In addition, a study of the redial and sporocyst tegument, i.e. the outer body wall of the parasite and also the region which makes the most intimate contact with host cells, is offered in Chapter III.

This study fully avails itself of the existing information on L. saxatilis and may be regarded as a counterpart to the ecological analysis of this host and its digenean parasites as presented by Davis (1972). Finally, it contributes to a wider research project on the host-parasite

association in the family Littorinidae. Other aspects of this work have been investigated by Lambert (1967), Lambert and Farley (1968), Platt (1968), Davis (1972), Davis and Farley (1973) and Mordsoy and Farley (1973).

CHAPTER II

THE HOST: THE DIGESTIVE GLAND CELLS OF UNINFECTED LITTORINA SAXATILIS

INTRODUCTION

The northern rough periwinkle, Littorina saxatilis (Olivi) is an intertidal prosobranch gastropod unique among the Littorinidae because of its viviparity. It is widely distributed and has been reported from the rocky shores of Europe, the British Isles and North America, ranging on the latter continent from Baffin Island to New Jersey.

Extensive studies have shown that there are numerous subspecies and varieties of L. saxatilis (Fischer-Piette et al., 1960, 1961, 1963, 1966, 1968; James, 1968a). These, together with other Littorinids and their digenean parasites have been reviewed and classified by James (1968b).

While there are various reports of L. saxatilis from the Atlantic coast of Nova Scotia (Gowanloch and Hayes, 1926; Bousfield, 1958), only recently has a more detailed investigation been undertaken, revealing notable differences in anatomical characters among varieties of this species as observed from nine separate populations in and around Halifax (Davis, 1972). In addition, Davis (1972) conducted a more comprehensive population study of L. saxatilis subsp. tenebrosa, on a sheltered rocky shore at Blue Rocks, Lunenburg County, Nova Scotia. This study included an eco-

logical survey and examination of the digenean fauna associated with these gastropods. Information in Davis' work pertaining to host age, distribution, numbers, reproductive patterns and feeding habits, as well as data on the parasites, provided the necessary background for the present study.

Various aspects of the general ecology, biology and physiology of L. saxatilis have been reviewed by Fretter and Graham (1962), Wilbur and Young (1966) and Purchon (1968). Members of this species are most common in semi-sheltered habitats, but can occur in reduced numbers where exposure is greater. They are generally found in rock crevices and cracks, ranging in position from about M.L.W.S. (mean low water spring) to E.H.W.S. (extreme high water spring) depending in large part on the nature of the shore and the tide range of the region. At Blue Rocks, for example, L. saxatilis occupies a very narrow band in the supralittoral zone and is completely submerged for no more than 1½ - 2 hours at each high tide. Their food consists mainly of blue-green algae in the spring and Urospora spp., Enteromorpha spp., algal detritus and diatoms in the summer.

L. saxatilis obtains its food by grazing over the wet surface of the substrate, and subsequently passes it along through the digestive system. A network of branching

tubules surrounded by connective tissue occupies the greater part of the visceral hump, and opens into the stomach by way of two main ducts. The tubules are collectively referred to as the digestive gland and it is this structure which represents the seat of digestion in the mollusc.

Considerable work has been done on the digestive gland epithelium of gastropod molluscs (Krijgsman, 1926; Graham, 1939; Fretter, 1948; Morton, 1955a, 1955b; Pugh, 1963; Andrews, 1965; Schmeckel and Wecksler, 1968). The digestive diverticula of bivalve molluscs have likewise been closely examined (Young, 1926; Owen, 1955; 1956; Ballantine and Morton, 1956; Sumner, 1966a, 1966b) and current literature suggests that more workers are focusing their attention on the bivalves (McQuiston, 1969; Morton, 1969; 1970; Owen, 1970; Pal, 1971).

Many authorities believe that the digestive epithelium of molluscs is composed of two cell types: the digestive cell and the excretory or secretory cell (Graham, 1932; Morton, 1955a, 1955b; Owen, 1955; 1956; Pugh, 1963; Andrews, 1965; Sumner, 1966a, 1966b). The digestive cell is invariably characterized by a columnar or club-shaped appearance and is equipped with those structures which render intracellular digestion possible. The excretory cell is identified by its smaller size, triangular shape when viewed in cross section, and a densely staining cytoplasm that re-

veals a highly elaborate network of rough endoplasmic reticulum as observed from electron micrographs.

More than two cell types have been reported from the digestive epithelium of certain mollusc species (Martoja, 1964; Schmeckel and Wecksler, 1968; Sugawara, 1969). However, a tendency by some authors to describe many different cell types in the epithelium and to present different descriptions for cells which are obviously identical, may be attributed to the fact that workers overlook the likelihood of phasic activity. This possibility is enhanced when we consider that identical cells from a single individual may be out of phase with each other (Purchon, 1971), hence a greater variety of cells may seem to exist within the single organism than in fact occurs.

Phasic activity was described by Millot (1937) for the pulmonate Jorunna tomentosa and later by Morton (1955a, 1955b) who identified successive phases of absorption, intracellular digestion and fragmentation in the digestive cells of different pulmonate species. Morton (1956) also demonstrated that the feeding and the phasic activity of the digestive system in the intertidal bivalve Lasaea rubra could be correlated with tide cycle. Recent work on phasic activity in molluscs has dealt exclusively with bivalves (Morton, 1969; 1970; McQuiston, 1969; Owen, 1970) except for a study by Nordsoy and Farley (1972), where phasic

activity was reported from the digestive gland cells of the intertidal littorinid gastropod Littorina littorea. This activity, however, could not be correlated with the state of the tide. Davis (1972) in a preliminary investigation, noted that the feeding activity and digestive functions of the intertidal prosobranch Littorina saxatilis could be correlated with the tide cycle. This chapter offers a comprehensive study of the digestive gland cells of Littorina saxatilis, tracing their structural variation over the tide cycle.

MATERIALS AND METHODS

Seven individuals of Littorina saxatilis were collected at hourly intervals throughout the tide cycle from the supralittoral zone of a sheltered rocky shore at Blue Rocks, Lunenburg County, Nova Scotia. After crushing the shells, the snail bodies were removed, transferred to cold sea water and examined briefly under a dissecting microscope. The visceral humps of infected snails were subsequently dissected and the tips discarded. One to two cubic millimeter portions of the digestive diverticula were then cut from the humps and prepared for light and electron microscopy.

Light Microscopy

For examination with the light microscope, the tissues were fixed in Masson's modification of Bouin's fluid (Foot, 1933), for four days, then washed in 70 percent ethanol for one day. Following dehydration in ethanol, they were embedded in Paraplast embedding medium (Sherwood Medical Industries Ltd., St. Louis, Missouri, U.S.A.) and sectioned at six to seven microns. Sections taken from the same block were cut serially and mounted on three slides. They were then examined after staining with either Heidenhain's iron haematoxylin stain, Gomori's aldehyde fuchsin, counterstained in Halmi's trichrome (Cameron and Steele, 1959), or according to the Mallory's Triple Stain Method. It is important to emphasize that of the three staining techniques

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employed, the last proved to be most effective. This is primarily due to its ability to differentiate cellular components on the basis of their coloring properties and also because of its relative ease in handling. By this method, nuclei stain red, connective tissue light blue, digestive vacuoles varying shades of blue and RNA intense red.

Electron Microscopy

In preparation for electron microscopy, the tissues were fixed in 4 percent glutaraldehyde buffered in filtered sea water at pH 7.3 for one hour. They were then rinsed and stored in Millonig's sodium phosphate buffer for forty-eigh hours. The tissues were subsequently postfixed for two hours in cold 0.9 percent osmium tetroxide solution in Millonig's buffer (pH 7.3). After dehydration, the material was embedded in Maraglass. Sections were obtained with glass knives on an L.K.B. ultratome and were picked up on copper grids (70 and 100 mesh). The sections were double stained with alcoholic uranyl acetate (Stempak and Ward, 1964) for fifteen minutes and with lead citrate (Reynolds, 1963) for ten minutes. All sections were examined in a Zeiss E.M. model 9S. Preliminary observations with the light microscope of thick sections using the toluidine blue staining method (Trump et al., 1961) always preceded final sectioning. This helped in block orientation, as wel as with localization of relevant structure. (For a more detailed elaboration of the techniques used, see Appendix I).

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OBSERVATIONS

Light Microscopy

The digestive gland of L. saxatilis is composed of two lobes, each of which opens into a series of blind tubules. The glandular epithelium is made up of the digestive and secretory cells, the morphology of which is similar to that described for other prosobranch gastropods (Graham, 1932; Pugh, 1963; and Merdsoy and Farley, 1972).

Secretory Cell

The secretory cells appear triangular in section. They usually occur in the crypts of the digestive tubules, although they may be present singly among the digestive cells. Variations in their size and shape occur during the tide cycle, but this is attributed not to any intrinsic change in such cells, but because of differences in compression by the adjacent digestive cells whose cellular contents, and thus cell volumes, fluctuate with tide height (see below).

The cytoplasm appears homogeneous and stains a deep red with the Mallory technique and an intense purple by the aldehyde-fuchsin method. A large nucleus is positioned halfway from the apical border (Fig. 2.1). Morphological changes such as a reduction in spherule number or an alteration in cell structure, which could be correlated with tide height, were not observed.

Digestive Cell

The digestive cell is larger than the secretory cell and is characterized by a variety of vesicular structures whose staining intensity, distribution, size and number fluctuate relative to time of collection over the tide cycle. A nucleus, somewhat smaller than that of the secretory cell is located basally, immediately adjacent to the basal lamina.

Distinct changes between hourly intervals are apparent and a rhythmic digestion cycle is detected for these cells. Such activity can be categorized into three phases: absorption, digestion and excretion (fragmentation), similar to that described for other gastropods (Morton, 1955a, 1955b; Merdsoy and Farley, 1973). While the separate phases are easily identifiable, intermediate stages can be observed throughout the digestion cycle.

Phasic Activity of the Digestive Cell

1. Absorption

At the start of absorption, the cells appear distinct and columnar. Their flat borders stain intensely and a series of vacuoles of diameters 1 - 3 μm, whose contents stain light-blue by the Mallory technique are observed in the apical third of the cells. Purple-staining inclusions,

as revealed by the aldehyde-fuchsin technique, correspond in position to the above vesicles. In contrast, the basal regions of the cells appear free of structures with only an occasional trace of minute structures extending as far back as the nucleus (Fig. 2.1, 2.2).

2. Digestion

As ingestion continues, the tubule lumen becomes reduced in diameter. The individual cells appear more distended and also somewhat shorter. They are packed with vacuoles and their contents stain varying shades of blue by the Mallory method and purple with the aldehyde-fuchsin technique. These vacuoles extend as much as 1/3 - 1/2 basally in the earlier stages and up to 2/3 basally towards the later part of this phase. Fusion between the smaller vacuoles is observed in the anterior-middle regions and their diameters range from 20 - 25 µm. (Fig. 2.3, 2.4).

Although the pigmented apical border, as described for the absorption phase, prevails during this stage, the staining becomes considerably less intense with the passage of time. Also, there occur numerous colored vesicles in the basal region, but these become less widespread, disappearing completely towards the end of the phase.

3. Excretion (Fragmentation)

Vacuoles gradually become reduced in number and more

concentrated in the apical 1/3 - 1/2 region, although they are observed in the basal area as well. Ultimately, very few (2-3) large vacuoles remain (Fig. 2.5, 2.6, 2.7). These are characterized by a homogeneous and densely-staining material, similar to that present in the lumen. Signs of disorder and possible fragmentation are present, although the latter condition is not clearly evident. Certainly varying degrees of vacuolation prevail, the more extreme cases being observed towards the end of the cycle (Fig. 2.8, 2.9). At this time, the cells begin to assume their original columnar appearance as described for the absorption phase.

Electron Microscopy

Secretory Cell

The examination of large numbers of electron micrographs supports and at the same time elaborates the findings with the light microscope.

The secretory cells appear pyramidal in shape, backed by a basal lamina and bands of smooth muscle and are characterized by an extensive network of granular endoplasmic reticulum (Fig. 2.10, 2.12). The flattened cisternae assume concentric and curvilinear forms in the middle and basal parts of the cell, while in the apical regions they appear as sparsely scattered profiles which at times are agranular.

Certain micrographs would suggest that during the excretory phase the endoplasmic reticulum is more fragmented and also more swollen than in the absorption and digestion phases, especially along the peripheral margins (Fig. 2.12). There is no evidence of a flagellum in the apical zone, but a microvillus border is clearly present (Fig. 2.11, 2.12). The individual microvilli are about 31 µm. in length and 1 µm. in diameter. Numerous dense granules ranging from 3 - 10 µm. in diameter, which appear spherical and are enclosed within a tightly applied limiting membrane occupy the anterior-middle area. Such granules resemble the zymogen granules of exocrine secretory cells in higher organisms. Material of staining density similar to the above structures is often seen concentrated between the microvilli, especially during the earlier phase of digestion (Fig. 2.11). Signs of pinocytosis or pinocytotic vesicles are not detected at any time. Mitochondria, varying in shape, from long elongated structures to smaller spherical forms are present throughout the cell, but are primarily concentrated in the apical part, (Fig. 2.11, 2.12). In the basal region, there occur one and sometimes two or three Golgi complexes. Their cisternae which may appear curved in section are distended at the peripheral margins and give rise to a series of electron lucent vesicles which are about 800°A in diameter. While such vesicles are found primarily around the Golgi apparatus, structures of similar size and staining properties ap-

pear in other parts of the cell.

The nucleus varies in shape, but is most often elongated and slightly lobed. It is large when compared to that of the digestive cell (Fig. 2.10) and also contains a large, centrally-positioned nucleolus. The nuclear sap appears moderately granular and densely staining chromatin is disposed along the nuclear envelop. Numerous non-membranous lamellated inclusions identical to the calcium spherites reported for Helix pomatia by Abolins-Krogis (1965) and for Littorina littorea by Merdsoy and Farley (1973) are visible in the basal and middle regions (Fig. 2.10, 2.12). At times they appear as large empty cavities with only some remnants of material. This is probably attributable to loss of such material during sectioning because of differences in cutting properties between it and other components of the cell. Occasionally, lipid spherules can be seen.

With the possible exceptions of the granular endoplasmic reticulum and the occurence of densely staining material between the microvilli in the earlier stages of digestion, no definite correlation could be made between structural variation in this cell and time of collection.

Digestive Cell

As observed with the light microscope, the digestive cell varies in shape, appearing long and columnar in the

absorption phase and gradually more distended and shorter as digestion proceeds. During the excretory phase, the cell reverts to its more elongated form. While there is clear evidence of vacuolation and possible disorganization within the cell at this time, there is no indication that the cell membrane ruptures.

The basal membrane also lies on a homogeneous basal lamina whose inner and outer limits are poorly defined. Distally, separate desmosomes attach to the adjacent cells and in general, there seems to be little interfolding between cells. The flagellum, originating from the apical region, projects into the lumen. This surface also bears a brush border, the microvilli of which are covered by a characteristic surface coat along their tips. The number and size of the microvilli vary, depending on the phase of digestion (Fig. 2.13). The length of the individual villus is about 15 µm. during the absorption stage, becoming reduced by as much as 4 µm. in the late digestion and excretory phases. Its average diameter is about 1 µm.

Considerable membrane activity occurs in the distal cytoplasm during the absorptive and early digestive phases (Fig. 2.14). Pinocytosis is taking place, with a gradual decrease and ultimately a complete stoppage during the excretory phase. At the same time, numerous pinocytotic vesicles with an electron density similar to that of the

lumen content can be seen. These range in diameters from 0.5 - 4 µm. and vary in morphology from simple spheres to doughnut-shaped vesicles. To explain the formation of the latter structures, it is believed that the surface membrane on either side of a microvillus invaginates such that the resulting folds fuse with each other to form a doughnut-shaped inclusion. The lumen content is now enclosed between an inner and outer membrane, with a bit of native cytoplasm contained in the centre (Fig. 2.14).

Mitochondria are common, but become visibly reduced in number as digestion proceeds. While they are scattered throughout the cell, they are most heavily concentrated in the apical region, especially during absorption, early digestion and late excretion. Lipid droplets exist throughout the cell and their number increases as digestion proceeds, reaching a peak during the late digestive and excretory phases. Moreover, they are usually in close association with certain large vacuoles (see below). Glycogen rosettes have been detected in the middle region during the digestive phase.

The basal region of the cell contains a nucleus which is smaller and more regular in shape than that of the secretory cell. It is characterized by an electron dense nuclear sap in which numerous dark granules are scattered. The nucleolus is located centrally. Several Golgi complexes

occur in the adjacent cytoplasm and their cisternae appear distended at the marginal ends, giving rise to a series of vesicles ranging from 1 - 3 µm. in diameter (Fig. 2.15). The presence of parallel membranes within the distended regions of the saccules, as well as in the associated vesicles, makes this type of Golgi unique in gastropods thus far. A similar complex however, has already been reported from the bivalve Cardium edule (Owen, 1970). Other vesicles considerably larger (6 µm. in diameter) have also been observed in the cell (Fig. 2.11). The latter are composed of medium-dense granules which appear homogeneous and are enclosed within a unit membrane. Fragmentary profiles of endoplasmic reticulum are sometimes visible in the digestive cell.

By far, the most conspicuous characteristic of the digestive cell is the presence of a wide variety of membrane-bound vesicles. These structures correspond to those already examined with the light microscope, and will now be described in greater detail. To facilitate reading, the macrovesicles have been arranged by type according to their contents and morphological characteristics, similar to the scheme adopted by Owen (1970).

TYPE 1

This type of vesicle has been observed primarily

during the absorption phase in the apical region. It is most often spherical and varies in diameter from about 4 - 15 µm. The visible content of this vesicle is characterized by a moderately electron dense granularity which appears to be primarily scattered along the periphery. (Fig. 2.11).

TYPE 2

This type of vesicle occurs primarily in the apical third of the cell during the absorption and digestion phases. It is characterized by a moderately dense granularity which may be unevenly distributed or may occupy the entire interior of the vesicle (Fig. 2.11, 2.16). In addition, bits of membrane, as well as minute spherules, have also been detected. Fusion between Type 2 vacuoles is common, consequently their diameters can range from 10 - 30 µm. They seem to coalesce with pinocytotic and Type 1 vesicles during absorption and are also associated with lipid droplets during the digestion phase.

TYPE 3

This type of vesicle is especially predominant during the digestive phase in the lower basal and mid-regions of the cell. It is characterized, in some cases, by a granularity similar to that of Type 2 vesicles. Numerous dense and somewhat larger granules, as well as

bits of membrane can also be observed from these vesicles. Type 3 vesicles are often in close association with Golgi complexes, and it is believed that the Golgi vesicles are ultimately absorbed by them. Fusion has also been observed between these vesicles and those of Type 2, as well as between themselves. Thus, the variations one observes in their contents and electron densities may be a consequence of the numerous associations which they have. Their diameters range from 7 - 40 µm. and this variation is to be expected (Fig. 2.16, 2.17).

TYPE 4

These vesicles are present almost exclusively during the excretory phase and are located mainly in the mid and apical parts of the cell, although they can occur basally as far back as the nucleus. The vesicles are irregular in shape and their contents which are in the form of a fine electron-lucent granularity, are usually concentrated in the middle portions. Unlike the other vesicles, fusion is limited and in some cases, their membranes are ruptured. Their diameters range from about 7 - 50 µm., the smaller vesicles usually occurring at the cell extremities. In general, their close contact with lipid droplets, their empty appearance and their large size, make them easily identifiable (Fig. 2.18, 2.19).

A diagram of the epithelial cells of the digestive gland of L. saxatilis is shown in Figure 2.20. While the characteristic structures of these cells are incorporated into the diagram, their varying quantitative and qualitative properties, usually associated with one or more of the digestive phases, have been disregarded. A summary, of the physical alterations of such structures both in the digestive and secretory cells showing how these relate to phasic activity, is offered in Table 1. Moreover, the phasic activity as originally recorded from the light microscopy findings for the digestive cell and later confirmed through the electron micrographs, falls into a definite sequence, one which is well correlated to the periodicity of the tide (Table 2.2). This becomes even more obvious when collection times are arranged into groups of two and the percentage numbers of snails for each of the phases are plotted against the height of the tide (Fig. 2.21).

Table 2.1 Summary of the morphological characteristics of the digestive and secretory cells of L. saxatilis at various stages of activity.

age	Function	Appearance of Digestive Cell			Appearance of Secretory Cell
		Apical	Medial	Basal	
1	Absorption	Numerous long microvilli. Much pinocytotic activity. Abundant mitochondria. Type 1 and Type 2 vesicles present.	Some Type 2 vesicles present. Upper limits are characterized by large empty spaces. Some mitochondria.	Sparsely scattered E.R. Large empty spaces.	Characterized by presence of abundant granular E.R. which appears as concentric and curvilinear forms. Lipid droplets, mitochondria, calcium spherites and one possibly two or three Golgi present. Zymogen granules concentrated in apical third of cell. Material with similar staining properties seem to occur between microvilli.
2	Digestion	Slight reduction in number and size of microvilli. Less pinocytotic activity. Type 2 vesicles predominate. Fewer mitochondria.	Type 2 vesicles present but Type 3 predominate. Golgi present and in close association with Type 3 vesicles. Lipid droplets abundant. Evidence of Type 4 vesicles.	Lipid droplets	
3	Excretion	Microvilli fewer in number and considerably shorter in size. No pinocytotic activity. Mitochondria present. Type 4 vesicles present.	Type 4 vesicles present, their membranes not always intact. Some evidence of Type 3 vesicles in earlier parts of stage. Large number of lipid droplets. Golgi complexes reduced in size and number, few signs of Golgi vesicles.	Type 4 vesicles may be packed against nucleus	Zymogen-like material not evident between the microvilli. E.R. is fragmented, where present in the form of parallel cisternae, their peripheral ends are swollen.

Table 2.2 Number of individuals of *L. saxatilis* displaying the various phases of digestion throughout the tide cycle.

Collection No.	Hours (AST)	Ht. tide above chart datum (ft.)	No. in absorptive phase	No. in digestive phase	No. in excretory phase	Total
1	08:30	7.3	5	0	0	5
2	09:30	6.9	4	3	0	7
3	10:30	5.9	0	7	0	7
4	11:30	4.3	1	5	0	6
5	12:30	2.7	0	7	0	7
6	13:30	1.5	0	5	1	6
7	14:30	1.0	0	6	0	6
8	15:30	1.1	0	4	2	6
9	16:30	1.8	0	2	4	6
10	17:30	3.1	0	0	7	7
11	18:30	4.7	0	2	5	7
12	19:30	6.3	1	3	3	7

Figure 2.21

Percentage of Littorina saxatilis in each phase of digestion, plotted against height of tide. Collections have been arranged in groups of two and water levels averaged for each group of two collections.

1 ..... snails in absorptive phase
2 ..... snails in digestive phase
3 ..... snails in excretory phase

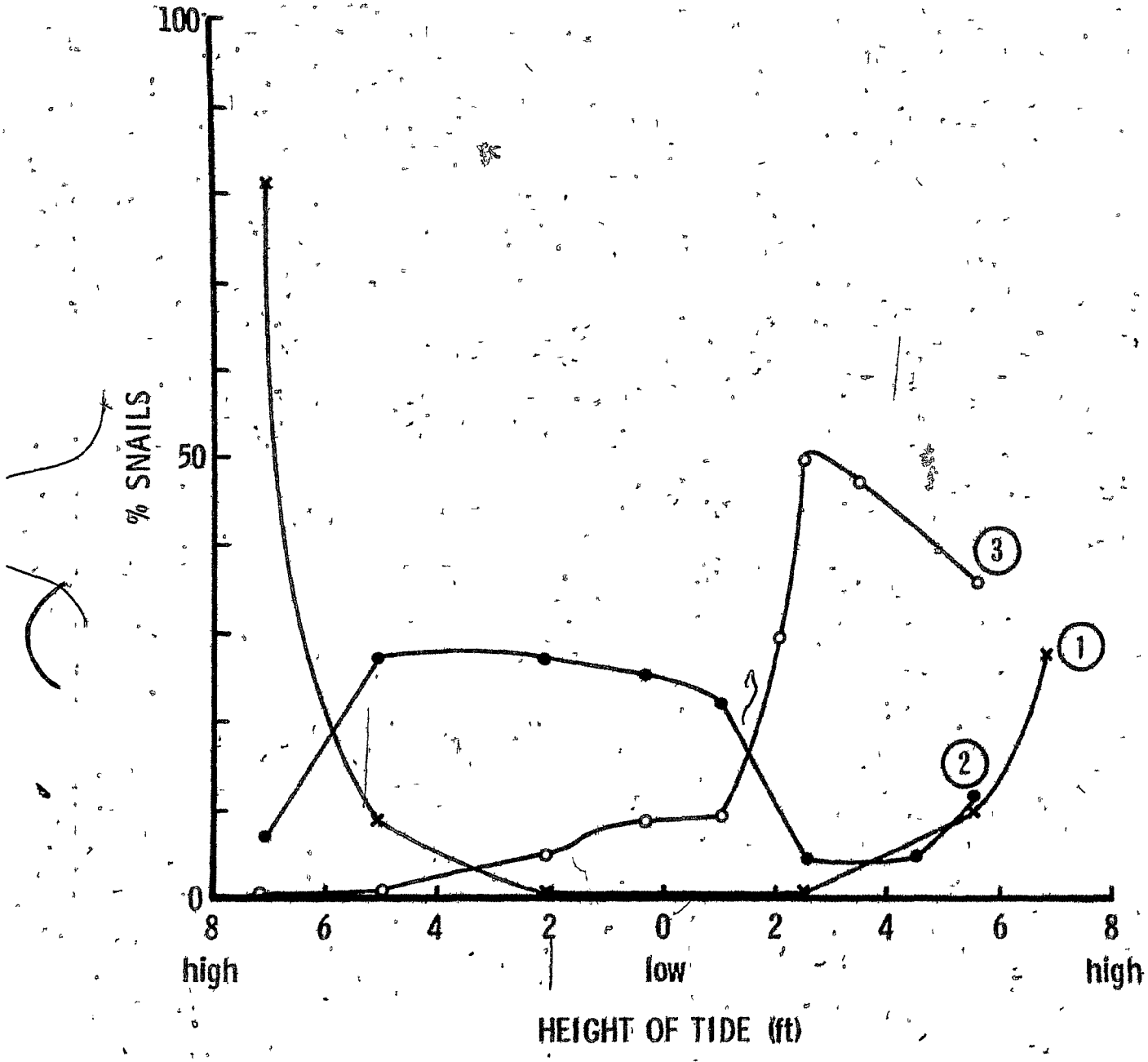

Fig. 21

DISCUSSION

In general, the present findings are in agreement with those of others who report two cell types from the digestive glands of molluscs. The secretory cell described in this study resembles the "B" cells of P. vulgata (Pugh, 1963), the excretory cells of P. canaliculata (Andrews, 1965), the basophil cells of Anodonta (Sumner, 1966), and the "C" cells of T. granosa (Schmeckel and Wecksler, 1968), as well as the secretory cells of L. rubra (McQuiston, 1969).

Although various functions have been attributed to this or similar such cells, the ultrastructure of the cell under consideration is such as to render a secretory role most likely. The importance of an extensive granular endoplasmic reticulum, as can be seen especially during the absorptive and digestive phase in L. saxatilis, has been well illustrated for other secretory cells (Fawcett, 1965). The apparent fragmentary and swollen appearance of this organelle during the excretory stage may represent an end to protein synthesis and at the same time, a period for reorganization and preparation for the new feeding and digestion cycle. Distension of endoplasmic reticulum cisternae in secretory cells from Lasaea rubra (McQuiston, 1969) has likewise been associated with the completion of the digestive cycle. The numerous dark granules described

from the lower middle and apical regions closely resemble the zymogens of exocrine secretory cells in the pancreas and salivary glands of higher organisms (Bell, et al., 1965). Moreover, the similarity in electron density of these granules and the material concentrated between the microvilli, suggests that such granules may be released into the lumen, possibly becoming activated when they come in contact with the appropriate triggering agents. The presence of such material between the microvilli during the absorptive and up to about the mid-digestive phase, corresponds to the time when extracellular digestion in the stomach lumen ordinarily occurs. Although no direct association can be shown between the Golgi complexes of these cells and the endoplasmic reticulum, the probability of its involvement in the cellular metabolism further supports the possibility of a secretory function for this cell, as does the presence of the large nucleolus (Novikoff and Holtzman, 1970).

The change in appearance of the digestive cell over the tide cycle, from a long columnar state in the absorptive phase, to a shorter and more distended form in the digestive phase, and again to a more elongated structure in the excretory stage, has already been discussed and attributed to the presence or absence of food (see OBSERVATIONS - Light Microscopy). Because of the morphology of

this cell, it is believed that the food passes across the apical border and is ultimately contained within the various digestive vacuoles.

Several interpretations have been proposed with respect to how the food is acquired and ultimately processed by the digestive cells of molluscs, and pino- or phagocytosis appear the most common methods by which the digestive cells absorb the food (Sumner, 1965; 1966; Merdsoy and Farley, 1973). It is generally believed that the ensuing vesicles fuse with larger digestive vacuoles located subapically, and in this way perform the function of a transport mechanism. An alternative to this method, proposed by McQuiston (1969) for Iasaea rubra, is that the pinocytotic vesicles may actually represent profiles of tubules which connect the lumen to large food vacuoles. Because of the presence of doughnut-shaped vesicles similar to those shown for Littorina littorea (Merdsoy and Farley, 1973), and as a result of fusion between these spherical pinocytotic vesicles with Type 2 vacuoles during absorption and early digestion, the findings support the occurrence of pinocytotic activity. The pinocytotic vesicles can be compared to Stage 1 endocytotic vesicles described by Meuleman (1972). The large number of mitochondria at these stages of digestion, especially as observed from the apical region, suggests the necessity for a much needed supply of energy during the intense membrane

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activity.

The sequence of digestion of food once inside the vacuoles, as well as the mechanism by which enzymes are synthesized and transported to the appropriate sites, is a topic which is as yet not clear.

In principle, it is agreed that food vesicles are a common feature of digestive cells in all molluscs. Based on their morphology and the nature of their contents, the findings show that there occur four types of vesicles. Their similarity in staining properties and electron densities, as well as the extent of fusion which was observed between them, suggests that what in fact prevails is a singular structure which goes through four stages.

In the present study, the Type 1 vesicles may well represent the earliest stages of food accumulation, the scant distribution of material within them, however, makes it difficult to say with certainty that they are not actually preliminary Type 2 forms. They are on the average the smallest of the macrovesicles and are present in the apical regions primarily during absorption, their absence being conspicuously obvious from the more basal parts of the cell and during the later stages. In addition, they closely resemble the Type 1 vesicles in the digestive cells of C. edule (Owen, 1970). The Type 2 vesicles resemble the vacuoles characterized by the "green granules" in digestive

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cells from S. putris (Sumner, 1966), as well as the large vacuoles described from the apical region of Anodonta (Sumner, 1966). They are also similar in their electron density to the phagolysosomes reported by McQuiston (1969) and to Type 2 vesicles examined by Owen (1970). By carrying out feeding experiments, these authors showed that such structures are indeed food-containing vacuoles. The occurrence of membranous elements in Type 2 vesicles as described here, is probably attributable to the inner spheres of the doughnut-shaped pinocytotic vesicles, and to the membranous material acquired after fusing with Type 3 vesicles. Such fusion may also help to explain the variation in electron density of Type 3 vesicles or of intermediate forms between Type 2 and Type 3.

Macrovesicles which resemble the Type 3 vesicles have been described by McQuiston (1969) and Owen (1970). These authors maintained that Golgi vesicles in digestive cells were actually lysosomes which fused with larger macrovesicles to give rise to phagolysosomes. The possibility that the Golgi vesicles are in fact primary lysosomes is enhanced by the findings of McQuiston (as reported by Owen, 1970), who showed that in the intertidal bivalve Lasaea rubra, the Golgi vesicles exhibited a positive test for acid phosphatase activity. It is widely accepted that the Golgi vesicles may participate in enzyme elaboration (Hill, 1965; Mollenhauer, 1965) and that in some cases, they may

be the source of primary lysosomes (De Duve and Watiaux, 1966). It is noteworthy that in the present work, Type 3 macrovesicles are always in close proximity to the Golgi complexes and especially to their vesicles. It is likely that the Golgi vesicles are in fact lysosomes which become absorbed by the Type 3 vesicles. It must be pointed out, however, that while there is a resemblance between the composition of the peripheral margins of the Golgi bodies and their vesicles, to the contents of the Type 3 vesicles, i.e. the membranous elements, it cannot be ascertained that these bits of membrane are contributed solely by the Golgi vesicles.

It is significant that Type 3 vesicles occur primarily during the digestion phase, a period when Type 2 vesicles are present and laden with food. This suggests that a predominance of activity occurs at different periods during the tide cycle and that the characteristic appearance of the cell at any one time is not an indication of its age, as has been suggested for the pulmonate Biomphalaria pfeifferi (Meuleman, 1972), but rather of a specific phase during the process of digestion.

The reduced vacuolar content of the large Type 4 vesicles suggests that this might represent the last forms of the combined Type 2 - Type 3 structures, thus marking the end of digestion. The contents, which appear to be

electron lucent are quite likely the by-products of digestion and will ultimately be extruded from the cell. The apparent absence of cellular fragmentation and thus the retention of the cell membrane suggests that the material must be released from the cell by other means. Because the vacuolar membranes are ruptured in some cases, there is a possibility that the wastes, which are now either in the form of a liquid or a finely particulate matter, are excreted by simple diffusion, or by a type of reverse pinocytosis. The presence of minute spherules in the lumen, as well as the apical concentration of mitochondria at such time, supports the latter possibility. Lipid droplets appear to be most abundant during the excretory phase. Similar observations were made for different mollusc species by other workers (McQuiston, 1969; Merdsoy and Farley, 1973). It is possible that such lipid droplets will ultimately contribute towards the synthesis of new structures as the next digestion cycle comes into effect.

Rhythmic feeding and digestion as related to tide cycle, have been demonstrated in the intertidal bivalve Lasaea rubra by Morton (1956) but could not be shown for the prosobranch gastropod Littorina littorea (Merdsoy and Farley, 1972). While phasic activity was recorded for the latter species, it showed no correlation with the tide cycle. This is probably a result of the position that L.

littorea occupies on the lower levels of the shore. The snails are submerged for many hours during the tide period and the time intervals between feeding and non-feeding are not sufficiently long to make such activity dependent on the tide. In contrast, L. saxatilis occupies a very narrow band in the supralittoral fringe of the shore and even on spring high tides they are completely submerged for no longer than two hours per tide cycle. Their habitat, therefore, makes them most suitable for a study of phasic activity.

Field observations on L. saxatilis showed that the degree to which the digestive gland is collapsed or full is directly related to the amount of food present in the digestive tubules, and this in turn was observed to be controlled by the tide. The fact that such a cycle is actually a response to the periodicity of the tide, is enhanced by the findings of Davis (1972), whose studies showed that the snails graze at high tide, remaining active until the rock surfaces become dry. The snails subsequently become inactive and do not feed again until they are submerged by the next flood tide. Davis also described the gross morphological alterations of the digestive gland during the tide cycle, noting as well the passage and ultimate changes of food from the gut. Such observations closely parallel present findings and the combined results, in conjunction with

the cellular changes of the digestive epithelium are shown diagrammatically in Figure 2.22. In brief, at high tide plus 2½ hours, faecal pellets are being voided from the rectum. At the same time, food is being absorbed by the digestive cells from the stomach contents. At high tide plus six hours, faecal pellets are no longer visible in the rectum, and the digestive cells are packed with digestive vacuoles. At high tide plus nine hours, the food in the stomach has diminished and faecal pellets once again accumulate in the rectum. Numerous empty-looking vacuoles are now visible in the digestive cells. Finally at about high tide plus eleven hours, all food is absent from the stomach. The digestive cells consist largely of empty-looking vacuoles and faecal pellets are packed in the rectum.

In summary, the digestive gland epithelium is composed of two types of cells: a digestive cell and a secretory cell. Light microscopy and electron microscopy reveal that the digestive cell is so equipped as to make intracellular digestion possible. In contrast, the morphology of the secretory cell is similar to the exocrine secretory cells found among higher organisms and is believed to have a related secretory function. Digestive activity in both types of cells has been observed to take place in a consistently rhythmic pattern. Such phasic activity is more pronounced in the digestive cell where three stages of digestion are recognized: absorption, digestion and excretion. Diges-

tive activity is seen to be closely correlated with and dependent on the periodicity of the tide.

In a study of the effects of five species of digenean trematodes on the digestive gland of L. saxatilis, James (1965) described the digestive epithelium at the light microscope level. The fact that he did not stress the significance of phasic activity tends to make his work somewhat incomplete. Moreover, questions can be raised whether the "disrupted" cells he described are a consequence of parasitism or are actually representative of a later phase in the digestive process. Unlike James, Meuleman (1972) takes into account the state of the cells when considering the parasitic influences, but since the pulmonate Biomphalaria pfeifferi is a constant feeder, all stages of digestion are represented by the cells at any one time. As in James' work, the actual effects of the parasite were not ascertained since the ultrastructure of a parasitized cell may assume the character or appearance of a cell normally associated with some phase of digestion.

L. saxatilis lends itself well to a parasitological study concerned with the effects on the digestive gland. All the digestive gland cells are in the same phase, at any one time, thus the different phases can be isolated by removing the snails from the shore at specific times during the tide cycle. Prior to such an investigation, however,

an initial expose of the applicable parasites is regarded as relevant.

Figure 2.1

Cross section of digestive gland tubules of L. saxatilis in the absorptive phase. Note the heavily pigmented apical border, as well as the concentration of digestive vacuoles in the apical third of the digestive cells. Note, too, the intensely stained secretory cells. The Mallory's Triple Stain Method renders the nuclei red, the connective tissue light blue, the digestive vacuoles varying shades of blue and RNA intense red.

ab - apical border lu - lumen
DC - digestive cell SC - secretory cell
(Bouin's, Mallory's Triple Stain) x175

Figure 2.2

Cross section of a digestive gland tubule in the absorptive phase taken at a higher magnification.

dv - digestive vacuole
(Bouin's, Mallory's Triple Stain) x450

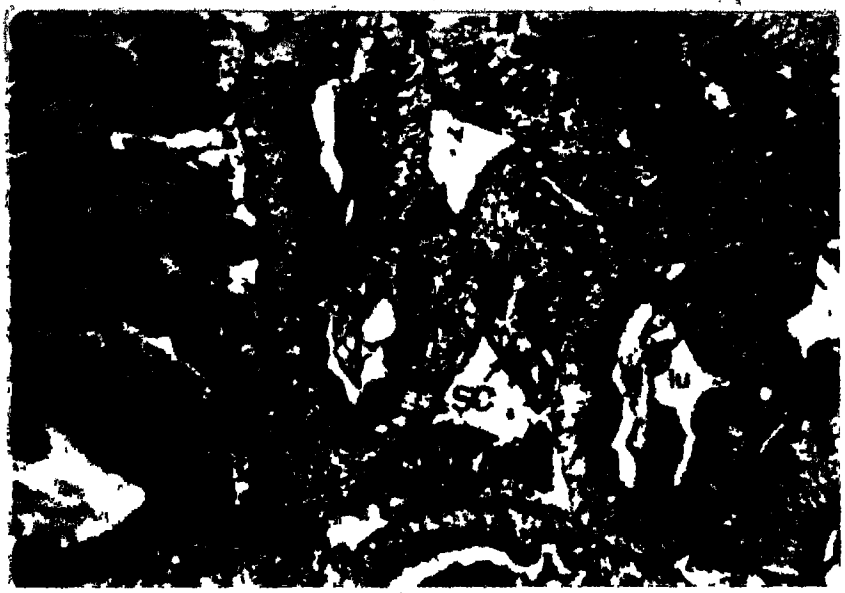


Figure 2.3

Cross section of a digestive gland tubule of L. saxatilis in the late absorption, very early digestion phase. The apical border is very heavily pigmented. The digestive vacuoles are small to medium in size (1 - 5 um.). They are loosely distributed, extending further basally than in the previous two figures.

nu - nucleus

(Bouin's, Aldehyde Fuchsin) x720

Figure 2.4

Cross section of a digestive gland tubule in the digestion phase. Note the number of food-laden vacuoles in the digestive cells. Coalescence of vacuoles takes place at this time. Note also the minute colored granules in the basal region (arrows).

(Bouin's, Mallory's Triple Stain) x450

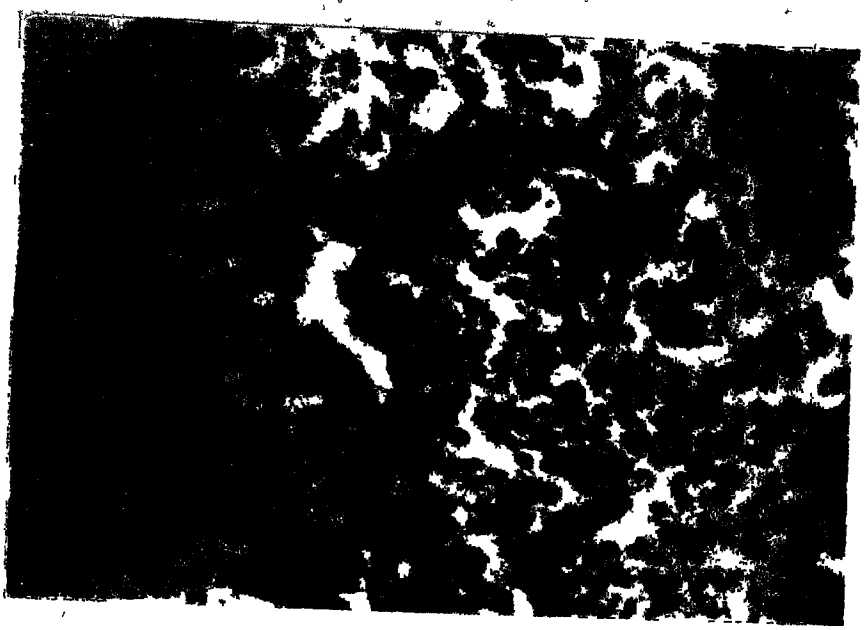


Figure 2.5

Cross section of a digestive gland tubule showing cells in the very late stages of digestion. Note the reduced number of digestive vacuoles concentrated in the apical half and the beginnings of large empty-looking vacuoles in the basal regions of the digestive cells (arrows).

SC - secretory cell

(Bouin's, Mallory's Triple Stain) x400

Figure 2.6

Cross section of digestive gland tubules in the early - mid excretory phase. Note the presence of only 1 - 3 large digestive vacuoles in the digestive cells. (See also Figure 2.7).

ct - connective tissue

(Bouin's, Mallory's Triple Stain) x210

Figure 2.7 (Figure 2.6)

Portion of Figure 2.6 taken at a higher magnification.

(Bouin's, Mallory's Triple Stain) x500

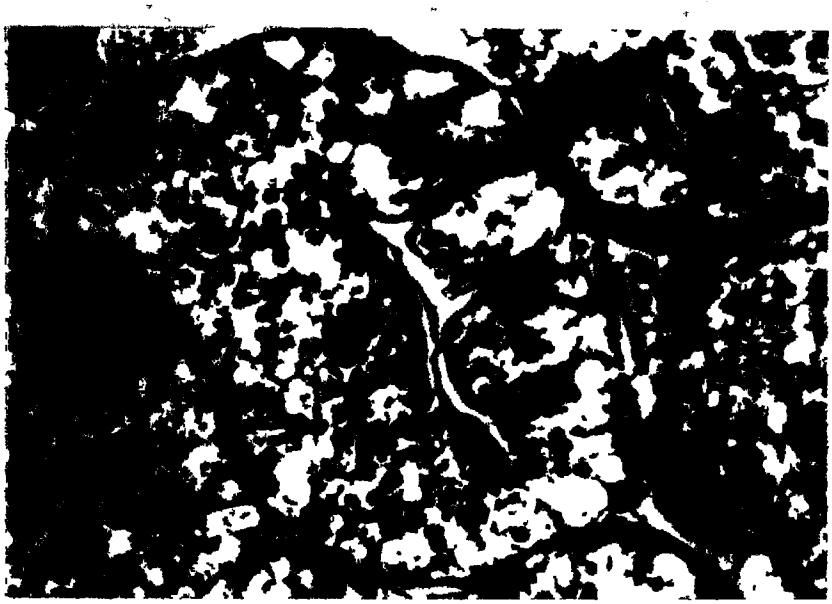


Figure 2.8

Cross section of digestive gland tubules during the late excretory phase. Note the absence of digestive vacuoles and the predominance of large empty-looking vacuoles containing the indigestible residues. The apical borders do not stain heavily and there is only a slight bluish coloration within them, possibly representing the last remnants of digestible material. Note also material destined to be excreted from the lumen of the stomach.

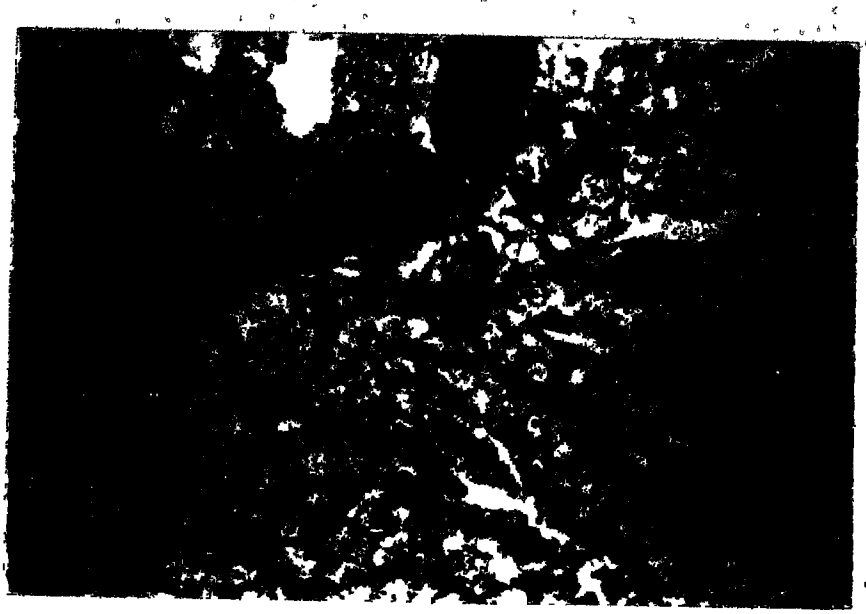
se - epithelial lining of the stomach
sl - stomach lumen

(Bouin's, Mallory's Triple Stain) x175

Figure 2.9

Cross section of digestive gland tubules in the late excretory phase taken at higher magnification. Arrows point to greenish residue contained within the vacuoles.

(Bouin's, Mallory's Triple Stain) x375



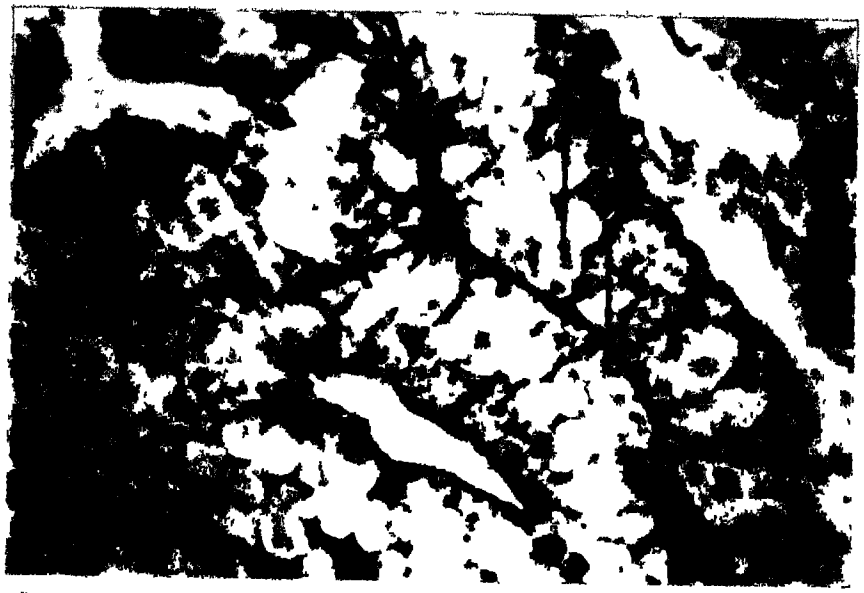


Figure 2.10

Electron micrograph of the basal portions of secretory and digestive cells of L. saxatilis in the early absorptive stage. Note extensive granular endoplasmic reticulum in the secretory cell at this time. Triangles denote possible remnants of previous digestive cycle.

bl - basal lamina
cs - calcium spherite
DC - digestive cell
ger - granular endoplasmic reticulum
li - lipid
n - nucleus
SC - secretory cell

x7,380

DC

Figure 2.11

Apical portions of secretory and digestive cells during the absorptive phase. Note large number of (secretory) zymogen granules and material of similar optical density concentrated between the microvilli of the secretory cells. The seeming granularity in the apical areas of the digestive cells, represents large numbers of pinocytotic vesicles and mitochondria (see Figure 2.14). Arrows point to zymogen granule concentration.

lu - lumen
m - microvilli
T1 - type 1 vesicle
T2 - type 2 vesicle
v - vesicle of unidentified origin
zg - zymogen granule

x7200

Figure 2.12

Electron micrograph of secretory cell during the late digestive, early excretory stage. Note fragmented endoplasmic reticulum and distended cisternae (arrows). Note absence of dense granularity between the microvilli.

cs - calcium spherite
li - lipid
m - microvilli
mi - mitochondrion
n - nucleus
nu - nucleolus

x6300

12

Figure 2.13

Electron micrograph of the apical portion of the digestive cell during the excretory stage. Note total absence of pinocytotic vesicles, but presence of mitochondria.

f - flagellum
mi - mitochondrion

x35,230

Figure 2.14

Electron micrograph of pinocytotic vesicles during the early absorption stage. Picture represents an enlargement of region just within apical border as seen from Figure 2.11. Note the numerous doughnut-shaped pinocytotic vesicles.

mi - mitochondrion
pv - pinocytotic vesicle

x192,500

pv
pv

Figure 2.15

Electron micrograph showing characteristic Golgi complex of a digestive cell during the digestion phase. Note membranous striations in the swollen peripheal margins of the Golgi apparatus as well as in the Golgi vesicles. Note too, the close proximity of this organelle to what seems to be a forming Type 3 vesicle. Arrow points to the early stage of a budding Golgi vesicle.

Ga - Golgi apparatus
Gv - Golgi vesicle
T3 - type 3 vesicle

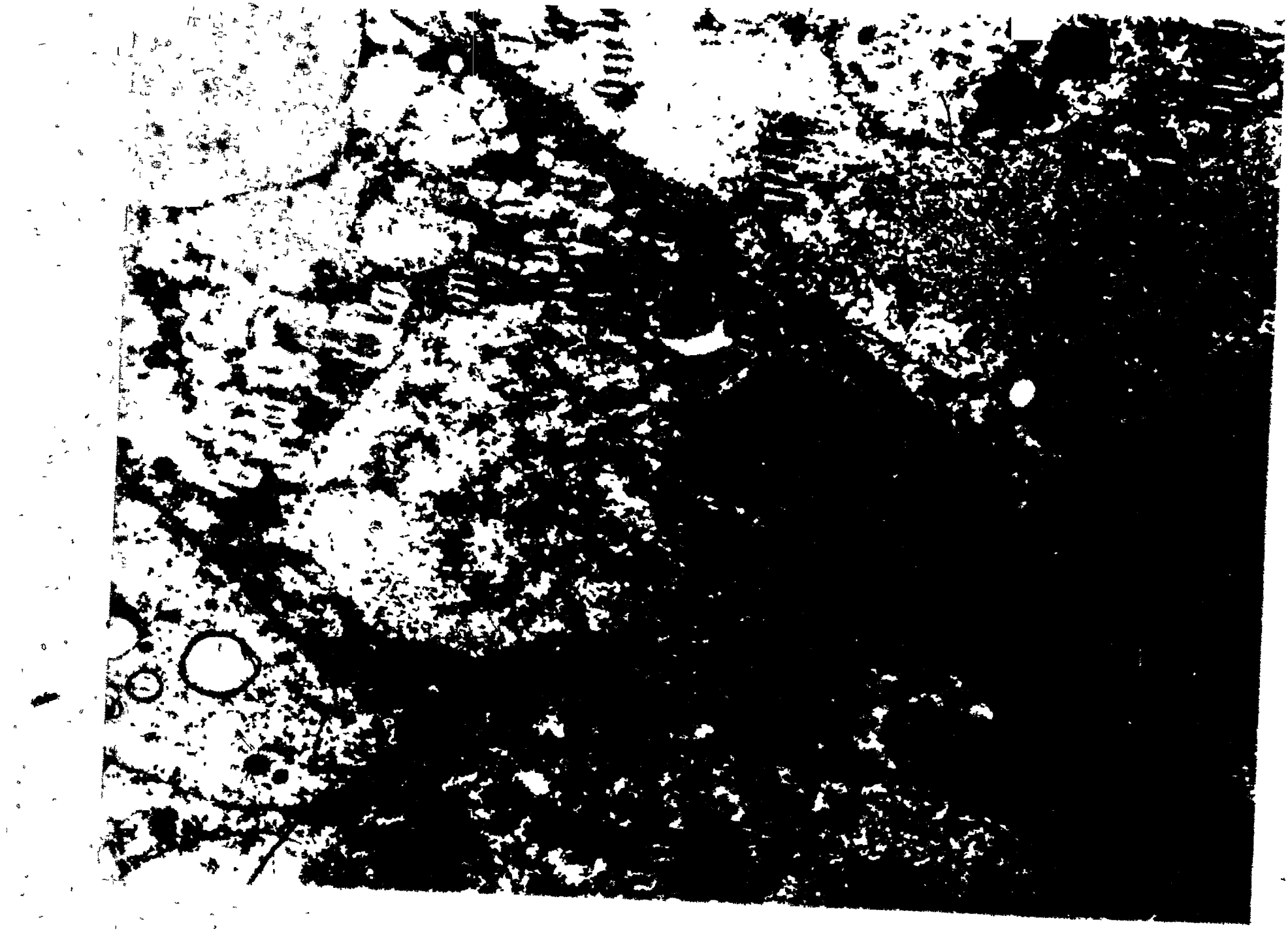
x18,800

Figure 2.16

Electron micrograph showing Type 2 and Type 3 vesicles from a digestive cell during the digestion phase. Note the presence of fusion between such vesicles.

li - lipid
T2 - type 2 vesicles
T3 - type 3 vesicles

x24,750



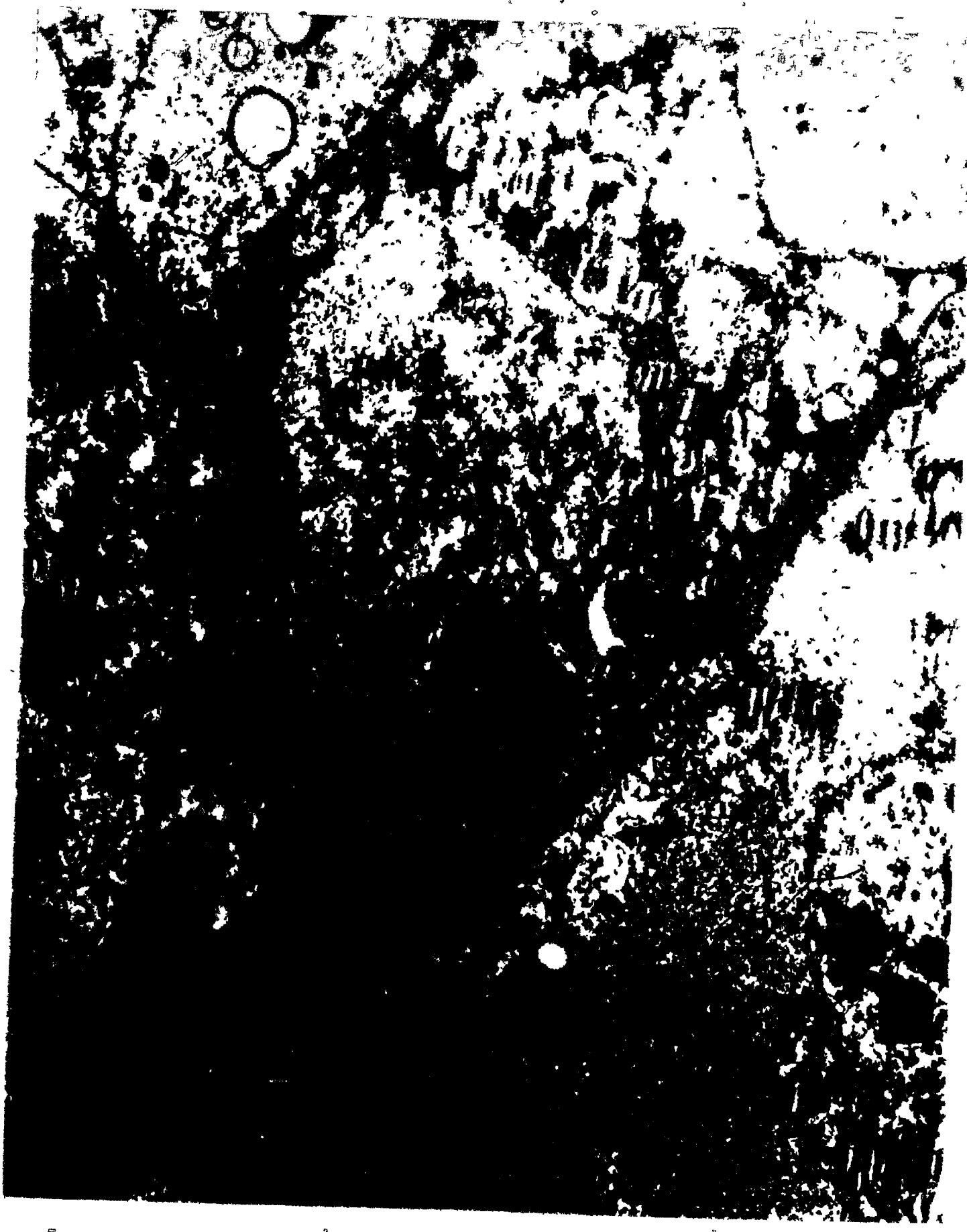


Figure 2.17

Electron micrograph of a digestive cell in the mid-stage of digestion showing fusion (arrow) between T3 vesicles. Note abundance of lipid droplets.

Gv - Golgi vesicle
li - lipid
T2 - type 2 vesicle
T3 - type 3 vesicle

x15,810

Figure 2.17

Electron micrograph of a digestive cell in the mid-stage of digestion showing fusion (arrow) between T3 vesicles. Note abundance of lipid droplets.

Gv - Golgi vesicle
li - lipid
T2 - type 2 vesicle
T3 - type 3 vesicle

x15,810

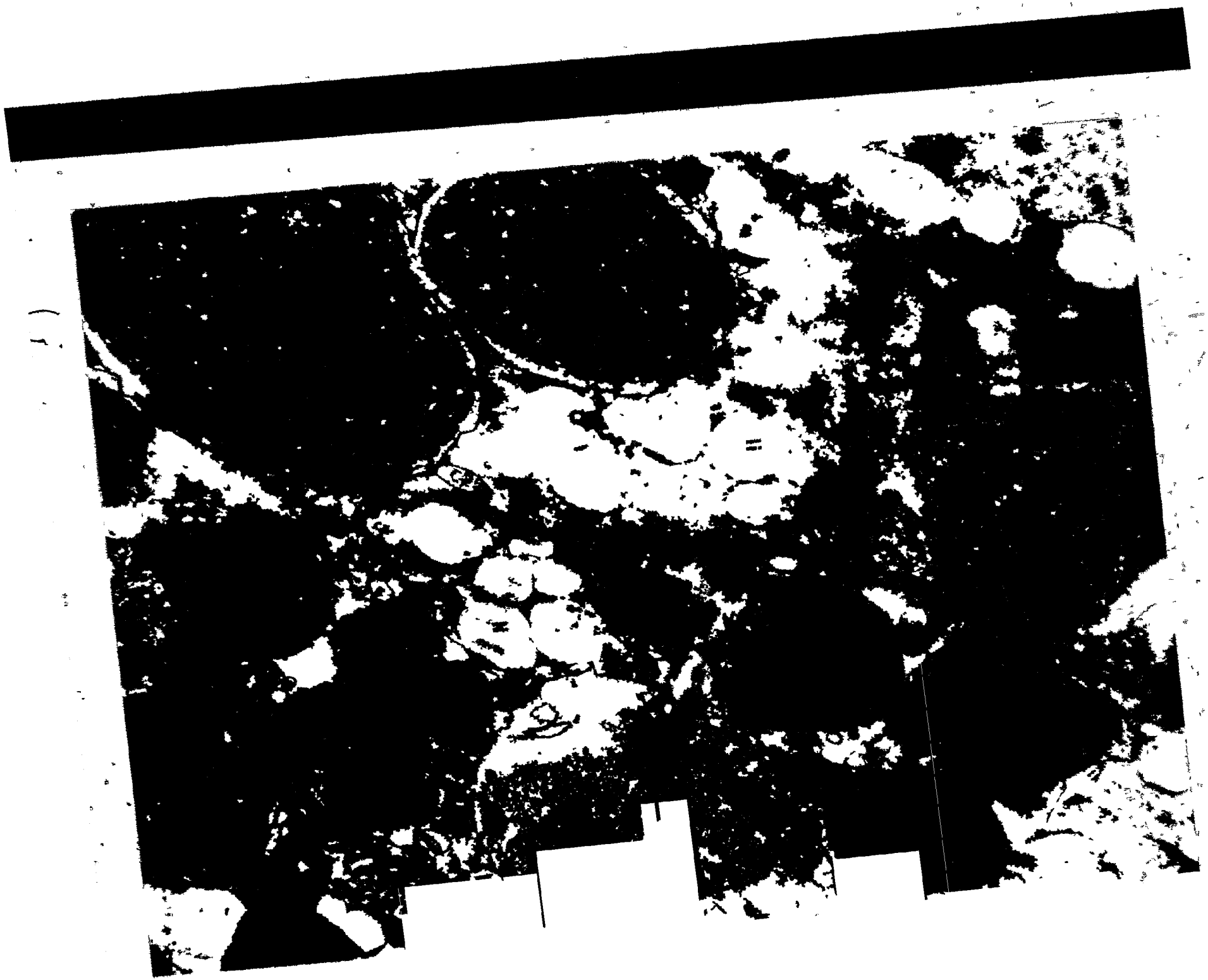


Figure 2.18

Electron micrograph showing mid-regions of digestive cells in the excretory phase. Note the very light granularity in T4 vesicle. This corresponds to the greenish residue observed in the vesicles in Figure 2.9. Note also the remnants of T3 vesicular content (arrows).

li - lipid
T4 - type 4 vesicles

x7380.

Figure 2.19

Electron micrograph of the apical regions of digestive cells in the excretory phase. Microvilli are reduced in size and endocytosis is absent. Lipid is less abundant than in mid and basal areas.

li - lipid

x7380

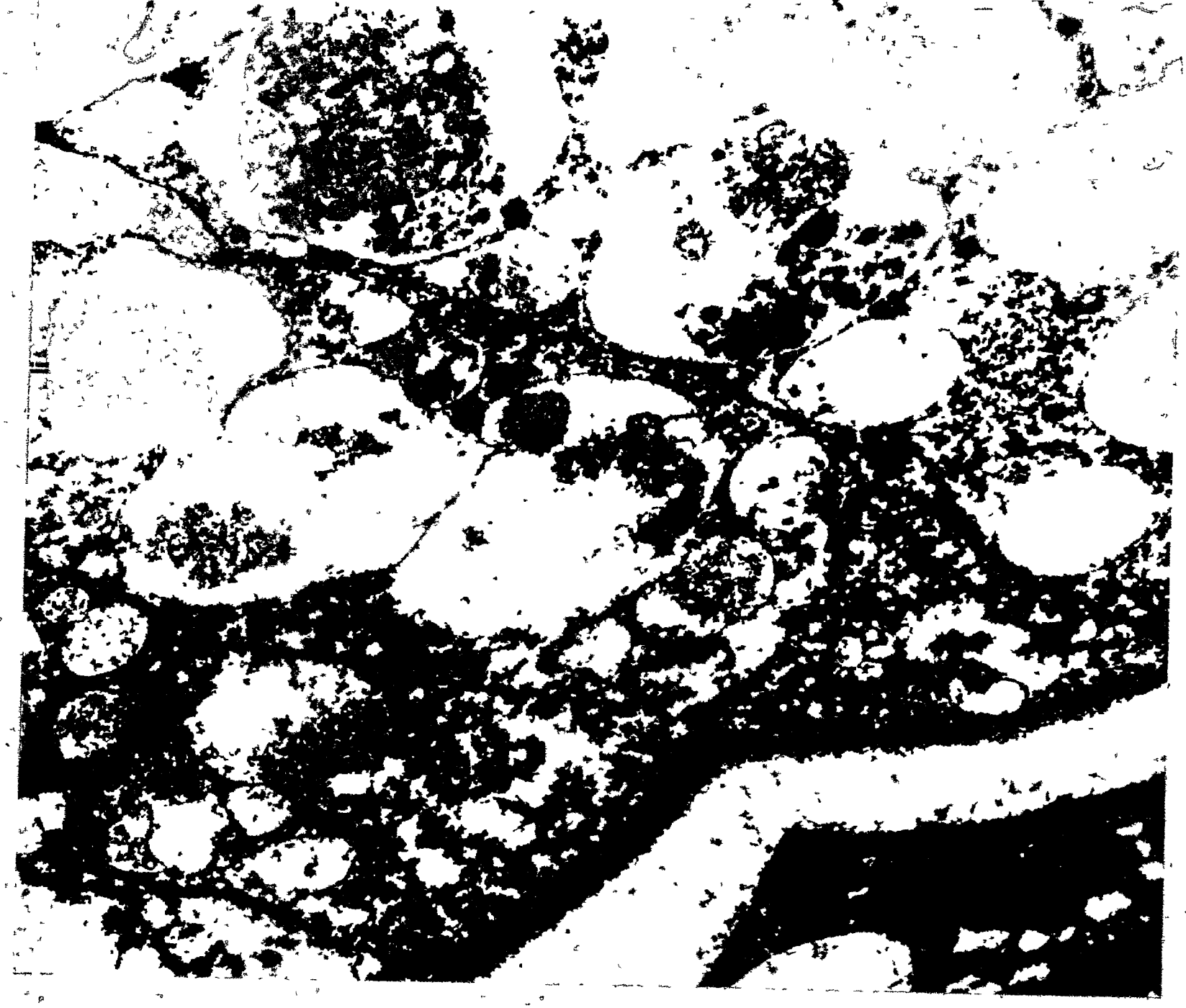


Figure 2.20

Diagram of the digestive and secretory cell of the digestive gland epithelium in Littorina saxatilis.

bm - basement membrane
cs - calcium spherite
Ga - Golgi apparatus
ger - granular endoplasmic reticulum
Gv- Golgi vesicle
li - lipid
m - microvilli
mi - mitochondrion
n - nucleus
nu - nucleolus
pv - pinocytotic vesicle
sd - septate desmosome
T1 - type 1 vesicle
T2 - type 2 vesicle
T3 - type 3 vesicle
T4 - type 4 vesicle
zg - zymogen granule

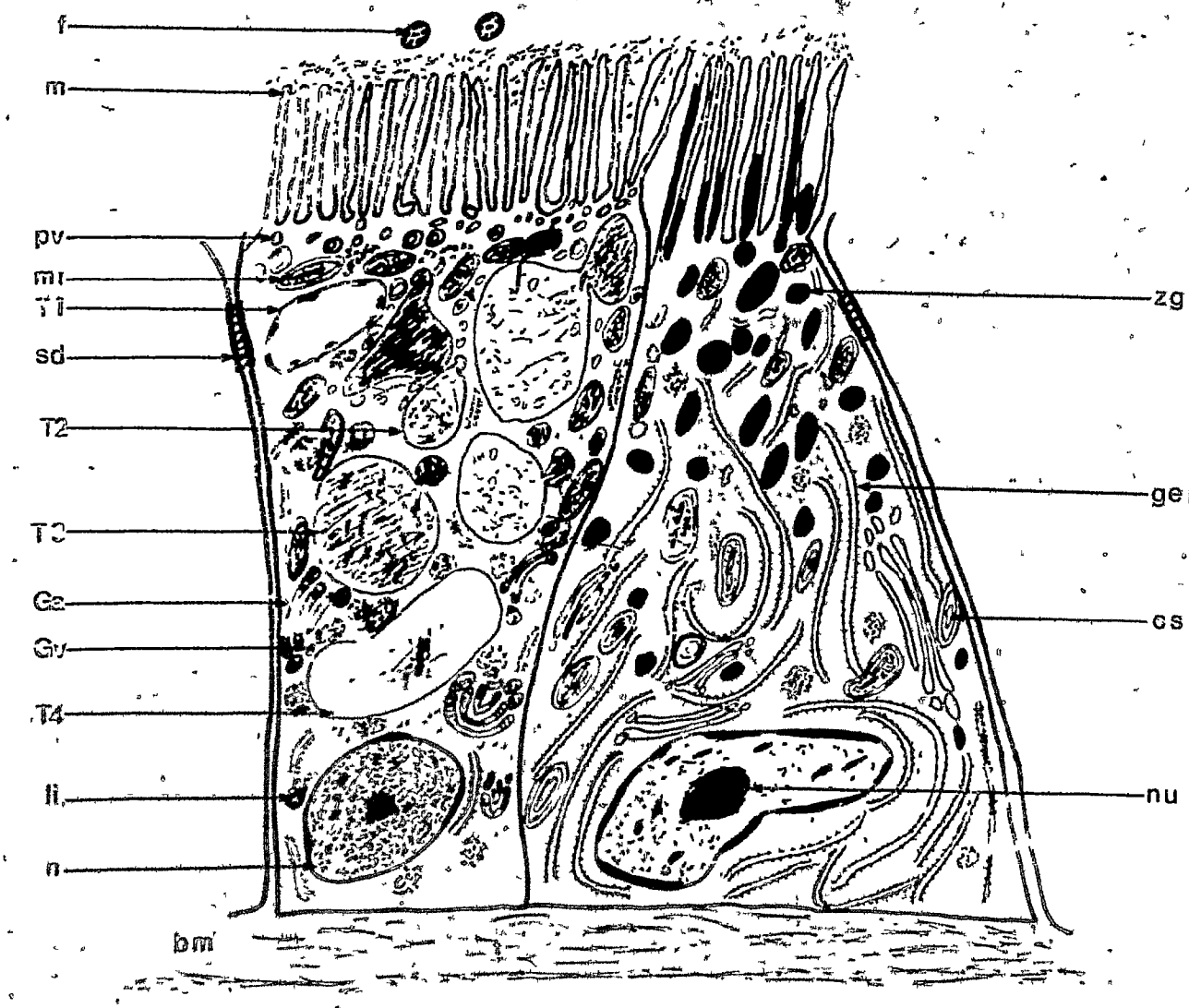


FIG.20

Figure 2.22

A series of diagrams showing the passage of food through the gut and the digestive gland cells in Littorina saxatilis in relation to the tide cycle.

1. High tide plus two and one half hours. The snails are submerged, active and feeding. Faecal pellets are being voided. The digestive gland is in the absorption phase.

2. High tide plus six hours. The snails are exposed and inactive. The mucus rod which is located in the stomach at the posterior end of the rotating food mass is now visible. The food gradually decreases in the oesophagus until there is none left. Few faecal pellets can be observed. The digestive gland is now in the digestion phase.

3. High tide plus nine hours. The snails are exposed and inactive. The rectum is being filled with fecal pellets as the stomach contents are processed. The mucus rod is most prominent at this time. The digestive gland is in the very late digestion, early excretory phase.

4. High tide plus eleven hours. The snails are exposed and inactive. They are soon to be submerged by the next flood tide. There is no food in the stomach by the end of this period. Faecal pellets are now packed in the rectum. The digestive gland is in the excretory phase.

ds - distal stomach
i - intestine
mr - mucus rod
po - posterior oesophagus
ps - proximal stomach
r - rectum

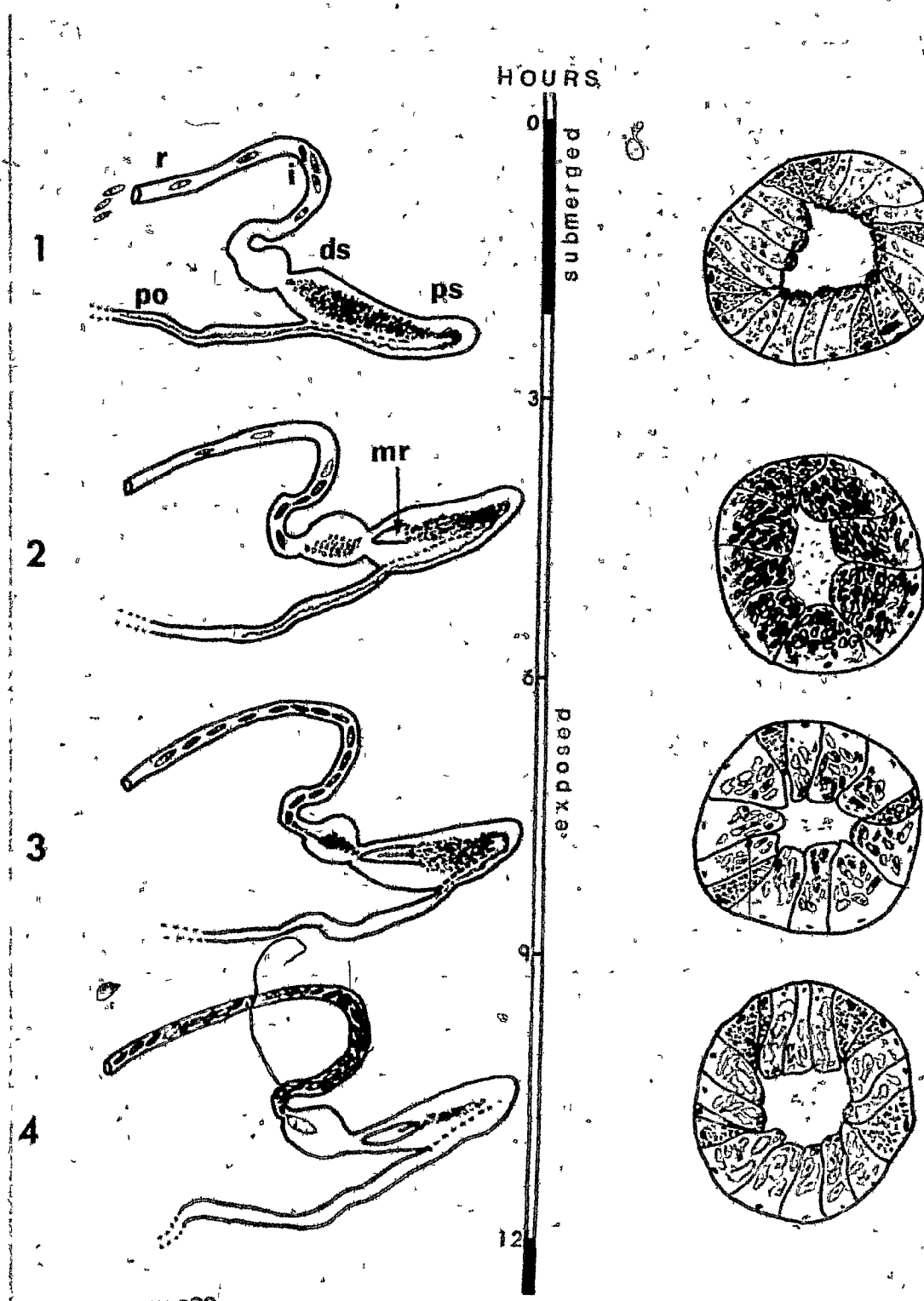


FIG22.

CHAPTER III

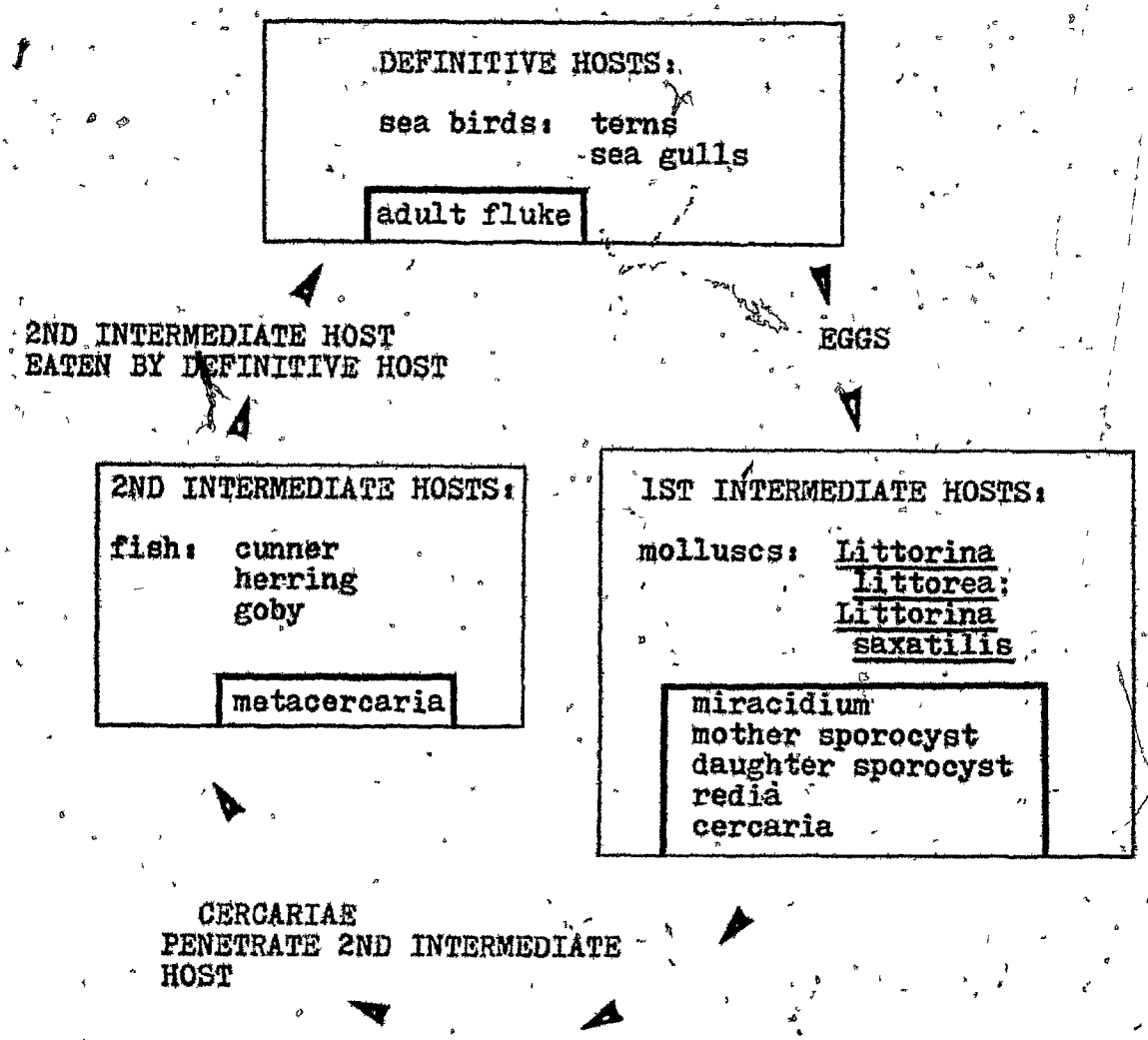
THE PARASITES: AN ULTRASTRUCTURAL STUDY OF THE TEGUMENTS OF *CRYPTOCOTYLE LINGUA* REDIAE AND *MICROPHALLUS SIMILIS* SPOROCYSTS

INTRODUCTION

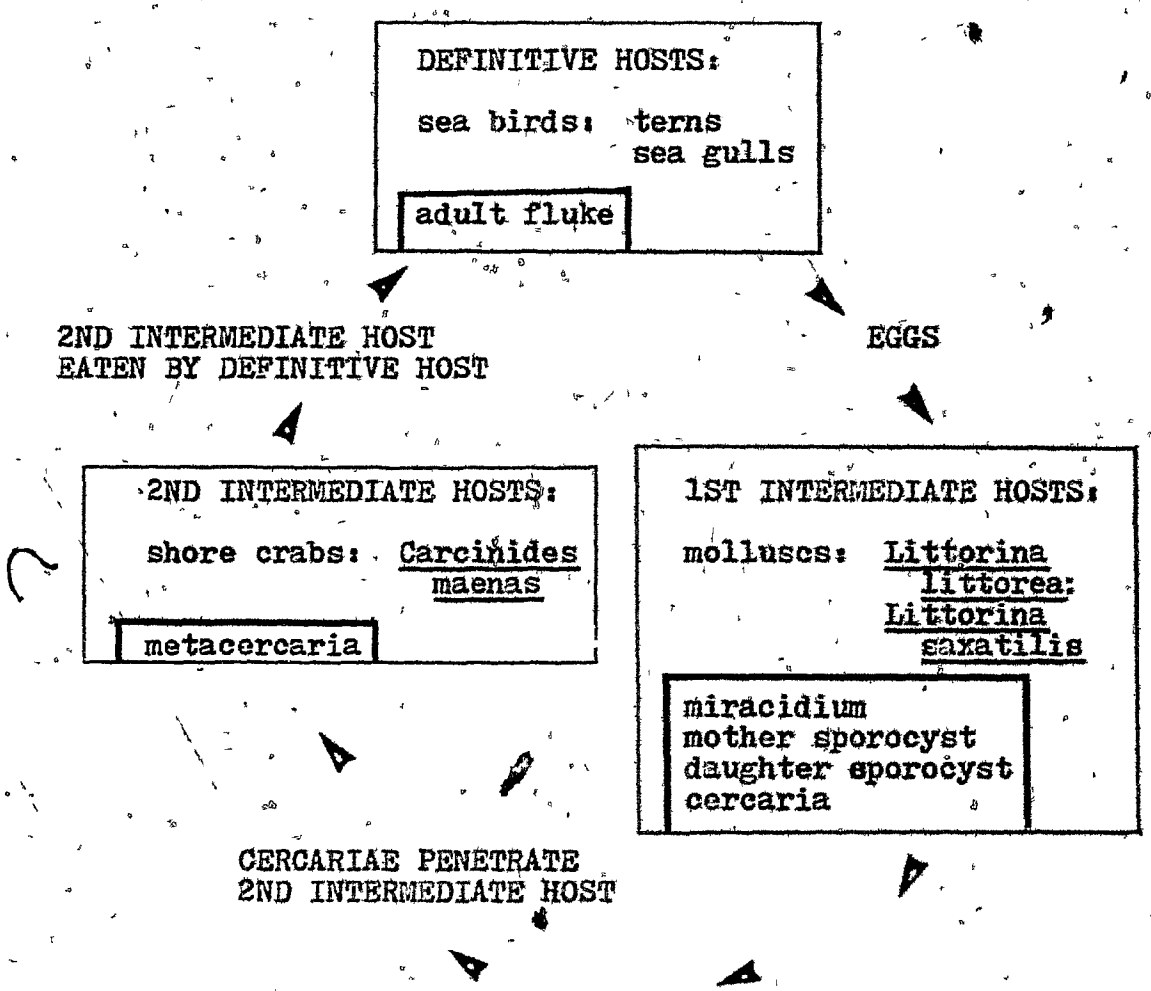
Digenetic trematodes require two or more hosts in order to complete their life cycles. Briefly, the eggs of the adult parasite are passed off in the feces of the final host and are either ingested by a snail or hatch into ciliated, pyriform-shaped miracidia which enter the gastropod by active penetration. Once inside, they transform into elongated sacs with no apparent internal structure, and it is these 'primary' or mother sporocysts which give rise to daughter sporocysts and/or rediae. The latter forms become lodged within the connective tissue of the digestive gland tubules and give rise to cercariae. Cercariae ultimately bore out of their molluscan host and eventually reach a final host where they develop into sexually mature forms.

The life cycles of *Cryptocotyle lingua* (Stunkard, 1930) and *Microphallus similis* (Stunkard, 1957) are considered on the following pages in the forms of schematic representations.

Cryptocotyle lingua:



Microphallus similis:



There are many morphological and physiological features which must have evolved in order to have allowed an organism to become adapted for a parasitic existence. A structure which has received considerable attention and continues to be the subject of many investigations is the helminth cuticle (Lee, 1966). Its importance is self-evident in so far as it represents that region which makes contact with the host. Recently, it has been discussed in the light of certain immunological factors (Smyth, 1973).

In contrast, to the nematode cuticle which is a highly proteinaceous, non-living covering (Jamuar, 1966; Kan and Davey, 1968a, 1968b), the platyhelminth cuticle or tegument is a cytoplasmic layer (Threadgold, 1963). It lies on a basal lamina and is connected by protoplasmic processes to tegument or parenchymal cells which rest in the general parenchyma of the body. While the epidermis of the adult stages of both the Trematoda (Threadgold, 1963, 1968; Bogitsch, 1968; Halton and Lyness, 1971) and the Cestoda (Rothman, 1963; Threadgold, 1965; Lumsden, 1966) have been examined extensively, there has been relatively little work done for the developing stages of these helminths. In the case of larval Digenea, a few studies feature more prominently than others. Bils and Martin (1966), Rees (1966) and Krupa et al (1967; 1968) examined the redial tegument, while James et al (1966), Starch and

Welsch (1970) and Bibby and Rees (1971) provided descriptions of the sporocyst tegument.

This study proposes to examine the ultrastructure of the teguments of Cryptocotyle lingua rediae and Microphallus similis sporocysts as observed from their host Littorina saxatilis, in the light of what is already known from other organisms.

MATERIALS AND METHODS

Individuals of Littorina saxatilis were collected every two hours throughout the tide cycle from the supralittoral zone of a sheltered rocky shore at Blue Rocks, Lunenburg County, Nova Scotia. The shells were crushed at once, and the snail bodies were removed and transferred to a petri dish containing cold sea water, whereupon they were examined immediately for parasitic infection under a dissecting microscope. The parasites were identified and two 1 - 2 sq. mm. samples of that portion of the visceral hump most heavily infected were isolated from the rest of the viscera. Ten snails, five infected with Cryptocotyle lingua and five infected with Microphallus similis were recovered at each interval and processed in preparation for light and electron microscopy. In addition, a number of infected snails were returned intact to the laboratory where they were immediately dissected. Subsequently, the developing stages of C. lingua and M. similis were isolated from their host and studied in the living state.

Light Microscopy

For examination with the light microscope, the tissues were fixed in Masson's modification of Bouin's fluid (Foot, 1933) for four days then washed in 70 percent ethanol for one day. Following dehydration, they were embedded in Paraplast embedding medium (Sherwood Medical Industries

Ltd., St. Louis, Missouri, U.S.A.) and sectioned at a thickness of six to seven microns. Ribboned sections of each specimen were mounted on slides and examined after staining by the Mallory's Triple Stain Method. Live specimens were studied by means of regular light and phase microscopy, as well as with a Namarski interference microscope.

Electron Microscopy

In preparation for electron microscopy, the tissues were fixed in 4 percent glutaraldehyde in buffered filtered sea water at pH 7.3 for one hour. They were then rinsed and stored in cold buffer (Millonig's phosphate buffer) for forty-eight hours. The tissues were subsequently post-fixed for two hours in cold 0.9 percent osmium tetroxide solution in Millonig's buffer (pH 7.3). After dehydration, the material was embedded in Maraglass. Sections were obtained with glass knives on an L.K.B. ultratome and were picked up on formvar-coated copper grids (70 mesh). The sections were double stained with alcoholic uranyl acetate (Stempak and Ward, 1964) for fifteen minutes and with lead citrate (Reynolds, 1963) for ten minutes. All sections were examined on a Zeiss E.M. model 9S. Preliminary light microscope observations of thick sections using the toluidine blue staining method (Trumpf et al., 1961) always preceded final sectioning. This helped to identify that region of the parasite to be viewed with the electron

microscope, as well as to orient the block in preparation for sectioning with the ultratome.

OBSERVATIONS

GENERAL

Cryptocotyle lingua rediae are pale grey sausage-shaped organisms which display considerable motility in their hosts and grow to a length of 0.5 - 1.5 mm. When studied in whole mount, the most conspicuous structures are the anterior sucker, the pharynx and the intestine (Fig. 3.1). The birth pore, located ventrally and immediately posterior to the sucker, and the excretory pores in the caudal third of the body are not visible. Different stages of cercarial development can be seen from Figure 3.1, the most mature organisms occurring in the apical region, and the embryonic forms located in the caudal part of the redia (Fig. 3.1, 3.2).

Microphallus similis sporocysts are pale-white in colour. These oval-shaped organisms are immobile and in general lack the high degree of structural development observed from rediae of C. lingua. They range from 100 - 600 µm, in length, and when fully mature, usually contain 10 - 12 cercariae (Fig. 3.3, 3.4).

TEGUMENT

Cryptocotyle lingua

The tegument of C. lingua rediae consists of an outer syncytial anucleated cytoplasmic layer which rests on a basal lamina (Fig. 3.5, 3.6) and is continuous with tegu-

ment cells lying in the general parenchyma by means of periodic cytoplasmic connections (Fig. 3.6).

The tegument surface is thrown into numerous folds along which there occurs a series of extensions that are occasionally branched (Fig. 3.6, 3.9, 3.10). These projections range from 0.5 - 1.0 µm. in size and vary in their distribution density, being most abundant towards the mid posterior regions and more sparsely scattered in the anterior zone.

The thickness of the tegument is between 0.15 - 1.0 µm. and is characterized by a light granularity in places. It contains few mitochondria, various unidentified membranous bodies, glycogen particles of the beta variety (150 - 200° A) and the occasional lipid inclusion (Fig. 3.6, 3.10, 3.12). In addition, membrane-bound vesicles, possibly the product of endocytosis, contain material of medium-heavy electron density similar to that found at various stages within the invaginated apical plasmamembrane (Fig. 3.8, 3.10, 3.12). Material considerably more electron dense can be seen enclosed within the tips of the microvilli (Fig. 3.6, 3.7), as well as in minute cytoplasmic vacuoles.

The basal membrane rests on a high undulating basal lamina with which it shares hemidesmosomes at regular in-

tervals (Fig. 3.7, 3.10). The lamina, whose contours correspond to the general topography of the outer plasma membrane, is amorphous, about 0.1 - 0.2 µm. thick and separates the outer layer from the musculature and tegument cells lying immediately beneath it (Fig. 3.10). Occasionally, invaginations of the basal lamina can be seen (Fig. 3.6) and these probably represent the sites of continuity between the outer syncytial area and the tegument cells.

Below the basal lamina is an outer layer of circular muscle and an inner one of longitudinal muscle (Fig. 3.10). Mitochondria, glycogen particles and lipid globules can often be seen embedded in the sarcolemma of the muscle cells. This highly developed musculature no doubt explains the motility displayed by the rediae within their host.

The tegument cells, which generally lie beneath, but closely applied to the musculature, can also be seen interspersed between the muscle cells (Fig. 3.5). Their large size and the intimate contact which they have with each other make it difficult to study them individually. They are characterized by elongated nuclei, each possessing condensed chromatin granules (Fig. 3.11). Mitochondria, glycogen particles and rosettes (alpha and beta) smooth endoplasmic reticulum and isolated ribosomes can usually be observed, while profiles of granular E.R. and Golgi

bodies are less common (Fig. 3.5, 3.10, 3.11). Electron dense granules within closely applied cell membranes, ranging from 500 - 2000° A in diameter are observed in some preparations. Their electron density is similar to material previously reported to be contained within the microvilli tips (Fig. 3.10).

Microphallus similis

While the sporocyst tegument of M. similis is basically similar to that of C. lingua rediae, certain differences do exist. It too, is composed of an outer syncytial anucleated cytoplasmic layer which rests on a basal lamina and is joined to a subtegument region by a number of protoplasmic connections (Fig. 3.13).

The sporocyst tegument displays a fairly regular topography in contrast to the highly convoluted redial surface, although invaginated pocket-like formations can be seen at times (Fig. 3.14). It varies in thickness from 2.0 - 7.0 µm. and its apical membrane is characterized by a series of finger-like invaginations ranging from 0.1 - 0.6 µm. in length (Fig. 3.13). These not only increase the surface area but also represent the sites of endocytotic activity. Their terminal ends usually appear swollen and vesicles containing host tissue can be seen pinching off (Fig. 3.13, 3.14, 3.16).

The matrix of the tegument is composed of a variable but usually electron-lucent ground substance. It contains dense granules (.1 - .5 µm. in diameter) bound by closely applied membranes (Fig. 3.13) and pinocytotic vesicles whose diameters range from .02 - .05 µm, and which are surrounded by a very light granular coat (Fig. 3.16). Abundant mitochondria and the occasional lipid inclusion as well as myelinated membranous whorls can also be seen from this region (Fig. 3.13, 3.14, 3.16). Glycogen particles are present but less common than in the redial tegument (Fig. 3.16).

The basal lamina is amorphous, measures 400 - 600° A and separates the syncytial layer from the subtegument portion (Fig. 3.13, 3.14). Occasionally, it projects slightly into the subtegument and provides for the presence of bridges which ensure a continuity between the two cytoplasmic regions (Fig. 3.13).

Immediately below and adjacent to the basal lamina is a loosely arranged outer layer of circular muscle fibers and an inner one of longitudinal fibers. These are poorly developed, especially so in the case of the older sporocysts which are about to release their cercariae (Fig. 3.13).

Beneath the musculature and separated from the brood chamber (germinal cells) by a distinct inner boundary is the subtegument cellular region. Unlike the tegument

cells of C. lingua the subtegument does not appear to be composed of separate and clearly defined cells, rather it assumes the form of a nucleated syncytium (Fig. 3.13, 3.14). The nucleated regions appear denser than the rest of the cytoplasm, displaying at times considerable granular endoplasmic reticulum (Fig. 3.13, 3.14, 3.15) as well as free polyribosomes (Fig. 3.17). This contrasts with the diffuse reticulate cytoplasm around them. The nucleated portions seem to be enclosed within a limiting membrane thus suggesting that they are in fact separate from the rest of the cytoplasmic material. More often, however, pseudopodial-like extensions emanate and connect them with other nucleated areas or project into the surrounding cytoplasm, thus allowing for fusion of the 'two' cytoplasms (Fig. 3.17).

Glycogen particles and rosettes, mitochondria, electron dense granules similar to those described from the tegument, numerous lipid inclusions and the occasional strand of smooth endoplasmic reticulum are often observed (Fig. 3.13, 3.14, 3.17). Golgi bodies characterized by short rounded cisternae which are relatively electron lucent and in the area of which there are numerous clear vesicles, also prevail in this nucleated region (Fig. 3.17).

DISCUSSION

The tegument of C. lingua rediae and M. similis sporocysts conforms to the basic organization of this structure as described previously for the different trematode stages (Threadgold, 1963; Bils and Martin, 1966; Lee, 1966) and for cestodes (Threadgold, 1962; 1968; Rothman, 1963; Lumsden, 1966).

In both organisms, the apical membrane gives rise to a series of minute structures, forming elongated, sometimes branched projections in rediae and finger-like invaginations in the sporocysts. Their role in increasing the exposed surface area, as well as participating in endocytosis, at least in the sporocysts and possibly in the rediae, is quite apparent. Moreover, their functions can be compared to those of the microtriches described from cestodes (Threadgold, 1962; Rothman, 1963) and various other apical irregularities including microvilli, branched processes, surface projections, etc., as reported from other trematode larvae (Rees, 1966; Krupa et al., 1967; Bibby and Rees, 1971).

Glycogen particles, often associated with tissues displaying high metabolic activity were observed from the teguments of both parasites. In addition, the mitochondria in the teguments probably provide the energy necessary for the extensive membrane activity. The redial tegument con-

tains fewer mitochondria, and this might be explained by the presence of a functioning gut which assumes to some extent the role of food acquisition, in this way relieving the tegument of the entire burden of food absorption.

Most workers thus far have examined the parasites after they had been removed from their hosts (James et al., 1966; Krupa et al., 1967; Rees, 1966; Reader, 1972). The present study however, takes into account the host-parasite interface, and examines the parasites in their native environments, i.e. immediately adjacent to host tissue. Because the parasites were not isolated from their food substrate, pinocytosis and pinocytotic vesicles were readily observed. This contrasts with the observations reported by some workers who initially separated the parasites from their hosts, thus completely failing to observe pinocytotic vesicles (Rees, 1966) or merely assuming the occurrence of pinocytosis on the basis of various structural modifications (Krupa et al., 1967; Reader, 1972) e.g. invaginations of the microvillis border.

The redial tegument cells and the nucleated subtegument cytoplasm of the sporocysts contain smooth endoplasmic reticulum characteristic of cells participating in the synthesis of lipids, and also granular endoplasmic reticulum known to be associated with protein synthesis. The presence of dense granules contained within tight-fitting

membranes, as observed from the tegument and subtegument regions in both parasites, may represent a form of secretory product. Because material of similar electron density has been observed from the tips of redial microvilli, and also because of the presence of Golgi bodies such granules may indeed be a type of secretory product functionally related to the Golgi body and/or to the granular endoplasmic reticulum. The presence of axial filaments in the microvilli and their possible association with the production of enzymes has already been considered in the case of the rat oviduct and jejunum epithelium (Hansen and Herman, 1962 - as cited by Brown and Bertke, 1969). Electron dense granules similar to zymogen-like granules have been reported from adult (Burton, 1964; Threadgold, 1967; Halton and Lyness, 1971) and larval trematodes (Southgate, 1970; Bibby and Rees, 1971) and these have usually been assigned a secretory role.

Alkaline phosphatase has been shown to have an important function in carbohydrate metabolism (Von Brand, 1952). Its presence in the tegument of other larval trematodes has been demonstrated (Cheng, 1964; James et al., 1967; Reader, 1972) and associated with an increase of glycogen and glucose in the parasite and a corresponding decrease from the host. It has not been definitely shown, however, that it is the dense granules discussed above

which represent the source of such an enzyme.

Krupa et al. noted the presence of acid phosphatase in lysosomes of C. lingua redial tegument cells. These structures may correspond to the dense granules observed in the rediae of C. lingua and sporocysts of M. similis. Likewise, Reader (1972) reported the presence of the enzyme in the cytoplasm of the redial tegument of Sphaeridotrema globulus, noting its abundance in the cores of the microvilli. It was also observed in what the author referred to as "small lysosome-like bodies".

There must be both influx and efflux across the apical tegument membranes of the parasites. Moreover, because the germinative cells develop in direct response to the availability of food for the parasites, it becomes increasingly clear that considerable nutrient must go through the tegument in order to reach the brood chamber. Unlike sporocysts, where all food must pass across the wall, the functioning gut of the rediae provides an additional source for the acquisition and processing of food. However, because the intestine does not continue to develop in proportion to the rest of the body, it ultimately comes to occupy only a fraction of the total volume in the mature redia (Stunkard, 1930). Therefore, the redia too, must gradually rely more heavily on the tegument for the acquisition of its food, especially in the older forms. It is also reasonable to

expect that there should be an increase in microvilli and membrane activity in the posterior third of the body, as has been observed, since this is the region where embryogenesis is taking place and one which requires considerable energy.

Finally, an aspect of digestion which warrants closer attention and has thus far been neglected concerns whether an imposed rhythm of feeding occurs in the parasites similar to that shown for L. saxatilis (Chapter 2), and one that can be correlated to the digestive state of the host. It is only recently that the question of the possible effects of host nutrition on a worm burden has been raised (Wright, 1971). The results of such studies might eventually help to explain the relative degrees of adaptation by different parasites to their hosts, on the basis of regulated or uncontrolled feeding.

In summary, the tegument of C. lingua rediae and M. similis sporocysts conforms to its organization as described for digenean trematodes by other workers. In both instances, the apical membrane gives rise to a series of minute structures. These assume the form of branched projections in the rediae and finger-like invaginations in the sporocysts. Observations indicate that endocytosis is common in both organisms. While M. similis sporocysts are totally dependent on such a mechanism for food pro-

curement, C. lingua rediae also possess a functioning gut which, to some extent, relieves the tegument of its role of food absorption. A basal lamina separates the tegument from tegument cell in C. lingua rediae and from a syncytial subtegument region in M. similis sporocysts. Cytoplasmic connections ensure a constant exchange between the teguments and the subtegument areas. The tegument is regarded as an important structure since it is that region of the parasite which makes the most intimate contact with the host.

Figure 3.1

Fresh mount of Cryptocotyle lingua redia containing the different stages of cercarial development. A progression in cercarial complexity can be traced through the redia from the early embryonic stages in the caudal region to almost mature forms in the apical region. Note the corrugated body wall and the greater thickness especially in the mid-basal portions (arrows). It is in these regions that major differentiation is taking place. Note too the relatively small gut (intestine) in relation to the rest of the body. Particulate matter of host origin can be observed within the intestinal lumen.

es - cercarial eye spot
i - intestine
os - oral sucker
p - pharynx
(Nomarski Interference)

x320

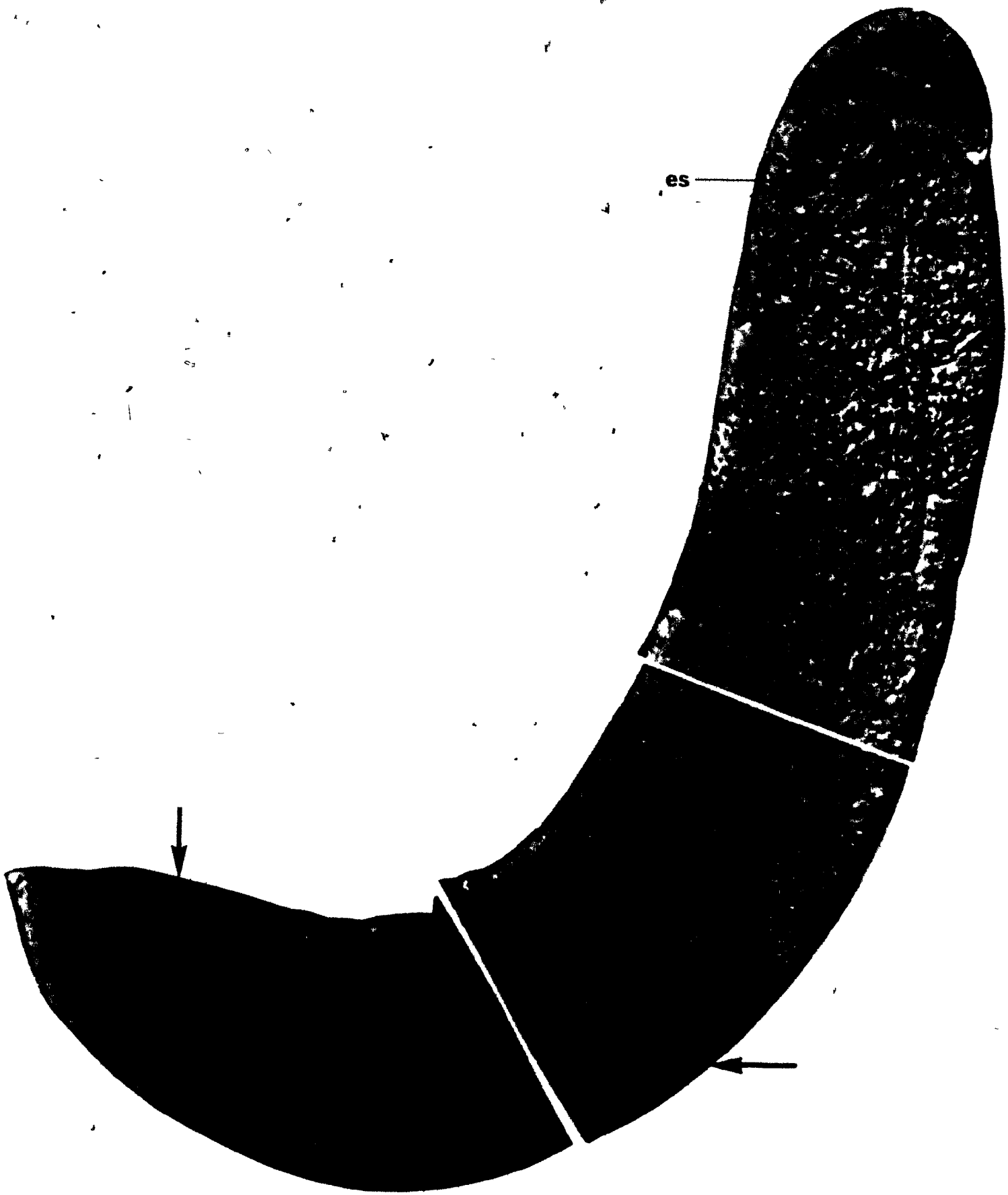


Fig. 1

Figure 3.2

Fresh mount of a mature C. lingua cercaria. Note the broad, long rhapalocercous tail, eye spots and oral sucker.

es - eye spot
os - oral sucker
(Nomarski Interference)

x240

Figure 3.3

Fresh mount of a mature Microphallus similis cercaria. Note the characteristic stylet and the oral sucker.

ep - excretory pore
os - oral sucker
pg - penetration glands
s - stylet
(Nomarski Interference)

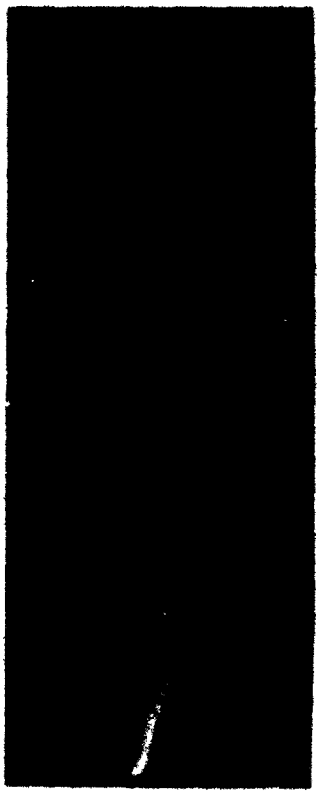
x252

Figure 3.4

Fresh mount of M. similis daughter sporocyst containing developing cercariae. Note the stylet of an immature cercaria. Unlike the C. lingua redia, there is no gut present in the sporocyst. The apparently clear layer immediately under the tegument is an artifact.

c - cercaria
s - stylet
(. Nomarski Interference)

x252



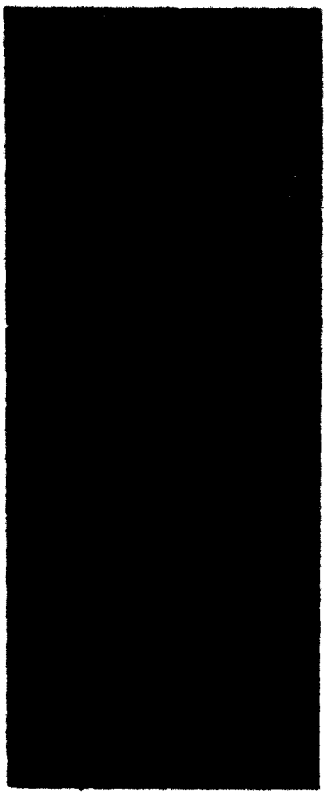


Figure 3.5

Cross section of C. lingua showing the different regions of the redial wall and the position of the parasite with respect to the host cells.

ab - apical border
cbl - cercarial basal lamina
CER - cercaria
ct - cercarial tegument
hbl - host basal lamina
gc - gland cell
n - nucleus
rbl - redial basal lamina
rm - redial muscle
rt - redial tegument
SC - secretory cell of host digestive gland
sg - secretory granule
tc - tegument cell

x10,000

Figure 3.6

Cross section through the redial body wall showing the invagination of the basal lamina (arrow). This represents the site for cytoplasmic continuity between the syncytial tegument and the tegument cell.

bl - basal lamina
li - lipid
mi - mitochondrion
sv - secretory vesicle

x26,700

Figure 3.7

Cross section through the redial tegument showing surface projections which enclose material of medium dense granularity, possibly the product of endocytosis (arrows). Note too, very small dense granules from the tips of the microvilli (thin arrows). The latter may have a secretory function.

bl - basal lamina
hd - hemi-desmosome
hct - host connective tissue
p - surface projection

x14,400

Figure 3.8

Enlargement of part of Figure 3.7 showing apical projections with medium dense granular material contained within them.

x111,340

Figure 3.9

Cross section through the apical membrane of a redia showing possible method of phagocytosis of host tissue. Note the invaginated outer membrane and the characteristic folding of a surface projection (arrow) engulfing extracellular material. Note too, the characteristic branching of a surface projection.

x102,550

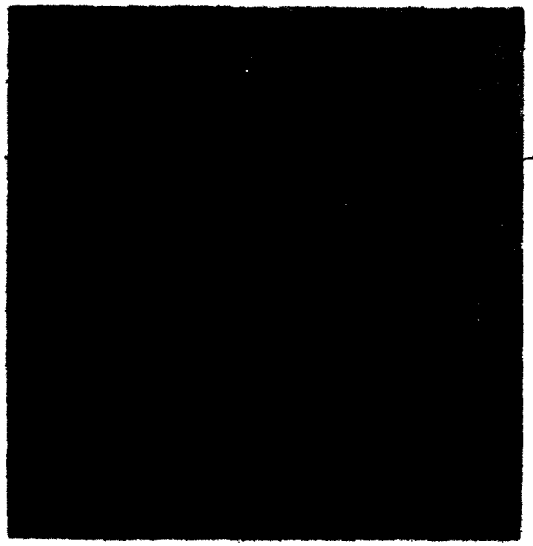


Figure 3.10

Cross section through the body wall of C. lingua redia. Mitochondria, glycogen particles and vesicles containing varying concentrations of material occur in the tegument. The latter may represent the endocytotic vesicles, the formation of which has been described in Figures 3.7 - 3.9. Note the highly folded basal lamina and the way its topography corresponds to the contours of the apical membrane. Note too, the highly developed musculature composed of the outer circular and inner longitudinal fibers. Glycogen particles and rosettes, profiles of smooth and granular endoplasmic reticulum and dense secretory granules contained within closely applied membranes, similar to those located in the tegument can be observed.

am - apical membrane
bl - basal lamina
cm - circular muscle
ev - endocytotic vesicle
ger - granular endoplasmic reticulum
gp - glycogen particle
gr - glycogen rosette
hd - hemi desmosome
lm - longitudinal muscle
mi - mitochondrion
ser - smooth endoplasmic reticulum
sv - secretory vesicle
t - tegument

x44,500

Figure 3.11

Electron micrograph showing a nucleus from a redial tegument cell. Note the elongated apparance of the nucleus and also the characteristic condensed chromatin.

l - lipid
n - nucleus

x7,200

Figure 3.12

Tangential section through the redial surface of C. lingua taken at high magnification. Note the abundant glycogen particles as well as the presence of what is quite probably endocytotic vesicles. The variation displayed by these structures in their electron densities, may actually be a reflection of the different states of digestion. Note the numerous membranous elements of unidentified origin (arrows).

ev - endocytotic vesicles
gp - glycogen particle

x71,000

Figure 3.13

Cross section of M. similis showing the different regions of the sporocysts wall and the position of the parasite with respect to host digestive gland cells and phagocytic cells. Note that the apical membrane gives rise to finger-like invaginations and does not display highly folded topography as is characteristic of the C. lingua redial tegument. Note the presence of dense secretory granules enclosed within tight-fitting membranes. Note too, the much more poorly developed musculature than that characteristic of C. lingua rediae. Heavy arrows indicate the location of a cytoplasmic connection between the tegument and subtegument regions.

ap - apical border
abo - amorphous border separating brood chamber from sporocyst subtegument.
ai - apical invaginations
bl - basal lamina
CER - cercaria
cm - circular muscle
ev - endocytotic vesicle
gg - glycogen particles
li - lipid
lm - longitudinal muscle
mi - mitochondrion
n - nucleus
PC - phagocytotic cell
SC - secretory cell
sv - secretory vesicle
t - tegument

x7,200

Figure 3.14

Cross section of M. similis sporocyst body wall. Note the electron dense region within which the nuclei are embedded in the subtegument. Note too, the pseudopodial-like extensions from the dense cytoplasmic areas and how they make contact with each other (also seen in Fig. 3.17).

Unlike the tegument cells of C. lingua rediae, the nuclei do not contain condensed chromatin along the inner membrane. The nuclear plasm is characterized by a homogeneous medium-light granularity interspersed in places with larger and more electron dense granules.

BC - brood chamber
bl - basal lamina
li - lipid
n - nucleus
pe - pseudopodial-like extension
pi - pocket-like invagination of apical membrane
t - tegument

x5,220

Figure 3.15

Cross section of a nucleus from M. similis sporocyst subtegument. Note extensive granular endoplasmic reticulum and mitochondria surrounding the nucleus.

ger - granular endoplasmic reticulum
mi - mitochondria
n - nucleus

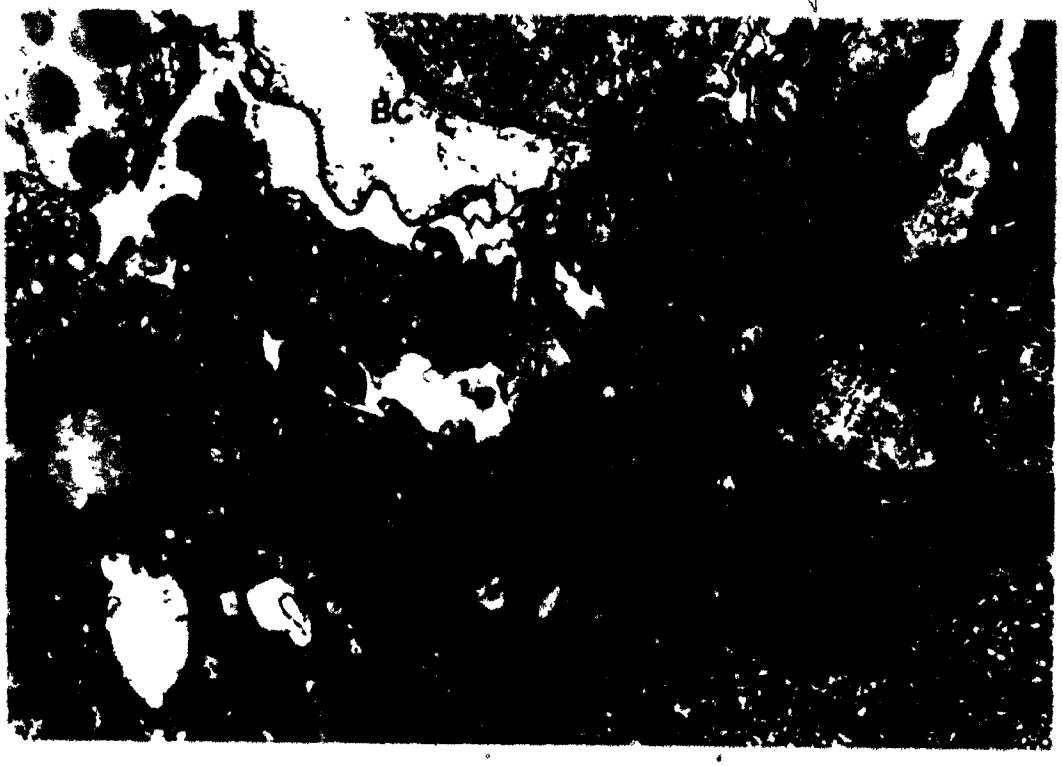
x22,310

Figure 3.16

Cross section of apical invaginations and tegument of M. similis sporocyst. Note endocytotic vesicles displaying characteristic light outer granularity and pinching off the terminal ends of the invaginated, finger-like protrusions. Note also the large number of mitochondria and the empty-looking membranous inclusions.

ai - apical invagination
ev - endocytotic vesicle
ger - granular endoplasmic reticulum
gp - glycogen particle
mi - mitochondrion

x34,760


BC

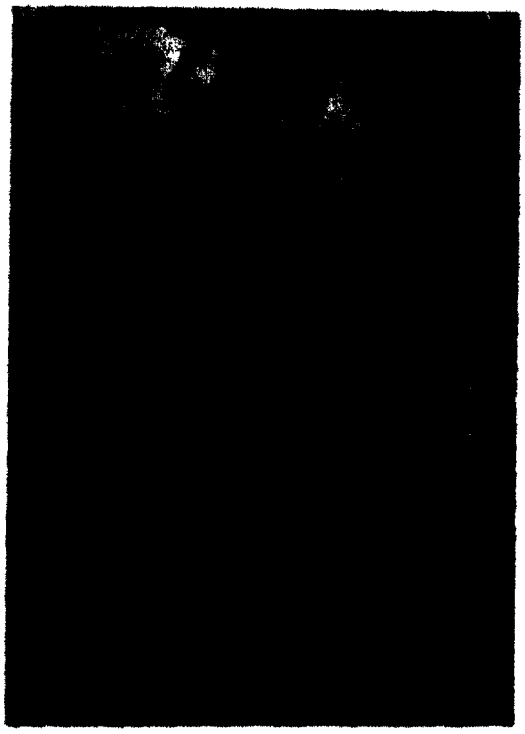


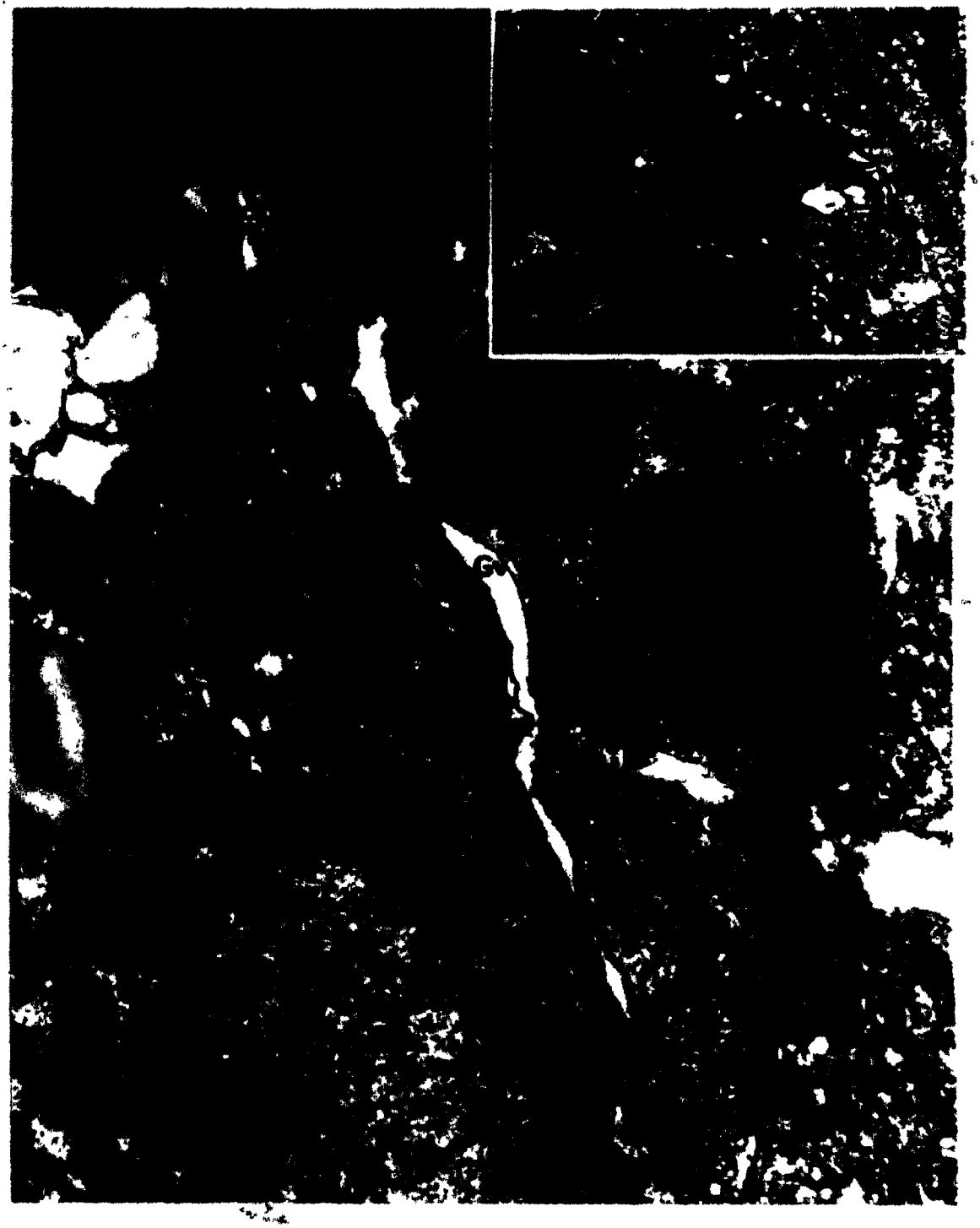
Figure 3.17

Cross section of the subtegument of an M. similis sporocyst. Note Golgi bodies in the region of the nucleus. Their cisternae are distended at their margins, giving rise to large electron lucent Golgi vesicles.

The arrows in the inset point out the location where the Golgi bodies occur in relation to the outer structures and regions of the sporocyst. Fusion between adjacent dense cytoplasmic regions is shown in the inset and is designated by the symbol F. The identical feature is shown in Figure 3.17 only at a higher magnification.

F - fusion between adjacent dense cytoplasmic regions
Gb - Golgi body
Gv - Golgi vesicle
gp - glycogen particles
gr - glycogen rosette
ser - smooth endoplasmic reticulum

x28,480
x3,240 (inset)



CHAPTER IV

THE INFLUENCE OF CRYPTOCOTYLE LINGUA AND MICROPHALLUS SIMILIS ON THE DIGESTIVE GLAND CELLS OF LITTORINA SAXATILIS

INTRODUCTION

The host parasite interactions between the parasitic larvae of digenetic trematodes and members of the phylum Mollusca have generated numerous investigations on the part of scientists. The work presented in this chapter was initiated and carried out in an effort to assist in establishing certain general principles governing the dynamics of such interrelationships.

Examination methods for studying host-parasite associations are undoubtedly varied, but for convenience sake they can be broadly categorized as follows: 1) ecological relationships, 2) biochemical and physiological relationships using quantitative methods, and 3) relationships at the tissue level. Many investigations have focused their attention on ecological relationships, both in the field (Rotschild, 1941a, 1941b; Lysaght, 1941; Berry, 1962; Sinderman and Farrin, 1962; Farley, 1967; James, 1968a, 1968b; Lambert and Farley, 1968; Davis, 1972) and under controlled laboratory conditions (McClelland and Bourns, 1969; Meuleman, 1972). These studies considered the effects of infection in the host on such processes as reproduction, growth and survival, and take into account

such variables as host sex, age and size. In contrast, the biochemical and physiological studies are fewer in number (Vernberg and Vernberg, 1967; Duerr, 1967; Watts, 1970; Davis and Farley, 1973), although relevant investigations concerned with the biochemistry and physiology of digenean trematodes (Friedl, 1961a, 1961b; Cheng, 1963a; Vernberg and Hunter, 1963; Read, 1968) or individual uninfected mollusc species (Fretter and Graham, 1962; Wilbur and Young, 1966; 1968; Emerson, 1967; Purchon, 1968; Lawrence and Giese, 1969) have likewise contributed extensively towards a better understanding of both groups of organisms and their influences upon each other.

By far the most common types of studies on trematode-mollusc interactions are those in which histological and histochemical methods have been employed in attempts to determine the forms of damage induced by the parasites on their hosts, and the nature of the exchange of materials between the respective organisms. The early pioneer work in this field was performed by Faust (1920), Agersborgh (1924), Hurst (1927), Rees (1931), Rees (1935), and the last decade has witnessed the output of a vast amount of literature on the subject (Cheng and Snyder, 1961; 1962a; 1962b; 1963; Cheng, 1963b; 1963c; 1965; Cheng and Burton, 1966; James, 1965; Porter, 1967; 1970; Reader, 1971a, 1971b). This has been reviewed comprehensively by Wright

(1966) and by Cheng (1967). It is noteworthy that up to the present time there have been no studies in depth on the effects of parasites on their molluscan hosts at the ultrastructural level. Only Meuleman (1972) has considered this method of examination, and her work is relatively brief in this respect. It is also fair to say that most of the emphasis thus far has been placed on the effects of the parasites on the digestive gland. This is to be expected, since as mentioned previously (Chapter I), it is in the interlobular haemocoel of the digestive gland that most trematode larvae ultimately lodge and tap their host's energy resources often-times to the point of exhaustion.

Rees (1936) proposed that there are three main causes of damage to the digestive gland - first, physiological degradation due mainly to the utilization of the host's food reserves or to the accumulation of the parasites' toxic substances (Cheng and Snyder, 1962a; Hurst, 1927; Rees, 1936); second, mechanical damage resulting from pressure of the parasites' germinal sacs on the lumen of the digestive gland and the formation of a 'blocking layer' which interferes with the normal passage of food through the digestive gland (Rees, 1936; James, 1965); third, direct ingestion of digestive gland cells, attributable primarily to the redial larvae, since unlike sporocysts, they are

equipped with a rudimentary gut and muscular 'pharynx' (Rees, 1936; Cheng, 1963c; Porter, 1970; Reader, 1971a). It is quite possible that any or all of these factors may be in operation for different associations, depending on the organisms involved (James, 1965; Rees, 1936).

Much attention has been devoted to recording gross morphological and composition changes in the parasitized digestive gland. Less time however, has been spent in trying to determine the exact pattern or sequence of events at the cellular level, i.e. the range of differences or changes which exist between an infected, but relatively healthy cell and a parasitized and totally degenerated cell. In this connection, it is important to pay heed to the digestive cells in their actual proximity to the parasites, since a cell which is immediately contiguous with the parasite's surface may exhibit considerable variation both in content and structure when compared to other cells which are merely 'nearby'. The problems of studying the extent of damage in digestive gland cells as induced by digenetic trematode larvae, become compounded when we recall the presence of phasic activity in the digestive gland cells of molluscs (Chapter II) and especially when all states of digestion may be represented in a single individual (Purchon, 1971).

In the past, the concept of phasic activity has been studied in bivalves but totally neglected in the case of gastropods. Moreover, no research has been conducted on the effects of parasites in their molluscan hosts in relation to such phasic activity. The present work will examine the effects of C. lingua and M. similis on the digestive gland cells at the host-parasite interface level (i.e. that region where host cells and parasite surfaces are in direct contact with each other) and will attempt to cope with those variables which result from the existence of phasic activity.

MATERIALS AND METHODS

Six individual snail samples of Littorina saxatilis were collected from the supralittoral fringe of a semi-sheltered rocky shore at Blue Rocks, Lunenburg County, Nova Scotia, between October 8th, 1971 and September 28th, 1972. In an effort to study the effects of Cryptocotyle lingua and Microphallus similis on the digestive gland cells, suitably infected or uninfected snails were selected and a series of histological, histochemical and ultrastructural techniques were employed, the procedures of which are described below.

A clear pattern of rhythmic activity for the digestive gland cells imposed by the periodicity of the tide having already been established (Chapter II), specimens were collected from the shore at two hour intervals over a half or complete tide cycle (see Table 4.1). When the snails were removed from the rocks, the shells were crushed and the bodies were rinsed in cold sea water and then checked for infection. Only uninfected individuals which would serve as controls, or those snails infected by one of the applicable parasites were retained.

The digestive gland was cut off from the rest of the viscera and dissected into two, sometimes three 1 - 2 cubic millimeter parts. These portions were subsequently immersed

Table 4.1 The relative numbers of Littorina saxatilis which were uninfected, infected with Cryptocotyle lingua and infected with Microphallus similis and an outline of the approaches used in the selection of snails comprising each of the samples.

Sample	Collection Date	State of Tide	Snails infected with C. lingua	Snails infected with M. similis	Uninfected Snails	Selection of Snails
1	Oct. 8/71	HT/LT	27	29	18	5 snails infected with C. lingua and 5 with M. similis collected each 2 hours. 3 uninfected snails collected each 2 hours.
2	Oct. 22/71	HT	15	13	14	Same as above except that 5 uninfected snails were collected each 2 hours.
3	Oct. 29/71	LT	12	14	13	Same as above.
4	Nov. 12/71	LT	12	16	15	Same as above.
5	June 28/72	HT	8	4	10	Snails were collected throughout the half tide but not at specific time intervals.
6	Sept. 28/72	HT/LT	15	10	12	Same as above but selected from among snails which had been collected throughout the entire tide cycle.

HT - high tide + 6 hours
LT - low tide + 6 hours
HT/LT - complete tide cycle, i.e. high tide + 6 hours and low tide + 6 hours

in separate vials which contained the appropriate fixatives. The vials were positioned into one of three wooden racks, depending on whether the tissue was a) uninfected, b) infected with C. lingua rediae or c) infected with M. similis sporocysts. While all the material for any particular test was treated in an identical manner, this method of isolating the different categories of tissues eliminated any confusion regarding what was to be examined.

Light Microscopy

Tissues to be examined with the light microscope were fixed in appropriate fixatives, dehydrated in ethanol, cleared in benzene and embedded in Paraplast (melting point 56 - 57°C). Serial sections, cut at a thickness of 7 µm. were stained, dehydrated, cleared in two changes of xylol and mounted either in Permount or DPX. Variations from this procedure will be pointed out in the following paragraphs. Both conventional, as well as phase microscopy were used.

For general routine investigations, tissues fixed in Masson's modification of Bouin's fluid (Foot, 1933) for four days and washed in 70 percent ethanol for one day, were stained by the Mallory's Triple Stain Method (Pantin, 1964).

Proteins: In order to identify the presence of and the changes in total protein content in the digestive gland

cells, the Mercury-Bromophenol Blue method was employed (Bonhag, 1955) after initial fixation in Bouin's fluid (Foot, 1933). (A comprehensive analysis of all the histological and histochemical methods used can be found in Appendix I).

Lipid: Two histochemical methods were employed for purposes of identifying lipids. Bound lipids were detected after the tissues were fixed in Baker's formol calcium and stained by the Acetone-Sudan Black method (Pearse, 1968).

In order to localize and identify neutral fats, tissues were fixed in formalin sea water at 4 - 5°C and then embedded in Cryoform. Sections were cut at a thickness of 15 µm. on an American Optical kryostat (Model 840) at -20°C and stained by the Oil red O method (Lillie, 1944) as recommended by Pearse (1968).

Carbohydrate: For the demonstration of carbohydrate-containing substances, the Periodic Acid-Schiff (PAS) (after McManus as cited by Pearse, 1968) reaction was employed on Paraplast-embedded tissue. One group of specimens was fixed in Baker's formol-calcium (Pantin, 1964), and another in alcohol at -73°C. To ensure that the positive PAS reaction was due to the specific oxidation of glycols, sections were exposed to Schiff's reagent without prior oxidation in periodic acid.

Glycogen was demonstrated both with the PAS reaction and the Best's Carmine technique (Ruthman, 1966). As for the detection of other carbohydrates, the tissues were fixed in Baker's formol-calcium or alcohol (-73°C). Controls were treated with saliva or 10 percent malt diastase in 0.9 percent NaCl for 45 minutes at 37°C in order to ensure that it was indeed glycogen which was being tested. In addition, samples of Guinea pig liver were treated concurrently in a similar manner. The latter acted as a known standard and thus provided a comparative basis for the histochemical methods.

For the identification of glucose, a modification of the method of Okamoto, Kadota and Ooyama was employed (Pearse, 1968). Tissues were fixed for 24 hours in methly alcohol saturated with barium hydroxide at 3°C. Unlike other prepared tissues, sections were mounted on glycerine-albuminized slides but were not flattened with water, in order to avoid displacement of the hexose.

Enzymes: In preparation for the examination of nuclei acids, tissues were fixed in Lillie's acetic-alcohol-formalin at 4°C for 24 hours (Pearse, 1968), and were subsequently stained by the Pyronin-Methyl Green technique (Jordan and Baker, 1955). Controls consisted of sections treated, before staining, in ribonuclease at 1 mg/ml in distilled water for one hour at 37°C.

Tests for acid phosphatase (Gomori, 1950) and alkaline phosphatase (Gomori, 1946) were performed as outlined by Pearse (1968). Tissues were initially fixed in cold buffered formalin-sea water at 4 - 5°C and embedded in Cryoform. They were then cut on the kryostat (-20°C) at a thickness of approximately 15 µm., incubated in the appropriate sodium B-glycerophosphate solution and treated with an 0.0004 M lead nitrate solution for acid phosphatase, or 2 percent calcium cobalt solution for alkaline phosphatase.

In order to localize the precipitates which resulted after the tissues were incubated in the substrate media, sections were immersed in a solution of ammonium sulphide. Sections which were not exposed to the sulphide during the normal course of staining served as suitable controls.

A resume of all the histochemical techniques applied appears in Table 4.2.

Electron Microscopy

All infected tissues examined with the electron microscope were treated in a manner similar to that already described in Chapter II for the unparasitized snails. As before, preliminary observations of thick sections (1 - 2 µm.) using the Toluidine Blue staining method (Trump et al., 1961) preceded final sectioning. Because of the difficulties encountered in obtaining large ultrathin sections, mainly due

to the cutting problems presented by the calcium spherules in the secretory cells, studies were performed on thick sections under the light microscope, and extra-fine trimming techniques were employed. Only in this way, was it possible to identify and isolate the host-parasite interfaces and at the same time recognize the various pathological states (see next paragraph). Further elaboration of the ultrastructural procedures can be found in Appendix I.

General Procedures

On the basis of the results obtained in Chapter II, it became firmly established that a rhythmic cycle of digestion occurs in the digestive gland cells of L. saxatilis. Studies on infected digestive gland tissue revealed that there are three distinct pathological states throughout the phases of digestion, both for digestive cells infected by C. lingua, as well as for those affected by M. similis. (For a further clarification on the criteria used in categorizing the pathological states, see OBSERVATIONS - GENERAL PATHOLOGY).

In an effort to acquire a rough approximation of the relative numbers of cells displaying the various pathological states for each of the phases of digestion, a routine study was performed on serially-sectioned infected tissues from specimens comprising Sample 1. All digestive cells forming part of a digestive gland tubule and at the same time having contact with the outer walls of larvae of either parasite

Table 4.2 A general outline of the various tests performed and the methods used in studying digestive gland cells from infected and uninfected Littorina saxatilis

Test	Sample	Methods	Comments
General	1	Bouin's Fluid (Foot, 1933); Mallory's Triple Stain Method; Electron microscopy; Quantitive studies.	
Protein	1	Bouin's Fluid; Mercury-Bromophenol blue.	
General Carbohydrates	2-5	Baker's formol-calcium; PAS, Ice Cold Alcohol (-73°C).	To ensure positive PAS test, a sample of slides were immersed in Schiff's reagent without prior exposure to periodic acid.
Glycogen	2-5	Baker's formol-calcium; PAS, Ice Cold Alcohol (-73°C); Best's Carmine Stain	Controls included treatment of tissue with 10 percent diastase in NaCl, and with saliva at 37°C. Comparisons were made to similarly processed Guinea pig liver.
Glucose	4-5	Methanol sat. with BaOH; Okamoto et al. Method.	
Bound lipids	2-3	Baker's formol-calcium; Acetone Sudan-Black.	
Neutral fats	6	Formalin in sea water; Oil Red O Lillie, 1944.	Kryostat cut sections (-20°C).
RNA/DNA	6	Lillie's Alcohol acetic formalin; Pyronin-Methyl Green.	Controls included treatment of sections in ribonuclease at 1 mg/ml distilled H_2O.
Acid Phosphatase	6	Formalin in sea water (buffered with sodium phosphate pH 7.3; Gomori (1950) Lead-nitrate.	Kryostat cut sections (-20°C); Control: Alternate treatment of slides with ammonium sulphide.

Table 4.2

Test	Sample	Method	Comments
Alkaline Phosphatase	6	Formalin in sea water (buffered with sodium phosphate pH 7.3); Gomori (1946) Calcium cobalt.	Kryostat cut sections (-20°C); Control: Alternate treatment of slides with ammonium sulphide.

were counted and placed in one of three categories according to the degree of damage exhibited. Because digestive cells are approximately 100 - 110 µm. in length, counts were made from every sixteenth section and then only from tubules located in regions of the sections not previously examined. This ensured that the same cells were not being counted more than once. An attempt to count at least 100 cells per specimen was made, but due to the presence of the occasional extremely heavily infected or lightly infected organism, this was not always possible.

While the above findings on the extent of damage of the digestive gland cells were achieved by means of conventional histological technique, they were corroborated and considerably expanded by the observations made from electron micrographs (see OBSERVATIONS - ULTRASTRUCTURE OF THE INFECTED DIGESTIVE GLAND CELLS). It was only after a vivid elucidation of the morphology and of the changes induced in the structural integrity of the digestive gland cells of C. lingua and M. similis, that it became possible to undertake the histochemical work (Samples 2 - 6). An outline of the various tests and procedures which collectively form the framework of this study is offered in Table 4.2.

OBSERVATIONS

GENERAL PATHOLOGY

The sequence of morphological changes in parasitized digestive cells, as observed under the light microscope, appeared to be the same for both C. lingua and M. similis in spite of the differing methods of infection and distribution within the host (Fig. 4.1 - 4.3). Below is a general description of the pathological changes which ensue in the digestive cells as a consequence of infection. Because of the cyclic form of activity characteristic of digestive cells, the description is intentionally broad and is meant to encompass all phases of digestion. The specific effects on the cells during the individual phases will be considered more fully under those observations concerned with histochemical and ultrastructural findings.

In the early stages of infection, no visible effects are detected, but as time passes the nuclei become displaced and migrate towards the apical end. Gradually the distal and lateral walls of these cells break down. Occasionally, a new distal membrane replaces the old one, giving the cells a box-like or squamous appearance. Upon degeneration of the lateral membranes, adjacent groups of parasitized cells may become confluent and assume a syncytial appearance.

The secretory cells do not undergo a similar breakdown

pattern, rather they seem to increase in number in the region of infection, occupying portions of the epithelium where once digestive cells prevailed (Fig. 4.4 - 4.5). The secretory cells will be considered in greater detail in later sections.

For purposes of clarification and also to provide a steady frame of reference, the digestive cells were grouped into three categories, according to the severity of damage which they exhibited. The morphological conditions described above, i.e. which could properly be attributed to all phases of digestion, were used as the main criteria for classification of the cells.

State 1. This state of pathology includes those cells which do not show any structural changes as observed by the use of routine histological methods. The reason that they are placed in this category however, is because they happen to be immediately adjacent to the parasite and thus qualify as "infected" cells. (Cells which form an interface with the parasite's outer surface). Present findings are not meant to imply that there are no parasitic effects when more sensitive methods of detection are employed (e.g. histochemical or ultrastructural techniques). (Fig. 4.6)

State 2. The nuclei from cells in this category become dislodged from their normal basal positions and migrate apically.

At such time, the basal regions of the cells stain faintly or not at all by the Mallory's Triple Stain Method. The cells in this state appear somewhat compressed and assume a squamous appearance (Fig. 4.6).

State 3. Cells in this state usually show a degeneration of the lateral as well as the apical membranes. This category is reserved for those cells which have lost their general integrity. Nuclei and other cellular contents may be shed into the lumen of the digestive gland tubule, and often the remnants of such cells contribute, by fusion with adjacent degenerating cells, towards the formation of a temporary syncytial layer (Fig. 4.6). In the latest stages, the syncytium itself breaks down until no further evidence of digestive cell structures prevail.

The quantitative study presented in the following paragraphs is based on the classification scheme described above. A further understanding of the extent of damage, as manifested by infected cells can be obtained from Figures 4.7 and 4.8.

In the case of Cryptocotyle lingua (Table 4.3), a greater number of cells was recorded from State 1 than from State 2 for the absorptive phase (45.0 percent vs. 30.7 percent) and the digestive phase (41.5 percent vs. 31.5 percent). An identical number of cells however, displaying States 1 and 2 were counted for the excretory phase (35.5 percent). When States 2 and 3 are compared, it is clearly evident that

fewer cells are characterized by the more severe forms of pathology. The respective percentages are 30.7 percent vs. 24.3 percent for the absorptive phase, 31.5 percent vs. 27.0 percent for the digestive phase and 35.5 percent vs. 29.0 percent for the excretory phase. Thus, the number of cells characterized by pathological State 1 are most numerous, while those displaying pathological States 2 and 3 become progressively reduced. The exception is the relative numbers between State 1 and State 2 during the excretory phase. This may be explained by the fact that fewer specimens were collected during the excretory phase (378) in relation to the other two phases and this might not have been a high enough number upon which to base conclusions. However, when the averaged totals of all phases are considered, a trend prevails because 42.1 percent of the cells occur in State 1, 31.7 percent in State 2 and 26.2 percent in State 3.

For cells infected by Microphallus similis (Table 4.4) quite the opposite appears to be the case, since a larger proportion of the cells are characterized by the more severe forms of pathology. This is obvious when, as above, we compare the relative numbers of cells in State 1 and State 3 - for the absorptive (16.8 percent vs. 63.2 percent), digestive (19.1 percent vs. 51.6 percent) and excretory phases (18.0 percent vs. 67.2 percent). Since there is no exception to this trend as was found for cells infected by C.

lingua, the averages obtained when the phases were considered collectively, are to be expected and are clearly reflected in terms of percentages: 18.6 percent of the cells in State 1, 24.9 percent in State 2 and 56.5 percent in State 3. The results, therefore, presented in Table 4.5 showing an inverse relationship between the numbers of cells in State 1 and State 3 for the two parasites (18.6 percent of the cells for M. similis vs. 42.1 percent of the cells for C. lingua in State 1, and 56.5 percent of the cells for M. similis and only 26.2 percent of the cells for C. lingua in State 3, are in accordance with the above findings. Finally, it is interesting to note the consistency which exists in the relative percentage values, representing any one pathological state for each of the phases, e.g. State 1 - M. similis: 16.8 percent, 19.1 percent and 18.0 percent for the absorptive, digestive and excretory phases respectively (Table 4.5). This tendency is generally appicable to both parasites.

In summary, it appears that in the case of C. lingua the greatest number of digestive cells display only the mildest form of disorder and that progressively fewer cells show the more severe states of pathology. This contrasts with digestive cells infected by M. similis which appear to be highest in the more severe states of pathology. The results therefore indicate that M. similis sporocysts induce greater damage to the digestive gland than do C. lingua rediae.

Table 4.3 The relative numbers and percentages of digestive cells in each of three states of pathology for the absorptive, digestive and excretory phases as observed from Littorina saxatilis infected with Cryptocotyle lingua rediae..

Phase of Digestion	Number of Infected Snails	Number of Tubules	Total Number of Cells	STATES OF PATHOLOGY: Cells in State 1 number	Cells in State 1 percentage	Cells in State 2 number	Cells in State 2 percentage	Cells in State 3 number	Cells in State 3 percentage
ABSORPTIVE	10	93	1126	507	45.0	346	30.7	273	24.3
DIGESTIVE	12	98	1269	526	41.5	400	31.5	343	27.0
EXCRETORY	3	33	378	134	35.5	134	35.5	110	29.0
Mean (TOTAL)			924	389	42.1	293.3	31.7	242	26.2

Table 4.4 The relative numbers and percentages of digestive cells in each of three states of pathology for the absorptive, digestive and excretory phases as observed from Littorina saxatilis infected with Microphallus similis sporocysts.

Phase of Digestion	Number of Infected Snails	Number of Tubules	Total Number of Cells	STATES OF PATHOLOGY: Cells in State 1 number	Cells in State 1 percentage	Cells in State 2 number	Cells in State 2 percentage	Cells in State 3 number	Cells in State 3 percentage
ABSORPTIVE	3	30	375	63	16.8	75	20.0	237	63.2
DIGESTIVE	19	131	1900	364	19.1	557	29.3	979	51.6
EXCRETORY	7	39	650	117	18.0	96	14.8	437	67.2
Mean (TOTAL)				181	18.6	242.7	24.9	551	56.5

Table 4.5 A comparison between the relative numbers and percentages of digestive cells in each of three states of pathology over the entire digestion cycle as observed from Littorina saxatilis infected with Cryptocotyle lingua rediae and Microphallus similis sporocysts.

Parasite	Number of Infected Snails	Number of Tubules	Total Number of Cells	STATES OF PATHOLOGY State 1 number	State 1 percentage	State 2 number	State 2 percentage	State 3 number	State 3 percentage
Cryptocotyle lingua	25	224	2773	1167	42.1	880	31.7	726	26.2
Microphallus similis	29	200	2925	544	18.6	728	24.9	1653	56.5

HISTOCHEMISTRY OF INFECTED DIGESTIVE GLAND CELLS

Having studied the general morphology of normal and parasitized digestive gland cells by the use of conventional histological methods, attention will now be focused on histochemical changes resulting from infections with Cryptocotyle lingua and Microphallus similis. To describe more precisely the actual condition of the digestive cells, reference will be made to the applicable phase of digestion, and when possible, to the state of pathology as determined on the basis of morphological criteria presented in the previous section.

Histochemical methods were employed in order to compare the qualitative differences in cellular constituents--i.e. proteins, lipids and carbohydrates in parasitized and unparasitized cells. The choices of tests were based on their abilities to reveal gross changes in cellular composition and are in general, similar to the methods applied by other workers who examined the state of the infected digestive gland in gastropods (Cheng, 1962; 1963a, 1963b; Cheng and Snyder, 1961; 1962a, 1962b; 1963; James, 1965; Porter, 1967; 1970; Reader, 1971a).

Because of the importance of acid and alkaline phosphatase to carbohydrate metabolism (Von Brand, 1952; Cheng, 1964), a routine study of these enzymes was performed.

A measure of activity of cells, particularly with respect to protein synthesis, is the abundance of cytoplasmic

and nuclear RNA. In order to acquire some insight into changes in the synthetic activity of the digestive gland cells, a test was performed on the nucleic acids - RNA and DNA.

An outline of the procedure employed for the various tests was presented in the section concerned with Materials and Methods. An elaboration of these techniques is offered in Appendix I. Finally, a review of all the histochemical results is presented in Table 4.6.

Protein (Mercury-Bromophenol Blue): The uninfected secretory cells appear to stain a deep reddish-purple during the absorptive phase and reddish-blue in the digestive and excretory phases. In contrast, the digestive cells display a purple or brownish tinge throughout all phases of digestion. Only the basal regions of the cells stain light-blue during the digestive phase suggesting the presence of proteins.

Both parasites are highly proteinaceous, as is observed from the intensity of the blue staining reaction with mercury-bromophenol blue. They cause little apparent change in the staining properties of the secretory cells, although a slight shift from reddish-purple to blue takes place in cells parasitized by M. similis. Both parasites cause the elimination of the light blue granularity from the basal parts of digestive cells which are in the digestive phase and in pathological States 2 and 3 (Fig. 4.9 - 4.10). Signs

of protein elimination are observed for those cells displaying pathological State 1 and infected with M. similis.

RNA - DNA (Pyronin-Methyl Green): The nuclei of unparasitized cells stain purple-red with pyronin, and this is mainly due to the intensity of staining of the nucleoli. Secretory cells contain large amounts of RNA and this is reflected throughout all phases of digestion with only a light decrease during the excretory phase.

In contrast, digestive cells stain only slightly with pyronin in the basal regions during the absorptive and digestive phases and to an even lesser extent when they are in the excretory phase.

Infected secretory cells, showing a lower content of RNA are usually located next to digestive cells displaying pathological State 3. A marked reduction in cytoplasmic RNA occurs from the basal portions of digestive cells in pathological State 2 during the absorptive and early digestive phases, while digestive cells in State 3 apparently have little or no stainable RNA (Fig. 4.11).

Numerous phagocytes characterized by large nuclei can be seen surrounding and often encapsulating the parasites (Fig. 4.12). This is more pronounced in those snails infected with M. similis. Of interest also, is the fact that the methyl green stain for DNA is more visible from the brood

chambers of both parasites and less from the body walls. This is probably a reflection of the extensive nuclear divisions which occur in the former region.

Carbohydrates (PAS, Best's Carmine, Okamoto-Glucose): Since unparasitized secretory cells stain only negligibly by the PAS method they can be considered relatively free of carbohydrates. In contrast, uninfected digestive cells display considerable amounts of carbohydrate within their cytoplasms as can be deduced from their capacity to stain reddish-purple after application of the PAS technique (Fig. 4.13a, 4.13b). The digestive cell's apical membrane stains intensely throughout digestion, and only slightly less so in the excretory phase. A PAS-positive, filamentous-like substance stains moderately and can be observed extending from just within the distal border to about 1/5 of the cell's length (Fig. 4.13a, 4.13b). This material is prominent during the absorptive and the early digestive phases, fading slightly in the late digestive and excretory phases. In the lower basal and middle regions of the cells, the PAS-positive material is scattered in clumps (Fig. 4.13a, 4.13b) somewhat diffusely, showing a decrease in staining intensity as the cells pass from the absorption through the digestive and into the excretory phase. The most basal part, i.e. that region immediately adjacent to the nucleus is characterised by a light concentrated form of PAS-positive granular material,

and shows no consistent change during the process of digestion.

For those sections which received prior treatment with saliva or malt diastase, the staining is seen to persist in the apical region, but is markedly reduced in the middle and lower half of the basal portion, and to some extent, in the most basal part (immediately adjacent to the nucleus - Fig. 4.14, 4.15) of the digestive cells. This, therefore, suggests that the latter areas consist primarily of glycogen. Glycogen assumes the form of granular clumps and is especially widespread within the meshwork of connective tissue covering the digestive gland tubules.

Digestive cells infected with C. lingua rediae and displaying pathological States 1 and 2, show a marked reduction in glycogen content throughout the digestion cycle. In contrast, there occurs an increase in other PAS-positive material from the basal regions of the digestive cells (Fig. 4.16). In the case of those cells which can be identified as being in pathological State 3, the glycogen content is absent and a decrease or total elimination of other PAS-positive substances likewise occur.

Digestive cells parasitized by M. similis sporocysts show an overall marked reduction in glycogen, as well as other PAS-positive material from the apical regions during

pathological State 1. Similar to cells infected by C. lingua, there is a corresponding increase in carbohydrate content in the most basal regions at this time. While some carbohydrate may be visible in the apical regions during the absorptive phase, less prevails during the digestive state, and only very minimal amounts occur when the cells are in the excretory phase. Cells in pathological State 2 show a total absence of glycogen during all phases of digestion. Some PAS-positive material is evident in the absorptive phase but this appears to be absent during the digestive and excretory phases (Fig. 4.17). Thus, less carbohydrate is detected in the basal regions at this time than in cells infected by C. lingua rediae and displaying pathological State 2. Cells infected by M. similis and characterized by pathological State 3 show negligible traces of carbohydrate during the absorptive and early digestive phases, particularly predominately in the basal regions.

Both parasites reduce, and at times, totally exhaust the glycogen content of the connective tissue, although this seems to be more acute in the case of M. similis. From the findings obtained by the Okamoto method for glucose, it appears that this hexose is always closely bound to the outer walls of the parasite when present in the connective tissue. Because of the faint staining achieved by this technique, however, no definite statements can be made regarding the

distribution of glucose.

Concurrent with the diminution of glycogen and other carbohydrates from the digestive cells, it appears that there is an increase of glycogen in the parasites. While embryonic forms do not display much PAS-positive material, an intense reaction for glycogen can be seen from mature cercariae, especially in the tails and oral suckers.

Bound Lipids and Neutral Fats (Acetone Sudan Black, Oil Red O): The acetone Sudan Black technique for bound lipids renders the uninfected digestive gland cells a deep brown-black color. Both the secretory and digestive cells stain intensely by this method but in the case of the latter cells, the apical membrane, the area just within the distal border, and the digestive vacuoles are more heavily stained, while the rest of the membrane is slightly less stained.

There is no fluctuation in staining intensity in digestive cells except in the region just within the apical border which appears very dense during absorption and less so as digestion proceeds through the digestive and excretory phases. While the intensity of color does not change within the vacuoles, their number varies in accordance with the phasic activity which is known to be taking place (Chapter II).

In the case of frozen Cryostat-cut sections stained with Oil Red O, neutral fat assumes the form of bright, red

droplets. These appear smaller and fewer during the absorptive phase, increasing in size and number mainly in the basal regions during the late digestive and especially in the excretory phases. Neutral lipid is more scarce in the secretory cells, showing no fluctuation in quantity over the digestive cycle.

For cells infected with either M. similis or C. lingua larvae, it was not possible to detect any changes in staining intensity for the various structures by the acetone-Sudan Black method. It seems that the only certain alterations are of a morphological nature, i.e. a gradual or complete breakdown of cellular structure, depending on the pathological states which the cells are in. The sequence of damage always begins with the vacuoles located most basally, and depending on the severity of the damage, may be followed by elimination of the more apically distributed vacuoles and ultimately of the apical border itself.

Because of the thickness of the Cryostat-cut sections (15 um.), it was not possible to identify the actual pathological states for the digestive cells. It seems, however, that in the presence of both parasites, there is an increase of neutral fat in the host's digestive cells, usually in the basal most regions, and this gradually spreads apically with the intensity of infection and heightened degree of pathology (Fig. 4.18). On occasion, a complete ab-

sence of neutral fat can be observed for digestive cells, even during the excretory phase, and this is believed to be applicable to cells which can be classed in pathological State 3.

Acid and Alkaline Phosphatase (Lead Nitrate, Calcium Cobalt): Acid phosphatase (Gomori, 1950), as observed under the light microscope, occurs in minute quantities in the uninfected digestive cells. The presence of this enzyme is indicated by a finely granular deposit of black precipitate and is found primarily in the basal regions. Alkaline phosphatase (Gomori, 1946) activity is likewise expressed in the form of granulation but it appears as somewhat larger, brownish-black deposits which are more widespread in the cytoplasm (Fig. 4.19). Because of the thickness of the sections, using the Cryostat technique, it was not possible to differentiate between digestive and secretory cells. Likewise, no consistent changes in enzyme concentration could be noted during the digestion cycle.

While both C. lingua and M. similis larvae display considerable alkaline phosphatase activity especially in their body walls (Fig. 4.19), less acid phosphatase is present. Due to distortion problems resulting from differential cutting and staining properties between host and parasites, changes in enzyme concentrations within the digestive gland cells could not be directly attributed to either parasite.

Thus while changes in the rate of synthesis or secretion of these enzymes may possibly take place within the digestive gland cells, the application of the present technique did not make their detection possible.

SYMBOLS FOR TABLE 4.6

P - protein
RNA - RNA
CBH - carbohydrate
Gl - glycogen
Bl - bound lipid
Nl - neutral lipid
AcP - acid phosphatase
AlP - alkaline phosphatase

++	intense reaction
+	moderate reaction
-	no reaction or negligible reaction
0	positive test, but not certain if there is a change in component being tested, with respect to other pathological states
a	apical
b	basal

Table 4.6 Results of histochemical tests of infected and uninfected digestive cells of Littorina saxatilis.

Phase	PATHOLOGICAL STATE 1								PATHOLOGICAL STATE 2								PATHOLOGICAL STATE 3							
	P	RNA	CBH	Gl	Bl	Nl	AcP	AlP	P	RNA	CBH	Gl	Bl	Nl	AcP	AlP	P	RNA	CBH	Gl	Bl	Nl	AcP	AlP
ABSORPTIVE	+	+	-b	-	0	+	0	0	-	-	+b	-	0	++	0	0	-	-	-b	-	0	-	0	0
DIGESTIVE	+	+	-b	-	0	+	0	0	-	-	+b	-	0	++	0	0	-	-	-b	-	0	-	0	0
EXCRETORY	-	-	-b	-	0	++	0	0	-	-	+b	-	0	++	0	0	-	-	-b	-	0	-	0	0
(C. lingua)																								
ABSORPTIVE	-	+	-b	-	0	+	0	0	-	-	-b	-	0	++	0	0	-	-	-b	-	0	-	0	0
DIGESTIVE	-	+	-b	-	0	+	0	0	-	-	-b	-	0	++	0	0	-	-	-b	-	0	-	0	0
EXCRETORY	-	-	-b	-	0	++	0	0	-	-	-b	-	0	++	0	0	-	-	-b	-	0	-	0	0
(M. similis)																								
ABSORPTIVE	+	+	±b/+a	++	++	+	+	+	PATHOLOGICAL STATES ARE NOT APPLICABLE															
DIGESTIVE	+	+	±b/+a	++	++	+	+	+																
EXCRETORY	-	-	±b/+a	+	++	++	+	+																
(uninfected)																								

ULTRASTRUCTURE OF THE INFECTED DIGESTIVE GLAND CELLS

Cells Infected with Cryptocotyle lingua

In studying the ultrastructural changes of the digestive cells, it was first necessary to identify the cells in their respective states of pathology based on the scheme proposed earlier. This was accomplished by initial examination of thick sections under the light microscope. As mentioned earlier, however, such a classification could not be applied to secretory cells.

Secretory Cell

Secretory cells whose basal laminae form interfaces with the outer walls of C. lingua rediae and which are usually located immediately adjacent to digestive cells exhibiting pathological State 1, show little change in their cellular constitutions. In contrast, cells affected for longer periods and usually located near digestive cells displaying pathological State 2, are characterized by some deterioration in their cellular composition.

The nuclei take on a folded appearance and give rise to blunt pseudopodial-like extensions (Fig. 4.20). Zymogen granules appear to be reduced in number and material of electron density similar to that of the zymogens and usually seen in the apical and microvillar region in uninfected cells, (Chapter II - Fig. 2.11) is likewise absent (Fig. 4.21).

In the more regressed state, it appears as if the E.R. cisternae are vesiculated, showing extreme signs of this condition as the state of the cells deteriorates still further (Fig. 4.22). At this time, many free, unattached ribosomes can be seen in the cytoplasm (Fig. 4.22). As observed from the micrographs, mitochondria appear more swollen or elongated than normal and at times show signs of rupturing (Fig. 4.22). Small nuclear profiles which are separate from the main nucleus can be seen and these probably represent cross sections of the nuclear extensions previously discussed. The possibility, however, that at least in some instances they are vacuoles which have become detached from the nucleus, cannot be excluded. In addition, a dense granulation can be observed at times. A shrivelling and advanced karyolysis appears to take place in the latest stages of degeneration (Fig. 4.23). Autophagic vacuoles can be seen throughout all stages of pathology and these seem to increase in number as the cell degenerates (Fig. 4.20, 4.24).

Digestive Cell

Pathological State 1 In general, cells displaying pathological State 1 as originally observed under the light microscope, show little evidence of morphological change at the ultrastructural level. There is,however, an increase in lipid droplets (Fig. 4.25) primarily in the basal regions, and this is especially apparent during the absorptive and

digestive phases. In cells displaying a slightly more severe form of pathology, the cisternae of the Golgi bodies appear more distended and also show signs of vesiculation (Fig. 4.26). Moreover, the normal characteristic striation pattern of these organelles and their associated vesicles (Chapter II - Fig. 15.2) is no longer as readily visible, although the vesicles themselves can still be observed.

Pathological State 2 - At this time, marked changes begin to appear at all levels. Initially, there is a gradual increase in lipid droplets in the basal regions, but as the pathology becomes more severe, the amount of lipid decreases. The nuclei are altered in shape and become reduced in size. There appears to be an increase in the number of Type 4 vesicles as early as the absorptive phase, and this persists throughout the process of digestion (Fig. 4.27). In contrast, there is a reduction in Type 3 vesicles and the latter are usually smaller than normal when they are present (Fig. 4.27). Judging from the prevalence of pinocytotic vesicles, there does not seem to be too much interference with the uptake of nutrients during the absorptive and digestive phases (Fig. 4.28), although there does seem to be a decrease in these vesicles as the cells show evidence of very late pathological States 2 or early pathological State 3 (Fig. 4.29). Vacuoles containing small membrane-bound vesicles and electron dense granules can also be seen, primarily in the basal regions during the di-

gestion and excretory phases. These probably assume the role of phagolysosomes (Fig. 4.27, 4.28).

Pathological State 3 Cells in this state may continue to show evidence of lipid droplets especially during the absorptive phase.

A cloudy or grainy effect is now characteristic of digestive cells, and this can be seen during all phases of digestion and from all regions (Fig. 4.21, 4.30). The internal framework is totally deteriorated and the cytoplasm shows extensive vacuolation. The mitochondria appear swollen and display varying degrees of cristolysis (Fig. 4.31). At such time, the nucleus displays an irregular topography and can be observed to be very shrivelled (Fig. 4.32).

Because of the breakdown in the outer cellular membrane, fusion between cells is evident and a syncytial condition ultimately predominates. No Type 3, and only the remnants of Type 4 vacuoles, prevail (Fig. 4.30, 4.32). Instead, phagolysosomes, as described for cells in pathological State 2, are common (Fig. 4.32).

Cells Infected with *Microphallus similis*

Secretory Cell

Unlike those cells parasitized by *C. lingua* rediae, secretory cells infected by *M. similis* sporocysts do not

display acute morphological alterations (Fig. 4.33 - 4.36) until the cell-parasite associations has existed for a considerably longer period of time.

Changes in secretory cells do not appear until the adjacent digestive cells are already exhibiting severe breakdown during pathological State 2 or early pathological State 3. At first, an accumulation of lipid can be observed throughout all phases of digestion (Fig. 4.36), little increase in this component is apparent however, as the severity of damage seems to become more intense. Evidence suggests that the granular E.R. cisternae become more dilated and that possibly the nucleoli become dispersed, giving rise to isolated concentrations of dense granules in the nucleoplasm (Fig. 4.37). At this time too, phagosomes, similar to the ones described for secretory cells parasitized by C. lingua rediae are also visible (Fig. 4.37). It is only in the latest stages of pathology that extensive vesiculation of the E.R. prevails (Fig. 38). This is most prominent during the absorptive and digestive phases since in unparasitized organisms, the cells in the excretory phase generally possess less granular E.R. (Chapter II - Fig. 2.12). At such time, many free, unattached ribosomes are visible from the cytoplasm, and mitochondria appear elongated and show evidence of cristolysis (Fig. 4.38). The nuclei assume a highly folded appearance, and the extensions

which arise may break off, giving rise to detached vacuoles. More likely, however, is that the nuclear extensions were cut in cross section, thus giving the impression that the small, nuclear profiles have become isolated (Fig. 4.39).

It is of special interest that a far greater number of calcium spherules can be observed from secretory cells affected by M. similis sporocysts, and these seem to increase up to a certain point, as the pathology becomes more severe (Fig. 4.40). In contrast, zymogen granules are especially prominent in the earlier stages of pathology (Fig. 4.35) but become reduced in number as cellular distortion is increased (i.e. in those cells which were adjacent to digestive cells exhibiting the characteristics of pathological State 3).

Digestive Cell

Pathological State 1 In contrast to digestive cells parasitized by C. lingua rediae, cells infected by M. similis sporocysts show a decrease in the number of Type 3 vesicles during the digestive and excretory phases. An increase in lipid globules is evident from the basal regions during all phases of digestion (Fig. 4.34), and there is no interference as yet with the cell's ability to absorb food through pinocytosis (Fig. 4.41).

Pathological State 2 The degenerative changes observed in digestive cells at this time are considerably more advanced than for those cells parasitized by C. lingua rediae, when the latter are categorized in pathological State 2. Pinocytotic activity is absent and apical borders break down (Fig. 4.42). At this time, there is a marked reduction in Type 3 vesicles and a corresponding rise in Type 4 vesicles during the digestive and excretory phases. In addition, empty-looking, irregularly shaped vesicles, possibly modifications of Type 4 vesicles are common (Fig. 4.43). Mitochondria usually appear swollen and show signs of extensive cristolysis (Fig. 4.43). In addition, large vacuoles, similar to the phagolysosomes described earlier from digestive cells infected with C. lingua rediae, are common at this time (Fig. 4.43). Now too, the nucleus begins to take on a folded appearance and eventually becomes reduced in size. The cisternae of the Golgi bodies display irregular foldings and the characteristic striated-like Golgi vesicles now appear to be absent or replaced by empty-looking vesicles (Fig. 4.44).

Pathological State 3 This state of pathology displays a complete breakdown in cellular composition. In general, the findings are similar to those observed in digestive cells in the late pathological State 3 and parasitized by C. lingua. The internal framework shows total deteriora-

tion. The most common feature throughout all phases of digestion is the predominance of phagolysosomes (Fig. 4.45, 4.46). Now, it is no longer possible to describe the apical and basal ends, since a breakdown of cell membranes occurs at both poles (Fig. 4.45, 4.46). The fusion of fragmented bits of digestive cells are a common feature (Fig. 4.47), as are isolated nucleated profiles of digestive cells (Fig. 4.39). Occasionally, nuclei are still visible in the ensuing syncytium which forms, and at such times they appear highly irregular in shape and much reduced in size, (Fig. 4.47).

DISCUSSION

A preliminary quantitative investigation was conducted on the infected digestive gland cells of Littorina saxatilis. This was done in an effort to shed some light on the extent of damage to the digestive gland, which could properly be attributed to M. similis sporocysts or C. lingua rediae. It must be pointed out here that throughout this chapter, the term 'infected' usually applies to those digestive gland cells which are contiguous with the parasite's outer surface and which can therefore be said to share an interface with the trematode larva.

Many investigations have demonstrated that in the case of infected snails, the sequence of structural degeneration of digestive gland cells, as observed under the light microscope, appears to follow a similar pattern irrespective of which parasite is inducing the damage (Rees, 1936; James, 1965; Wright, 1966). The present findings suggest that this is also applicable to digestive cells infected by C. lingua rediae and M. similis sporocysts. Digestive cells were therefore categorized into one of three pathological states. The criteria used in so grouping them was based on the extent of morphological alterations or disruptions that they manifested, as revealed under the light microscope using histological techniques.

The results indicate that the majority of digestive cells infected by C. lingua rediae display only the mildest form of disorder and that progressively fewer cells can be recorded as the severity of damage becomes more acute. In contrast, a larger percentage of digestive cells infected by M. similis sporocysts, exhibit greater degrees of cellular disruption. This suggests that M. similis is more damaging to its host.

The observations presented in this study reflect differing life styles and needs of M. similis and C. lingua in the same host species. Unlike M. similis sporocysts, C. lingua rediae are motile and do not become permanently concentrated in one region, but rather, are uniformly distributed throughout the tubules of the digestive gland. By virtue of their ability to move, it is possible that they may be capable of responding to some type of host signal that governs the duration which they remain in one spot. In this way, the specific length of time during which the rediae can form an interface with the digestive cells and tap them of nutrition, may be controlled. However, since some cells were identified as being in extremely degenerate condition, i.e. pathological State 3, total and irreversible deterioration does occur in some digestive cells infected with C. lingua rediae, and these cannot be disregarded. In this respect, it is possible that L. saxatilis possess a

surplus of digestive cells and are thus able to lose a certain proportion of the organ to the parasite, a suggestion put forth by Sinitsin (1931) and Davis and Farley (1973). It is interesting to note that C. lingua infects only sexually spent adults (Davis, 1972). Taking this into consideration, if the statement by McClelland and Bourns (1969) is correct that "it is more expensive in nutritional terms for a snail to produce eggs than it is to produce cercariae", then infected snails no longer have need for that portion of the digestive gland which may have been required to supply additional nutrition at the time when L. saxatilis were producing their young. Such factors also help to explain why no reduction was detected in the digestion rate or in the assimilation efficiency for L. saxatilis which were parasitized by C. lingua (Davis and Farley, 1973).

Unlike C. lingua rediae, M. similis sporocysts are not motile and thus remain concentrated in one region of the digestive gland. They reproduce asexually in a rapid manner and gradually replace the digestive gland cells completely. A "blocking layer", similar to that reported by Rees (1936) and James (1965) isolates the more distal parts of the digestive gland from its blood and food supply. It is, therefore, not surprising that the host dies in as little as four months after initial infection (Davis, 1972). Moreover, cercarial development is rapid in M. similis sporocysts and once the tegument ruptures all the mature cercariae

are released simultaneously. This contrasts sharply with C. lingua where cercariae emerge via specialized birth pores. In general, these findings support Davis' (1972) contentions that C. lingua enjoys a more compatible association with its host than does M. similis.

While the quantitative work suggested that M. similis produced more extensive damage to the digestive gland, it was only after the application of histochemical and ultrastructural techniques that it became possible to understand the sequence of alterations which was taking place in the digestive gland cells and the relative rates of degeneration which the cells were undergoing, depending on the parasite with which they were infected. At all times, however, it was necessary to consider the significance of phasic activity and the possibility that the digestive cells vary in quantitative and qualitative respects depending on their phase of digestion.

The concept of phasic activity in digestive cells of bivalves has received considerable attention (Morton, 1956; Morton, 1969; 1970; McQuiston, 1969; Owen, 1969) but only one such study has thus far been documented for a gastropod mollusc, Littorina littorea (Merdsoy and Farley, 1973). Because of the position of L. littorea on the lower levels of the shore, Merdsoy and Farley (1973) were unable to show a correlation between the different phases of digestion and

the periodicity of the tide. In contrast to L. littorea, L. saxatilis occupies a narrow band on the supralittoral fringe of the shore and is submerged for no more than two hours per tide cycle. As a result, by collecting the snails at specific times, it was possible to identify the distinct phases of digestion and to correlate them to the different tide levels.

In a study on the digestive cells of L. saxatilis, James (1965) indicated that there was a gradual decrease in "food vacuoles" and an increase in "excretory vacuoles" in starved or parasitized snails. The vacuoles which James described are comparable to the Type 3 and Type 4 vesicles discussed in the present dissertation. Moreover, their numbers (Chapter II) are seen to fluctuate over the tide cycle so that by the time the digestive cells are in the excretory phase, only Type 4 vesicles prevail. In brief, it was shown that each phase of digestion is characterized by a distinct assembly of vesicular structures which reflects on the extent of digestion that has already taken place. The question can therefore be raised whether the cells which James (1965) described were merely displaying the characteristic vacuolation ordinarily seen for this species during the excretory phase or expressing a condition which had been imposed on them by the parasitic larvae.

Unlike James, Meuleman (1972) considered the importance of phasic activity in her study of the effects of Schistosoma

mansoni on the digestive gland of Biomphalaria pfeifferi, although she suggested that the predominating morphological features at any particular time reflect the age of the digestive cell, rather than its phase of digestion. The problem was, however, that the digestive cells of Biomphalaria pfeifferi, a fresh water species, display all ages (phases) of digestion at any one time and are not synchronized as in the Littorinids. The fact that at any one time all the digestive cells of Littorina saxatilis are in the same phase, allows one to differentiate between parasitic effects and normal cellular activity. Therefore, in order to eliminate confusion regarding the morphological features as determined by the phase of digestion and as induced by the parasite, the criteria chosen for the pathological states were such that they could apply to cells irrespective of digestive phase.

One point brought out in James' work was that the digestive cells immediately adjacent to the stomach were not as severely affected as other cells which were more remote. James did not, however, adequately differentiate between the effects of parasites on cells which are in close proximity to the parasite and those located more distally which may suffer a form of "starvation autolysis". In contrast, present observations indicate that cells from the same digestive tubule may show marked differences in their response

to parasitic stress, depending on their relative proximity to the parasite. Such observations suggest that it is necessary to define the exact location of cells with respect to the parasite when discussing "the parasitic effects to digestive gland cells". For my work, cells which were in direct contact, and therefore, sharing an interface with the parasite, were considered to be infected and served as the subject for this thesis.

Many authors have employed histochemical and biochemical means to study the damage inflicted by digenetic trematodes on the digestive gland cells. In the present study, by applying various histochemical techniques, an effort was made to understand those changes that occur in the composition of digestive gland cells which could properly be attributed to Cryptocotyle lingua rediae and Microphallus similis sporocysts. However, because the cells manifested qualitative differences throughout the digestive cycle, it was essential to determine what was happening to the cells during each of the phases of digestion in non-parasitized individuals. Because the importance of phasic activity has been overlooked, past work has contributed towards conflicting and misleading information in this field.

Most of the investigations pertaining to the utilization of proteins and amino acids have been done on trematodes which infect freshwater molluscs (Friedl, 1961a, 1961b,

1961c; Cheng, 1963c, 1967; Dusanic and Lewert, 1963). No one has yet examined the changes in protein content, specifically as it relates to the digestive gland cells in molluscs infected with digenetic trematodes. In my work, protein was observed in small amounts in the basal regions of the digestive cells during the digestive phase. It is difficult to determine which parasite is drawing more protein from the cells, although it appears as if the reduction in proteins is conspicuous at an earlier state (pathological State 1) for cells affected by M. similis as compared to cells influenced by C. lingua rediae (pathological State 2). In addition, cells affected by either parasite during the digestive phase and exhibiting pathological State 2, show a marked reduction in RNA. Such findings not only indicate that RNA is eliminated when cells are in pathological State 2, but also suggest that protein synthesis may no longer be taking place at this time.

Numerous studies have been made of changes in the carbohydrate contents of digestive gland cells which were affected by larval trematodes. Such investigations have been comprehensively reviewed by Cheng (1967) and more recently by Erasmus (1972). The present findings indicate that while secretory cells stain negligibly for carbohydrates, digestive cells do contain large amounts of this component in their cytoplasm. The middle and lower basal regions contain

primarily glycogen, while the apical and basal regions include other carbohydrate material. Glycogen is totally absent during the excretory phase while other PAS-positive material becomes slightly reduced as digestion proceeds from the absorptive to the excretory phase.

Numerous workers have reported that the glycogen content of host cells becomes reduced when the digestive gland is infected with digenetic trematodes (Cheng, 1962; Cheng and Snyder, 1962a; James, 1965; Cheng and Burton, 1966; Porter et al., 1967; Porter, 1970). Even though there was no mention of phasic activity, the findings of these authors suggest a general situation which compares favourably with my own findings. In the present work, digestive cells displaying pathological States 1 and 2 and infected by C. lingua rediae showed a marked reduction in glycogen but a corresponding increase of other PAS-positive material in the basal regions. While similar observations were recorded for glycogen from cells infected by M. similis sporocysts, no increase in carbohydrate could be noted from the basal portions of cells which were identified as being in pathological State 2. In their paper, Cheng and Snyder (1962a) suggested that the gradual reduction of glycogen in the digestive cells results from the breakdown of this polysaccharide to glucose which in turn passes across the cell membrane and the parasite's wall to become incorporated in the developing cer-

cariae as glycogen. Such a possibility has received support from other workers (James, 1965; James and Bowers, 1967a; 1967b; Reader, 1971a) and may be applicable to my findings. It cannot be stated for certain that it is indeed glucose which is the carbohydrate prevailing in the basal parts. If this be the case, however, one could conclude that the exhaustion of glycogen and the uptake of glucose by the parasites is faster in those cells infected by M. similis sporocysts. It is of interest that there appears to be a marked increase of glycogen in digenetic trematodes which are adjacent to digestive cells characterized by an absence of this polysaccharide.

Various authors have indicated that there is an increase in acid and alkaline phosphatase in cells affected by digenetic trematodes larvae, suggesting that the heightened enzymatic activity may be associated with a rise in carbohydrate metabolism (Cheng, 1964; James, 1965; Porter, 1967; 1970). While these enzymes have been identified in digestive cells in the present work, no definite statements regarding changes in their activity can be made.

Many studies have been conducted in order to determine the presence of fats in digestive gland cells of infected and uninfected snails (Cheng, 1967). Cheng and Snyder (1962b) have observed that the digestive gland cells of parasitized snails display a marked increase in the number

and size of neutral fat droplets and in the quantity of fatty acids. They reported, however, that as the infection became more severe, the neutral fats were broken down into simple fatty acids and that the latter were ultimately absorbed by the parasite. While an initial increase and gradual depletion of fats has been documented by other workers (James, 1965; James, 1967a; Porter, 1967; 1971) for digestive cells, it has conflicted somewhat with the findings of Cheng (1965) who did not observe a gradual decrease in fatty acids. Moreover, Reader (1971a) did not observe an initial increase in neutral fats, but noted a decrease in this component with no change in fatty acids for digestive gland cells affected by trematode sporocysts. In my findings, neutral fats seem to increase as digestion proceeds and to be most abundant during the excretory phase. The present work also suggests that there is a rise in neutral lipids for cells affected by either C. lingua rediae or M. similis sporocysts. This can be seen to persist until the cells are characterized by pathological State 3. The initial increase in fats may indicate a decrease in the available oxygen and interference with the proper metabolism of lipids, which may in turn depend on the Krebs citric acid cycle and the aerobic metabolism of carbohydrates (Von Brand, 1966). The ultimate reduction of neutral lipid may be a result of enzymatic activity induced by the parasites. A parasite-emitted lipase has already been demonstrated by Cheng

(1965) and may also be secreted by either C. lingua, M. similis or both parasites.

Only Meuleman (1972) has considered the pathological effects at the ultrastructural level of a digenetic trematode on the digestive gland cells of its host. She provided a comparison between those changes induced by the parasite and the alterations which resulted in the cells after the snails had been starved. Because many of the disruptions in the digestive gland cells which might have been attributed to the parasite were also observed in uninfected specimens, she was unable to make any definite statements regarding the actual influences of the parasite.

In the comparative ultrastructural study on the relative effects of the two parasites on the digestive cells of L. saxatilis, it appears as if M. similis sporocysts induce their damage at an earlier stage in the infection process than do C. lingua rediae. Cells characterized by pathological State 1 and infected by M. similis sporocysts, manifest slight changes which are not ordinarily seen in cells affected by C. lingua rediae. Wile in both instances there appears to be a rise in the amount of lipid, cells affected by the sporocysts appear to contain fewer Type 3 vesicles. Such observations suggest that in the case of those cells sharing an interface with the sporocysts, Type 1 and Type 2 vesicles may become depleted of their food con-

tent rapidly, and are thus no longer available for purposes of fusing with the Golgi vesicles in order to form Type 3 vesicles. Another possibility is that the Golgi bodies no longer give rise to the characteristic striated-like vesicles which participate in the digestion process. Rather, they may now become involved in the formation of phago- or autolysosomes.

Autophagy is an intracellular process by which certain cytoplasmic constituents become enclosed within a phagolysosome or autolysosome and are subsequently digested. Autolysosomes have been shown to contain acid hydrolases, enzymes considered to be "lysosomal" or "lytic" (Novikoff, 1959). Moreover, an increase in the number of autolysosomes has been observed in cells that were exposed to a variety of damaging influences (Holtzman and Novikoff, 1965; Lane and Novikoff, 1965). It is, therefore, to be expected that if M. similis sporocysts subject the digestive cells to greater stress and do so at an earlier stage during the infection process then do C. lingua rediae, the Golgi bodies and their vesicles may become more involved with the protection of the cell through the formation of autolysosomes rather than with the process of digestion.

When digestive cells are characterized by pathological State 2 and the effects by C. lingua and M. similis are compared, some differences can also be observed. Electron micro-

graphs for example, suggest that pinocytosis remains functional in cells affected by C. lingua rediae, but no longer takes place in cells influenced by M. similis. Moreover, in the latter case, a complete breakdown of the microvillar border takes place towards the end of this state. Phagolysosomes are common during this state and appear more numerous in cells infected by M. similis sporocysts. It is only at this time that Type 3 vesicles are reduced in size and number, in cells infected by C. lingua rediae. Cells affected by either C. lingua or M. similis display similar forms of degeneracy during pathological State 3.

Most studies on the effects of parasites on the digestive gland are concerned with changes in the digestive cells and less with the histopathology of secretory cells. In my work, both histochemical and ultrastructural results suggest that secretory cells persist in a healthy, or unaltered state, long after digestive cells begin to display morphological abberances. Some investigators have indicated that the number of secretory cells increase when snails are parasitized by digenetic trematode larvae (James, 1965; Wright, 1966) and this has been observed in my work, especially for snails parasitized by M. similis sporocysts. It is also noteworthy that marked changes begin to appear in secretory cells affected by C. lingua sooner than they do in cells influenced by M. similis. Fretter and Graham (1962) have

suggested that the function of the secretory cells is to extract metabolic excretory products from the visceal haemocoel and to pass it off into the lumens of the digestive tubules.

Rees (1936) and Cheng and Snyder (1962a) have pointed out that the parasitic excreta which are released into the haemocoel may be a principle cause of cell degradation or lysis. In the present work, it has been shown that M. similis sporocysts are more damaging to the digestive cells than are C. lingua rediae. It is possible, therefore, that the damage induced by the sporocysts is indirect, and is caused primarily by the toxic nature of the accumulated larval wastes in the haemocoel. If this be the case, and if the secretory cells do perform the vital function of cleansing the haemocoel of undesirable wastes as suggested by Rees (1936) and Cheng and Snyder (1962), then it is reasonable to expect that snails infected by M. similis may rely more heavily on the secretory cells than do snails parasitized by C. lingua. Thus, a greater increase in the number and in the resistance of such cells, for snails parasitized by M. similis may reflect a molluscan response to cope with a more pronounced stress than it experiences from infection with C. lingua rediae.

It is of interest that secretory cells affected by M. similis display a greater number of calcium spherules than

cells in unparasitized snails. The presence of calcium spherules has been reported from similar cells by other workers (Abolins-Kragis, 1961; 1963; 1965; 1968; Merdsoy and Farley, 1973), and it is believed that one of their functions is associated with the provision of calcium for purposes of repair and regeneration of the gastropod shell. To prove this point, Abolins-Krogis (1970) damaged the shell of Helix pomatia L., and observed a heightened degree of activity in the gland cells. Because fragility of the gastropod shell has been associated with infection of snails by digenetic trematodes (Pan, 1965; Wright, 1966), it is quite possible that M. similis, unlike C. lingua, contributes towards the deterioration of the shell in L. saxatilis.

The boundary lines which demarcate the normal cell, the adapted cell, the injured cell and the dead cell, are difficult to define and there are as yet no clear descriptions which can properly be applied to differentiate these states. The importance, however, of the electron microscope as a tool in studying cellular morphology and the structural changes imposed on cells is indisputable. It is, nevertheless, particularly important especially in this study, to be aware that the adverse influences of the parasites are initially exerted at the molecular level and that biochemical changes occur long before injuries become visible.

The structural manifestations of cell injury have often been referred to by a variety of terms. In this connection, Robbins and Angell (1971) have reviewed the applicable terminology and have concluded that the term 'degeneration' should be reserved for such cells as are injured but capable of reverting to their normal condition once the adverse stimulus has been removed. Bessis (1964) often referred to 'cell pathology' and described the period during which cells show reversible deterioration as the "period of pathology". In addition, Bessis also differentiated between the "period of death" and the "period of necrosis", the latter phrase referring to those disintegrations which take place after the cell has already died. In contrast, Robbins and Angell (1971) accepted the term 'necrosis' to include those changes "indicative of cell death". In short, the problem seems to be one of semantics.

In the present work, few changes appear to take place in cells displaying pathological State 1. While there appears to be some lipid infiltration and a possible shift in the function of the Golgi body, the membranes and organelles remain intact so that the term 'degenerate', as defined by Robbins and Angell (1971) can be readily applied. In contrast, cells exhibiting pathological State 3 when infected by either parasite are characterized by a complete deterioration. Cell membranes appear ruptured, the organelles are either disintegrated or absent and nuclei are charac-

terized by varying degrees of pyknosis. In general, the term 'necrosis' can now be applied to these cells in accordance with the descriptions provided for cell death by Trump et al. (1962), Goldsmith (1966) and Robbins and Angell (1971).

Digestive cells in pathological State 2 and affected by C. lingua rediae display various morphological changes some of which may be construed as corrigible or reversible. In contrast, digestive cells affected by M. similis and characterized as being in pathological State 2 showed marked changes, such as breakdown in the apical border. Besides the absence of pinocytosis at this time, mitochondria appear swollen and show signs of cristolysis and nuclei appear highly irregular at first, and later display varying forms of pyknosis. Such morphological changes are compatible with those normally attributed to dying cells (Robbins and Angell, 1971) and are considered as permanent or 'irreversible'. While the exact time in the sequence of events when cells reach this "turning point" i.e., the stage where they will be destined to die or are already dead remains unclear, it is important to be aware that it must happen while cells are in pathological State 2. It is also likely that cells affected by C. lingua rediae may remain in a degenerate state longer than cells influenced by M. similis. In other words, once cells are characterized by pathological State 2, those infected by M. similis will die sooner than the ones infected by C. lingua.

There are numerous aspects of this work which warrant close attention, but are beyond the scope of this thesis. Consideration may be given to host responses, defence mechanisms, phasic activity of parasites, etc. Furthermore, enzymatic and histochemical methods at the electron microscope level, as well as autoradiography techniques to study the passage of food at the cell-parasite interface should be considered as potential tools for this kind of work. Investigations and approaches of these kinds, will, I feel, lend further support to the present findings.

Figure 4.1

Fresh preparation of Littorina saxatilis infected with Cryptocotyle lingua. The C. lingua rediae are not aggregated in one particular region of the host, but are widely distributed among the digestive gland tubules. When observed in the living state, the latter exhibit considerable movement.

dt - digestive tubule
r - redia

x10

Figure 4.2

Fresh preparation of L. saxatilis infected with Microphallus similis. Unlike C. lingua rediae, M. similis sporocysts are concentrated in one region of the digestive gland and appear as a pale-white patch. This tendency to localize in one region may be related to the lack of mobility displayed by the sporocysts.

dg - digestive gland
s - sporocysts

x10

Figure 4.3

Fresh preparation of L. saxatilis snails showing a) an uninfected gravid female b) a snail infected with C. lingua and c) a snail infected with M. similis. Note the characteristic distributions of the parasites as described for Figures 4.1 and 4.2 and the dark-brown uninfected digestive gland in the uninfected snail. Numerous rediae can be observed in the water and attached to the digestive epithelium of their host (b).

dg - digestive gland
r - rediae
s - sporocysts
ys - young shells

x10.

Figure 4.4

Cross section of digestive gland tubules of L. saxatilis infected with M. similis sporocysts. Note how the larvae are concentrated in the upper regions of the picture. Note too, the presence of secretory cells, especially common at the level where contacts exist between the cells and the parasitic larvae.

dc - developing cercaria
lu - lumen of digestive gland
DC - digestive cells
I - interface
S - sporocyst
SC - secretory cell

(Bouin's, Mallory's Triple Stain)

x200

Figure 4.5

An enlargement of part of Figure 4.4 showing interfaces between sporocysts and digestive gland cells. Note general disruptions in cells which are immediately adjacent to the sporocysts' outer walls, in contrast to the relative cellular integrity displayed by cells (arrows) more remote from the actual points of contact.

I - interface
S - sporocyst
S3 - digestive cells in pathological State 3

(Bouin's, Mallory's Triple Stain)

x450

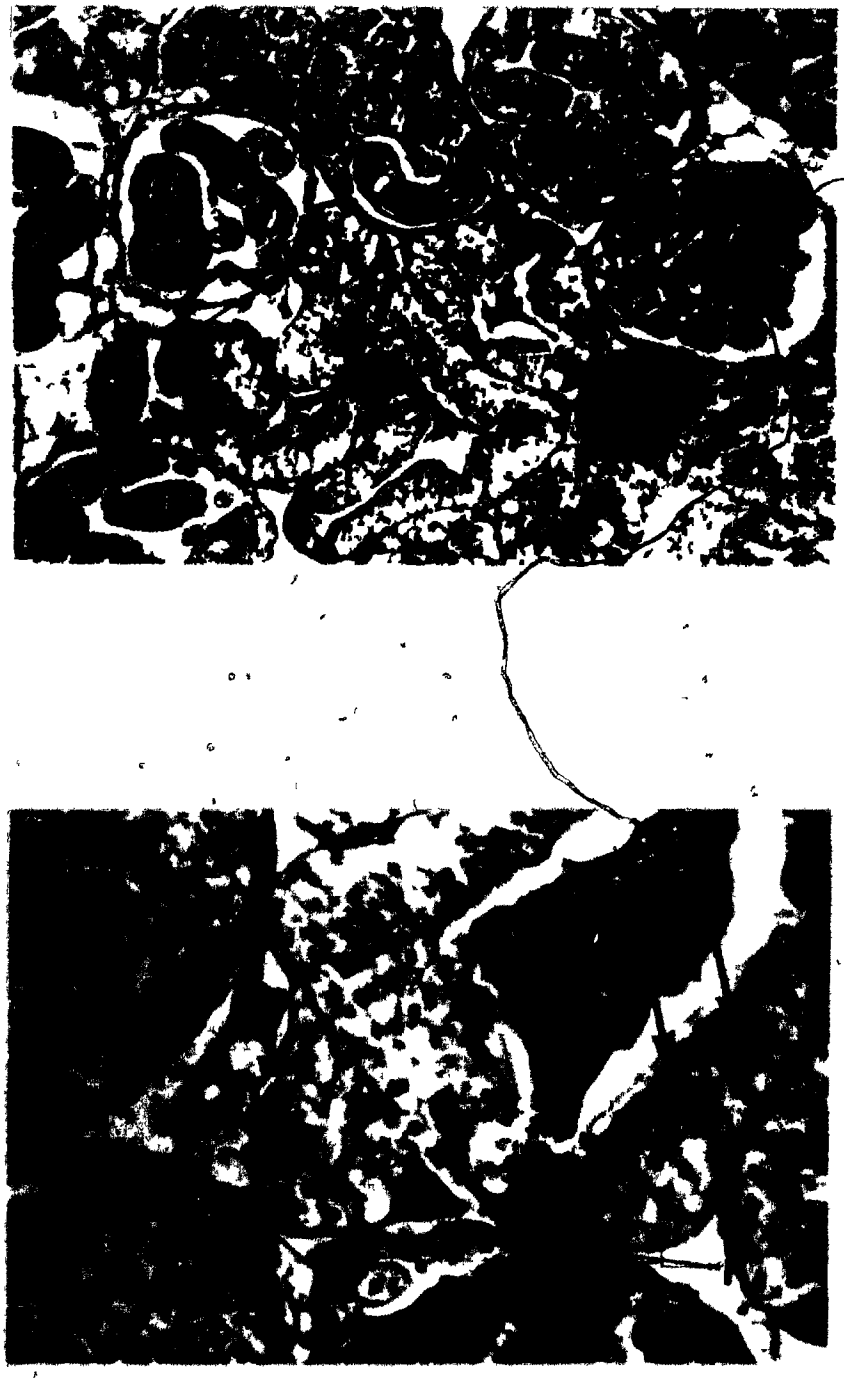


Figure 4.6

Diagramatic sketches of the digestive cells of L. saxatilis displaying three states of pathology that are applicable to all phases of digestion and which are induced by M. similis sporocysts and C. lingua rediae.

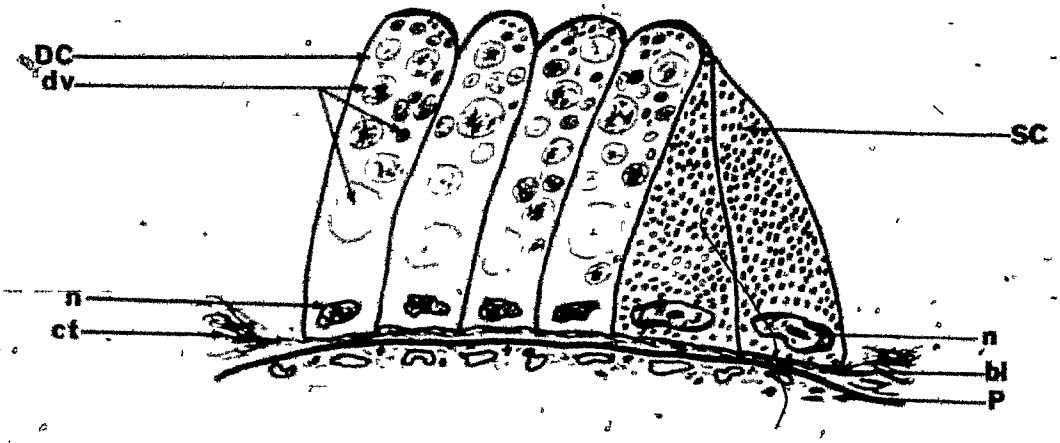
State 1: No structural changes are observed.

State 2: Nuclei become dislodged and migrate apically. General empty appearance is visible in the basal region. Cells are compressed and assume a squamous appearance.

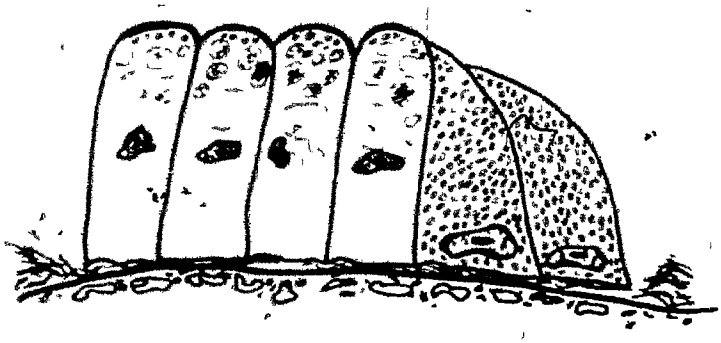
State 3: Degeneration of the lateral and apical membranes. Absence of much cellular material (as revealed by the Mallory's Triple Stain Method). Digestive vacuoles can often be seen in the digestive tubule lumen after cell membranes have ruptured. (This sketch does not show the occurrence of a syncytial layer - a feature commonly present after the breakdown of the lateral and apical membranes).

bl - basal lamina
ct - connective tissue
DC - digestive cell
dv - digestive vacuoles (idealized diagram of vacuoles to represent all three phases of digestion)
n - nucleus
SC - secretory cell
P - parasite

STATE 1.



STATE 2.



STATE 3.

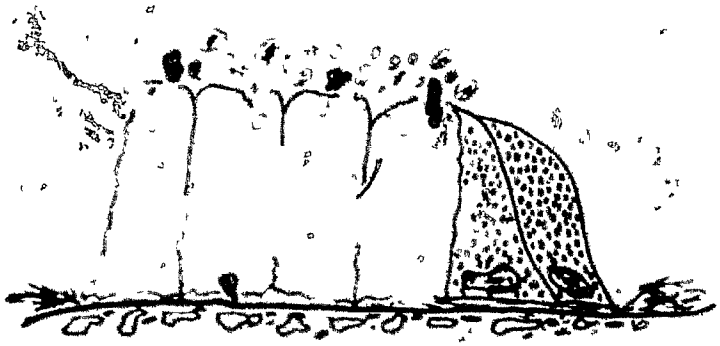


Figure 4.7

Cross section of digestive gland tubules of L. saxatilis infected with C. lingua rediae. Unlike M. similis sporocysts, and as seen from pictures of whole snails (Fig. 4.1 - 4.3), the rediae are dispersed between the tubules and not completely concentrated in one area. This can be seen by the presence of digestive tubules on all sides of the parasitic larvae. Fewer gross distortions (State 3) can be seen in digestive cells despite the fact that the cells and parasites have intimate contact in many places.

DC - digestive cells
R - redia

(Bouin's, Mallory's Triple Stain)

x180

Figure 4.8

Cross section of digestive gland tubules of L. saxatilis infected with C. lingua rediae. As in Figure 4.7, the intermingling of larvae with some digestive gland tubules is observed, but unlike above, some cells can be seen displaying more severe effects of the parasite (as characterized by State 3).

S3 - digestive cells displaying pathological State 3

(Bouin's, Mallory's Triple Stain)

x320

Figure 4.9

Cross section of a digestive gland tubule of L. saxatilis infected with C. lingua rediae and stained with mercury-bromophenol blue to reveal proteins. Note the absence of blue color from the basal regions of digestive cells displaying pathological State 2. In contrast, note the faint blue color from the basal regions of digestive cells which are adjacent to the parasite and which can be categorized into pathological State 1. The rediae can be observed to stain intensely with mercury-bromophenol blue.

lu - lumen of digestive gland tubule
n - nucleus
P1 - digestive cell displaying pathological State 1
P2 - digestive cell displaying pathological State 2
R - C. lingua rediae
SC - secretory cell

(Bouin's fluid, Foot, 1933; Mercury-bromophenol blue)

protein - blue

x400

Figure 4.10a

Cross section of a digestive gland tubule of L. saxatilis infected with M. similis sporocysts and stained with mercury-bromophenol blue to reveal proteins. Note the absence of blue color from the basal regions of digestive cells which are immediately adjacent to the sporocyst (light arrow). In contrast, note the violet-blue color of the basal regions of cells which are not immediately adjacent to the parasite (heavy arrow). Because of a slight color shift at the time when the photograph was taken, the protein content is now identified by a violet-blue instead of blue color.

DC - digestive cell
S - sporocyst

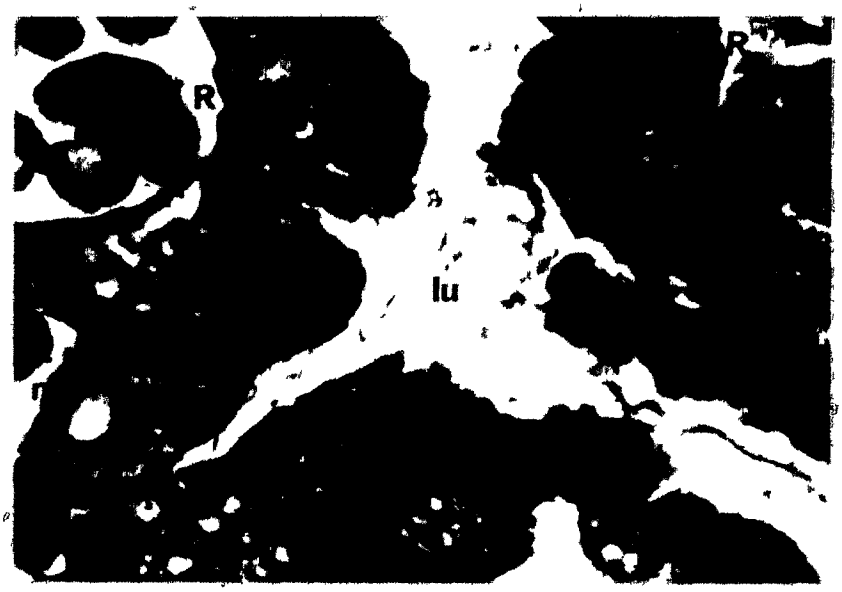
(Bouin's fluid, 1933; Mercury-bromophenol blue)
x400

Figure 4.10b

Similar to Figure 4.10a except that it is taken at a higher magnification to show the violet-blue staining of the uninfected digestive cells (arrow).

bb - basal border

(Bouin's fluid, 1933; Mercury-bromophenol blue)
x800


R
R
lu

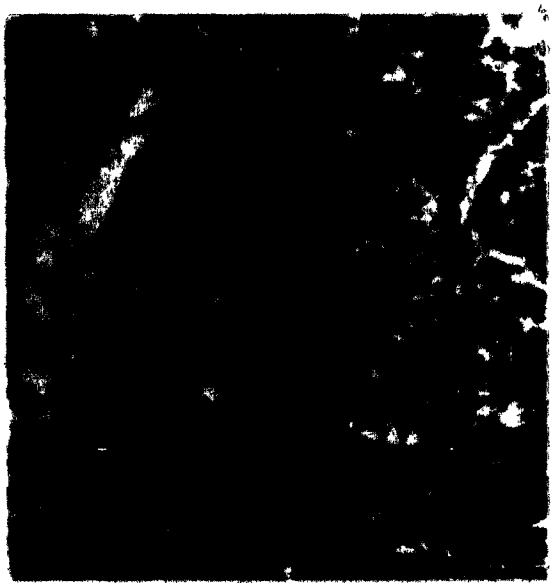


Figure 4.11

Cross section of digestive gland tubules of L. saxatilis infected with C. lingua rediae and stained with pyronin-methyl green to reveal nucleic acid. Note the absence of RNA in the basal regions of cells considered to be in pathological State 2. Note also the relatively large amounts of RNA in the secretory cells and in the rediae.

P2 - digestive cells displaying pathological State 2
R - redia
SC - secretory cell

(Lillie's acetic-alcohol-formalin, Pyronin-methyl green)

RNA - purple-red
DNA - deep blue

x400

Figure 4.12

Cross section of digestive gland tubules of L. saxatilis infected with C. lingua and treated with ribonuclease prior to staining with pyronin-methyl green. At this time, note the absence of RNA. Note too, the deep blue stain, revealing the presence of DNA, a component not affected by RNA se. Arrows point to phagocytes within the connective tissue.

DC - digestive cells
lu - lumen
S - sporocyst

(Lillie's acetic-alcohol-formalin, Pyronin-methyl green)
control: ribonuclease

x400

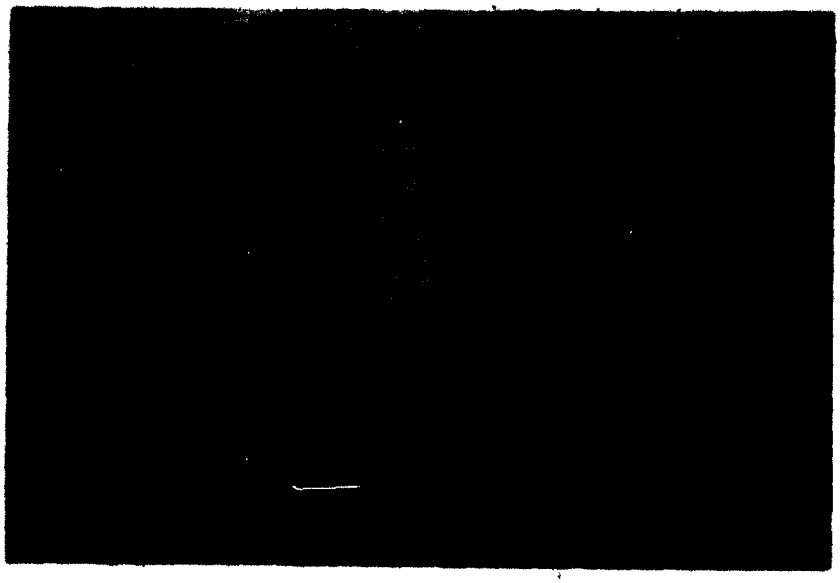


Figure 4.13a

Cross section of uninfected digestive gland tubules of L. saxatilis in the absorptive phase and stained by the PAS method to identify carbohydrates. The apical borders of digestive cells can be observed to stain intensely by the PAS method. Note, however, a slightly more moderate staining reaction just within the distal border. Note also the PAS-positive material which is scattered in clumps in the mid-basal region (arrows). The most basal region, i.e. the region immediately adjacent to the nucleus is characterized by a very light PAS-positive granularity, while the nucleus and nucleolus stain a light blue. In contrast to digestive cells, the secretory cells appear to stain blue, and show little evidence of PAS-positive material.

ab - apical border
DC - digestive cell
lu - lumen
n - nucleus
nu - nucleolus
SC - secretory cell

(alcohol at -73°C, Periodic Acid-Schiff counter-stained with Harris Haematoxylin)

PAS
carbohydrate - purplish-red
nuclei - blue-black

x400

Figure 4.13b

Similar to Figure 4.13a only taken at a higher magnification. Arrows point to PAS-positive clusters in middle and lower basal regions of the digestive cells.

n - nucleus

(alcohol at -73°C, Periodic Acid-Schiff counter-stained with Harris Haematoxylin)

x720

Figure 4.14

Cross section of uninfected digestive gland tubules of L. saxatilis in the absorptive phase, treated with malt diastase and stained by the PAS method to reveal the distribution of glycogen. Note the overall elimination of PAS-positive material from the middle portions and the lower half of the basal regions of the digestive cells, suggesting that the heavily stained PAS-positive clumps previously identified (Figures 4.13a, 4.13b) are glycogen. In contrast, note that the apical borders continue to stain intensely while the regions just within the distal borders retain their moderate staining capacity as well. The secretory cells stain negligibly by the PAS method.

ab - apical border
DC - digestive cell
SC - secretory cell

(alcohol at -73°C, Periodic Acid-Schiff counter-stained with Harris Haematoxylin)

control: malt diastase

x720

Figure 4.15

Cross section of digestive gland tubules of L. saxatilis in the digestive phase, infected with C. lingua rediae and stained by the PAS method without prior exposure of the tissues to periodic acid. Tissues fail to stain by the PAS method, indicating that a positive PAS reaction (Fig. 4.10a) is due to the specific oxidation of glycols. At this time, only material stained by the haematoxylin counterstain can be distinguished.

DC - digestive cell SC - secretory cell
R - redia

(alcohol at -73°C, Acid Schiff counter-stained with Harris Haematoxylin)

control: no periodic acid

x200

Figure 4.16

Cross section of a digestive gland tubule of L. saxatilis in the digestive phase, infected by C. lingua rediae and stained by the PAS method. Note the absence of PAS-positive material from the middle and basal regions (arrows) of digestive cells displaying pathological State 2. Material located in similar positions was previously identified as glycogen in uninfected cells (Fig. 4.13b, 4.14). Note too, a fine PAS-positive granularity in the basal regions, not ordinarily observed in uninfected cells (Fig. 4.13b).

ab - apical border
lu - lumen
n - nucleus
P2 - digestive cell displaying pathological State 2
R - redia
SC - secretory cell

(alcohol at -73°C, Periodic Acid-Schiff, counter-stained with Harris Haematoxylin)

x700

Figure 4.17

Cross section of a digestive gland tubule of L. saxatilis in the digestive phase, infected by M. similis sporocysts and stained by the PAS method. Note the absence of PAS-positive material from the middle and basal regions of digestive cells in pathological States 1 and 2 (as for Fig. 4.16). Material located in similar regions was previously identified as glycogen in uninfected cells (Fig. 4.13b, 4.14). Unlike for cells affected by C. lingua rediae (Fig. 4.16), there is a reduction of PAS-positive material from the basal regions of cells in pathological States 1 and 2. Note the complete elimination of PAS-positive material in cells displaying pathological State 3.

ab - apical border
lu - lumen
n - nucleus
P1 - digestive cell displaying pathological State 1
P2 - digestive cell displaying pathological State 2
P3 - digestive cell displaying pathological State 3
S - sporocyst

(alcohol at -73°C, Periodic Acid-Schiff, counter-stained with Harris Haematoxylin)

x800

Figure 4.18

Cross section of a digestive gland tubule of L. saxatilis in the digestive phase, infected with C. lingua rediae and stained by the Oil Red O method to reveal lipid. Note the large amounts of lipid, predominately in the basal regions of digestive cells - a feature not ordinarily common for unparasitized cells at this time.

ab - apical border
bb - basal border

(formalin sea water, Oil Red O, counter-stained in Mayer's Haemalum)

lipid - red

x680

Figure 4.19

Cross section of a digestive gland tubule of L. saxatilis in the digestive phase, infected with C. lingua rediae and stained by the calcium cobalt method (Gomori, 1946), to reveal alkaline phosphatase activity (light arrow). Note the dense granules concentrated primarily in the basal regions of the digestive gland cells. Note too, that rediae stain intensely for alkaline phosphatase (heavy arrow).

(buffered formalin sea water, calcium cobalt)

alkaline phosphatase - black

x720

Figure 4.20

Electron micrograph of the basal region of a secretory cell of L. saxatilis in the digestive phase, parasitized by a C. lingua redia. Note the highly folded appearance of the nucleus. Note too, an autophagic vacuole which contains small membrane-bound vesicles.

av - autophagic vacuole
i - invaginations of the redial tegument
I - interface between host cell and parasite tegument
n - nucleus

x6660

Figure 4.21

Electron micrograph of secretory cells of L. saxatilis during the digestive phase, parasitized by a C. lingua redia. Note the small isolated portion of a nucleus, immediately adjacent to the main nucleus (arrow). This is a common feature at such time and probably represents a cross section of a nuclear extension. Note too, the relatively few zymogen granules and the absence of material of similar electron density to the zymogens from the microvillar region (see Chapter II - Figure 2.11). An isolated portion of a digestive cell displaying a general empty appearance, characteristic of pathological State 3 can also be seen.

ab - apical border
DC - digestive cell
mv - microvilli
n - nucleus
SC - secretory cell
zg - zymogen granule

x5940

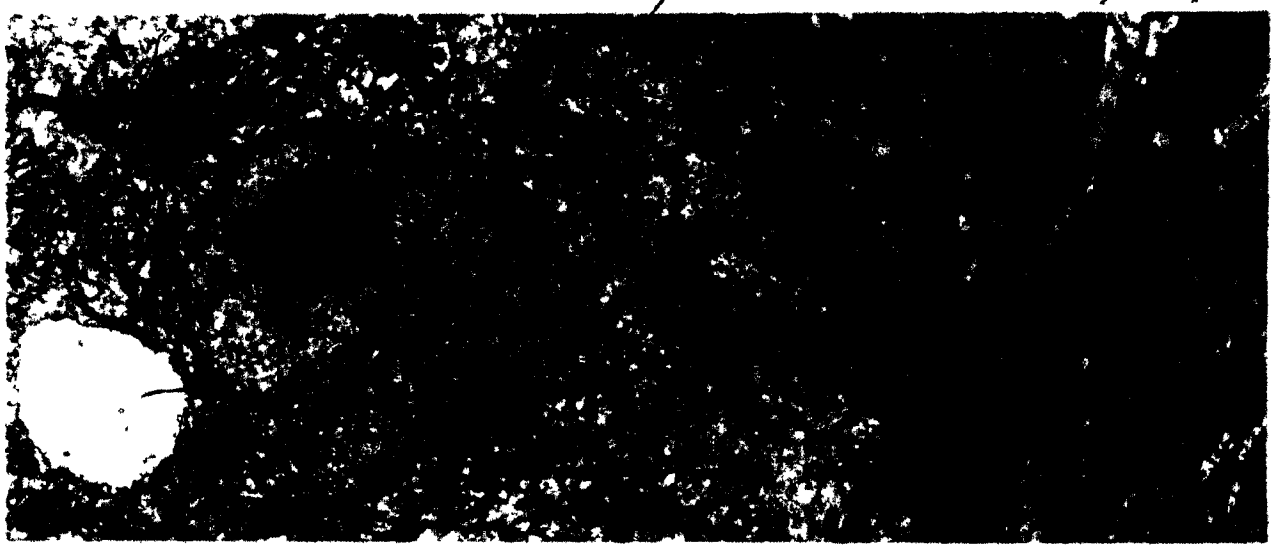


Figure 4.22

Electron micrograph of the mid region of a secretory cell of L. saxatilis, parasitized by a C. lingua redia. Note the absence of long parallel E.R. cisternae and the prevalence of numerous granular E.R. vesicles. Note too the large number of unattached ribosomes (arrows). Note the elongated mitochondria and possible signs of their rupturing.

m - mitochondrion
V - granular E.R. vesicles

x33,030

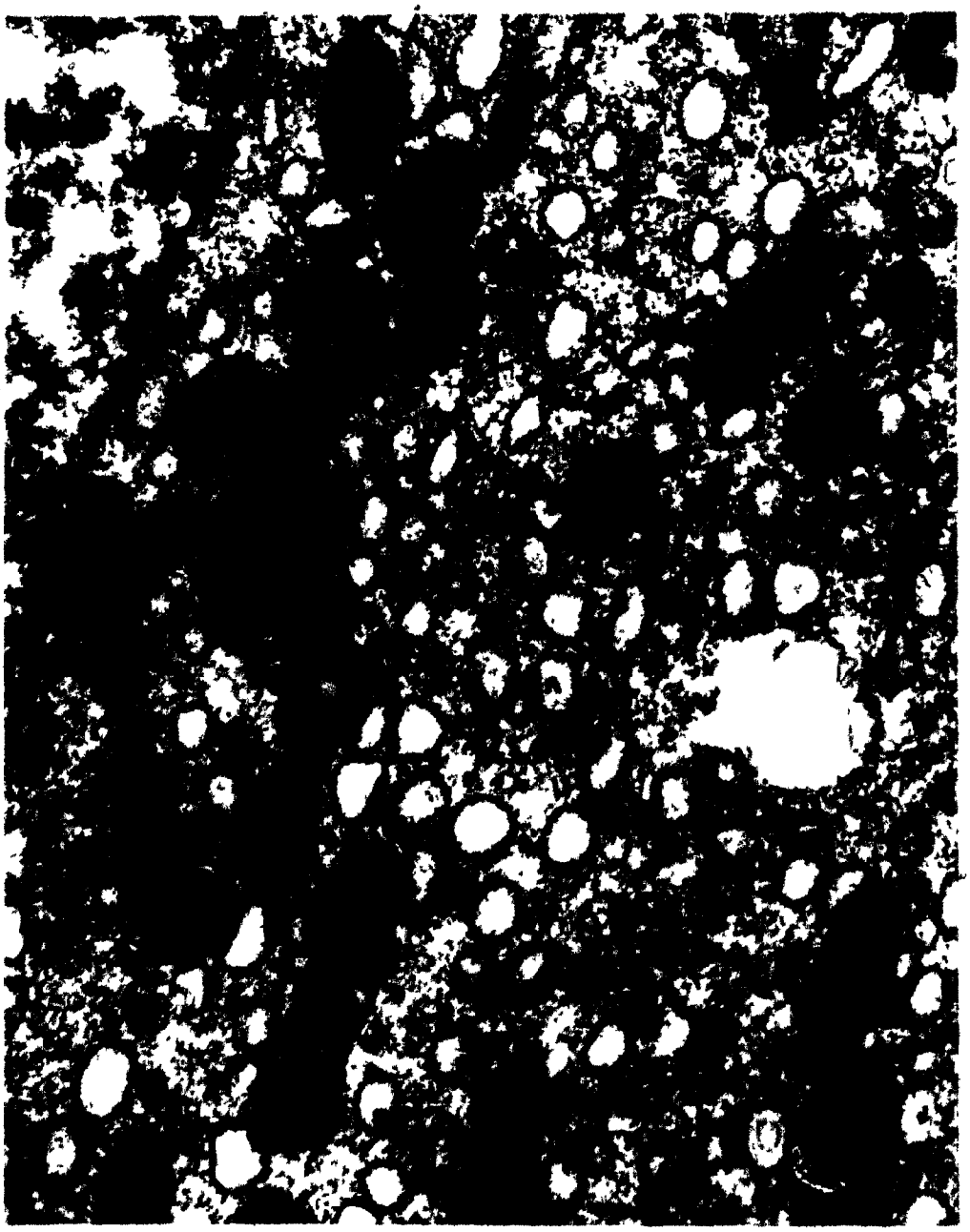


Figure 4.23

Electron micrograph of secretory and digestive cells, parasitized by C. lingua rediae. Note the shrivelled state of some of the nuclei and the dense granulation which they exhibit at this time. Note too, the extensive vesiculation of the granular E.R. Digestive cells, showing various states of pathology can likewise be seen.

BC - brood chamber of the parasite
DC - digestive cell
I - interface between secretory cell and parasite
n - nucleus
SC - secretory cell
t - redial tegument

x5760

Figure 4.24

Electron micrograph of the basal portion of a secretory Cell parasitised by a C. lingua redia. Note the autophagic vacuole and the way in which the latter seems to engulf cellular material. Note too, the irregularly shaped nucleus which shows signs of extreme degeneration.

n - nucleus
av - autophagic vacuole

x5500

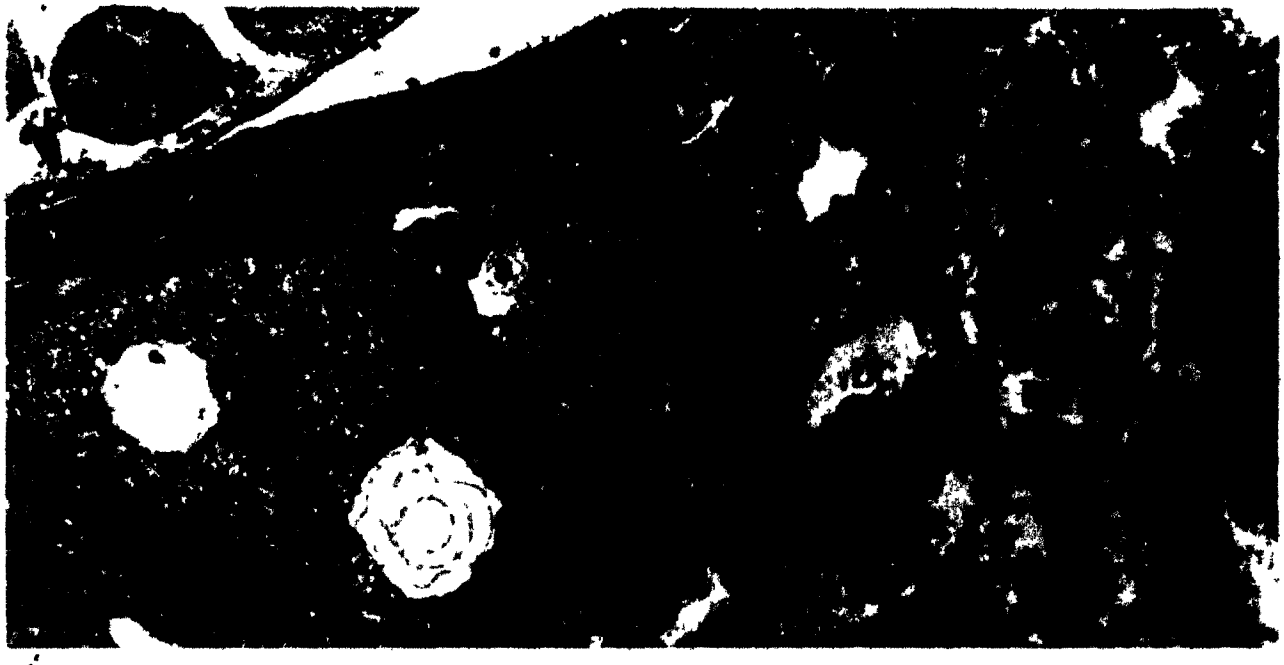


Figure 4.25

Electron micrograph of the mid region of an L. saxatilis digestive cell in the digestion phase parasitized by a C. lingua redia and exhibiting the early stages of pathology. Note that the cell membranes appear to be intact, and also the relatively normal condition of the T3 vacuoles. Note the large number of lipid droplets at this time.

Gv - Golgi vesicle
l - lipid
T3 - Type 3 vacuole

x11,600

Figure 4.26

Electron micrograph of the basal region of an L. saxatilis digestive cell, parasitized by a C. lingua redia and exhibiting properties characteristic of pathological State 1. Note how some of the cisternae of the Golgi bodies appear distended and give rise to clear structures (arrows). Note the accumulation of densely concentrated material along the cell's inner membrane at the level of cell-parasite interface. The latter is similar in electron density to the contents enclosed within the invaginated portions of the surface projections of the redial tegument and may represent host substance which will be phagocytized.

dm - dense material along inner boundary of digestive cell
Gb - Golgi body
GC - glycogen cell in the connective tissue
I - interface
is - invagination of surface projection (containing electron dense material)
n - nucleus
t - redial tegument

x6,120

Figure 4.27

Electron micrograph of the mid and basal portions of digestive cells in the digestive phase, parasitized by C. lingua rediae. Note the predominance of T4 vacuoles and the relative absence of T3 vacuoles. The few remaining T3 vacuoles are smaller than the ones usually observed from unparasitized cells.

DC - digestive cell
n - nucleus
pl - phagolysosome
SC - secretory cell
T3 - Type 3 vacuole
T 4 - Type 4 vacuole

x2,800

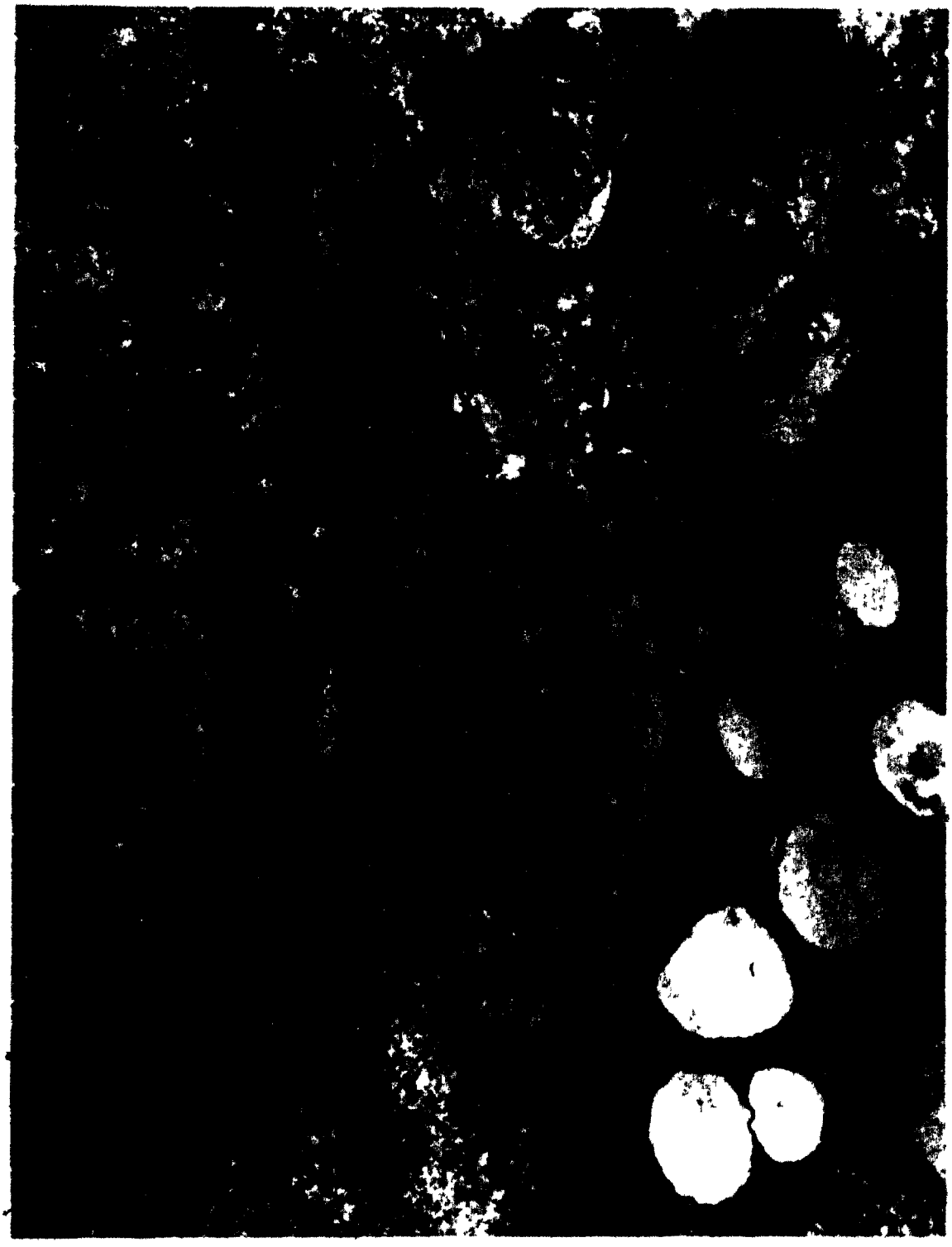


Figure 4.28

Electron micrograph of the apical regions of digestive cells of L. saxatilis in the early phase of digestion and which are parasitized by C. lingua rediae. Pinocytotic vesicles and mitochondria are visible within the apical border. As in Figure 4.27, T4 vacuoles prevail, while the T 3 vacuoles are few in number. Note the presence of a phagolysosome in the more basal region.

li - lipid
m - mitochondrion
mv - microvilli
pl - phagolysosome
pv - pinocytotic vesicle
T3 - Type 3 vacuole
T4 - Type 4 vacuole

x7,960

Figure 4.29

Electron micrograph of the apical region of a digestive cell of L. saxatilis in the early phase of digestion and parasitized by a C. lingua redia. Unlike Figure 4.28, pinocytotic vesicles are not present and the apical border is characterized by an irregularly folded membrane. The presence of dense membrane-bound vesicles may represent an advanced form of lysosomal activity at this time.

dv - dense vesicle

x7,920

28

Figure 4.30

Electron micrograph of the mid regions of digestive cells of L. saxatilis in the absorptive phase and parasitised by C. lingua rediae. Note the light grainy quality and the relative emptiness of these cells. Note also the folded and ruptured membranes (arrows) of the cells. Numerous small electron lucent vesicles can also be seen in the micrograph.

T4 - Type 4 vacuole v - vesicle

x6,660

Figure 4.31

Electron micrograph of the apical regions of digestive cells of L. saxatilis in the absorptive phase and parasitized by C. lingua rediae. Note the general deterioration of the mitochondria. Swelling, internal vesiculation and a reduction in the number of cristae can be seen in the micrograph.

m - mitochondrion

x10,260

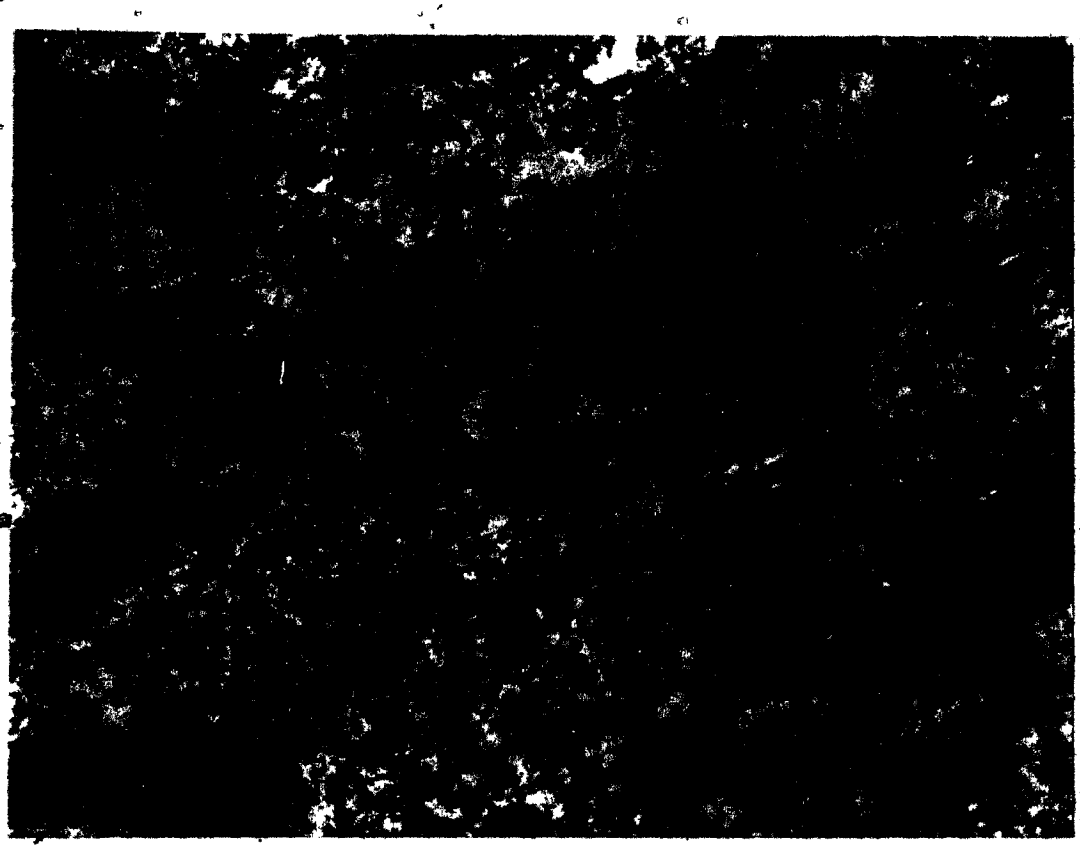
Figure 4.32

Electron micrograph of L. saxatilis digestive cells infected by C. lingua rediae, showing extreme deterioration. Note the extensive breakdown of cellular membranes (arrows), the large number of phagolysosomes and the irregularly shaped nuclei. The latter are usually much reduced in size and appear very dense. Fusion of adjacent digestive cell fragments is apparent in the micrograph.

l - lipid pl - phagolysosome

n - nuclei

x6,000



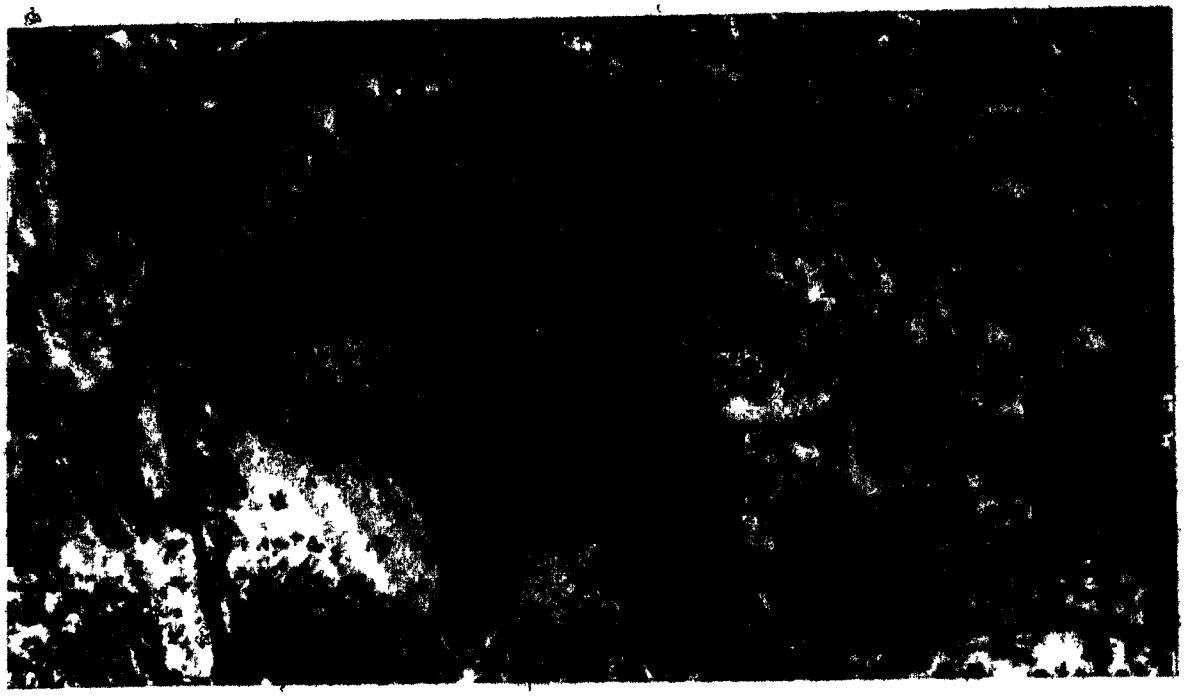


Figure 4.33

Electron micrograph of the basal regions of secretory cells of L. saxatilis in the early absorptive phase and parasitised by M. similis sporocysts. The granular E.R. cisternae do not show signs of swelling or vesiculation and in general, there is no observable evidence to suggest that the cells have suffered any form of damage.

cs - calcium spherule
DC - digestive cell
SC - secretory cell

x20,000

Figure 4.34

Electron micrograph of the basal regions of secretory and digestive cells in the early absorptive phase and parasitised by M. similis sporocysts. Note the relatively normal state of the secretory cells. In contrast, note the large accumulations of lipid, apically to the nuclei, a feature characteristic of digestive cells displaying pathological State 1. Note also a portion of a digestive cell to the right of the secretory cells, characterised by a large phagolysosome, suggesting therefore that the latter may be in pathological State 3.

cs - calcium spherule
DC - digestive cell
l - lipid
n - nucleus
pl - phagolysosome
SC - secretory cell

x5,940

Figure 4.35

Electron micrograph of the apical regions of secretory and digestive cells in the early absorptive phase parasitised by M. similis sporocysts. Note the large number of zymogen granules in the secretory cells as well as material of similar electron density to that of the granules dispersed between the microvilli (arrows). Note the presence of large phagolysosomes as well as ruptured T4 vacuoles within the digestive cell (to the right of the secretory cells). Note also the irregular folds and ruptures of cell membranes (to the left of the secretory cells). The latter conditions are characteristic of cells displaying pathological State 3.

cs - calcium spherule
DC - digestive cell
pl - phagolysosome
SC - secretory cell
T4 - Type 4 vacuole
zg - zymogen granule

x6,840

D

Figure 4.36

Electron micrograph of secretory cells in the absorptive phase, parasitized by M. similis sporocysts. Note the large number of lipid droplets.

cs - calcium spherule
DC - digestive cell
ger - granular endoplasmic reticulum
l - lipid
SC - secretory cell

x6,060

Figure 4.37

Electron micrograph of the basal regions of secretory and digestive cells of L. saxatilis infected with M. similis sporocysts. Note the absence of nucleoli from the nuclei and the scattered concentrations of electron dense granules within the nucleoplasm. A phagolysosome can also be seen in the micrograph.

DC - digestive cell
dg - dense granule
n - nucleus
p - phagosome
pl - phagolysosome (of a digestive cell)
SC - secretory cell

x6,000

Figure 4.38

Electron micrograph of the mid region of a secretory cell in the absorptive phase which is infected by M. similis sporocysts. Note the extensive vesiculation of the granular endoplasmic reticulum, as well as the large number of detached ribosomes. Note too, the elongated mitochondria which in some instances show a reduction in the number of cristae and signs of possibly ruptured membranes.

m - mitochondrion
r - detached ribosomes
v - granular endoplasmic reticulum vesicles

x34,710

Figure 4.39

Electron micrograph of secretory cells of L. saxatilis infected by M. similis sporocysts. Note the highly folded nucleus of the secretory cell. Note also the relatively smaller nuclei (arrows) which may represent the cross sections of nuclear extensions. The remains of what was formerly a digestive cell can be seen in the middle of the photograph.

DCF - remaining portion of a digestive cell
n - nucleus

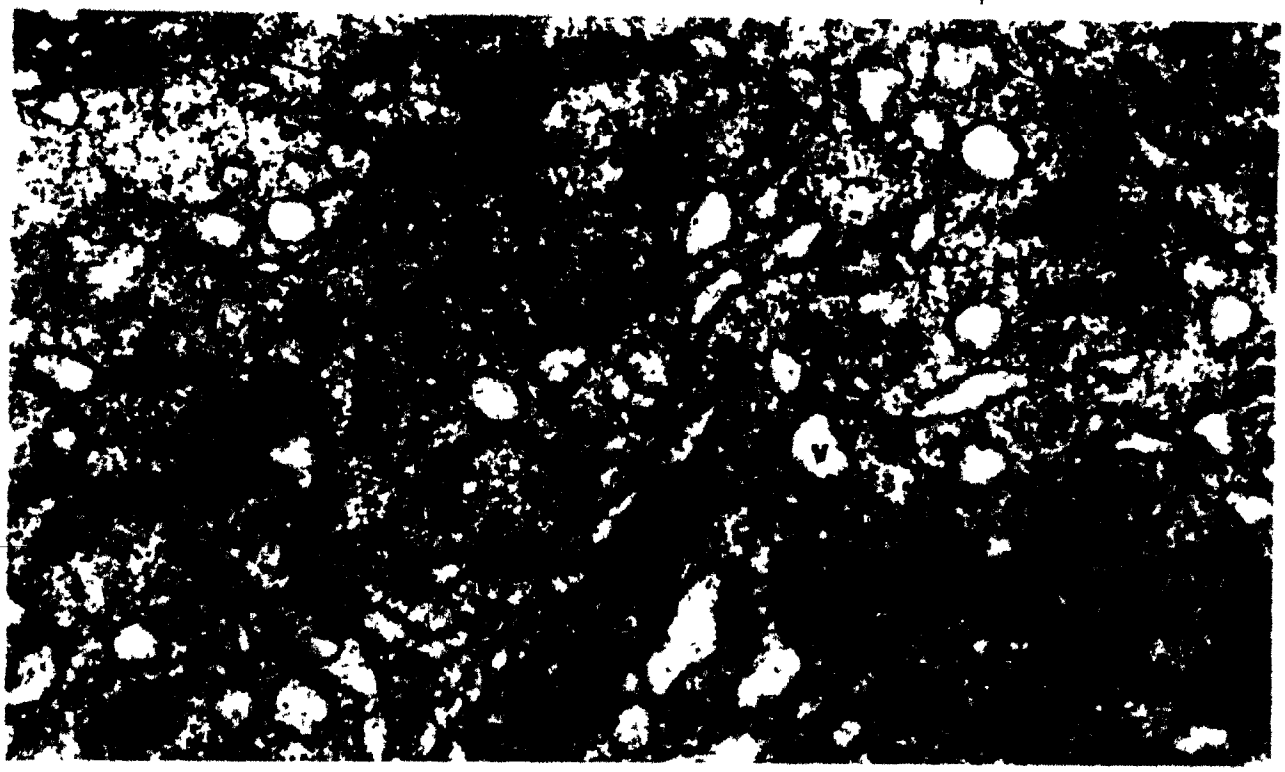
x4,500

Figure 4.40

Electron micrograph of a secretory cell of L. saxatilis infected with M. similis sporocysts. Note the large number of calcium spherules.

cs - calcium spherule

x5,940



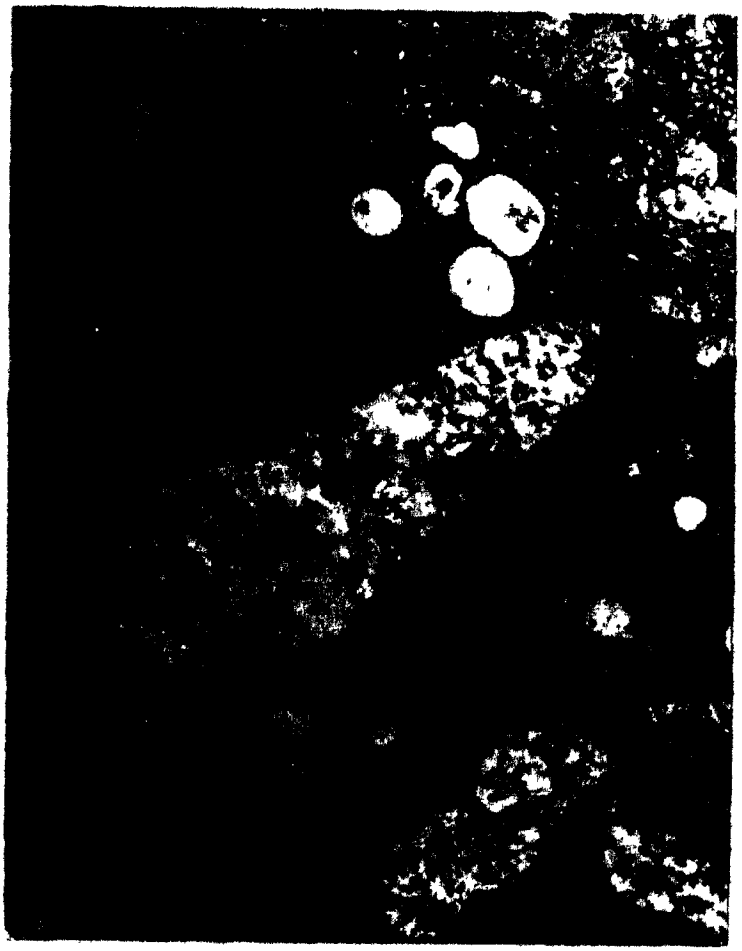


Figure 4.41

Electron micrograph of the apical region of a digestive cell in the absorptive phase and parasitized by M. similis sporocysts. Note the presence of pinocytotic vesicles in the distal end. Note also, that the apical border and the microvilli are intact.

m - mitochondrion
mi - microvillus
pv - pinocytotic vesicle

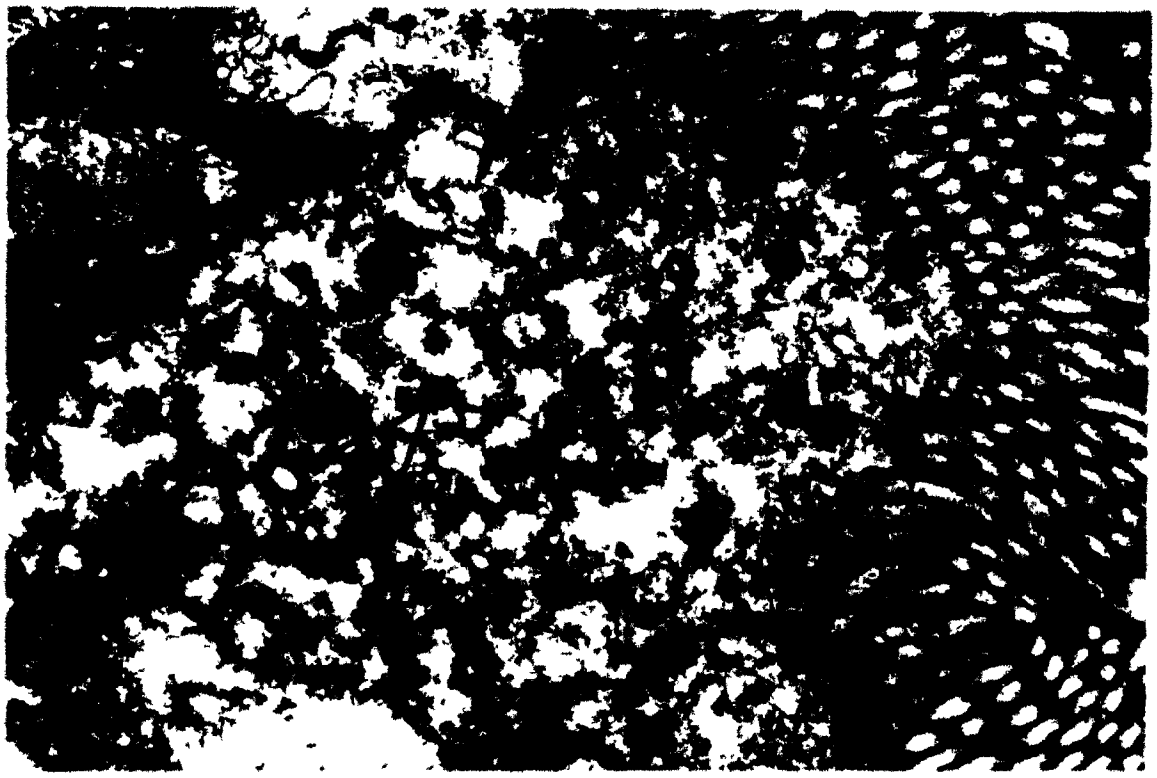
x24,120

Figure 4.42

Electron micrograph of the apical region of a digestive cell in the absorptive phase and parasitized by M. similis sporocysts. Note the absence of pinocytotic vesicles. Note also the beginnings of membrane breakdown along the apical and lateral borders (arrows).

m - mitochondrion
mi - microvillus

x26,700



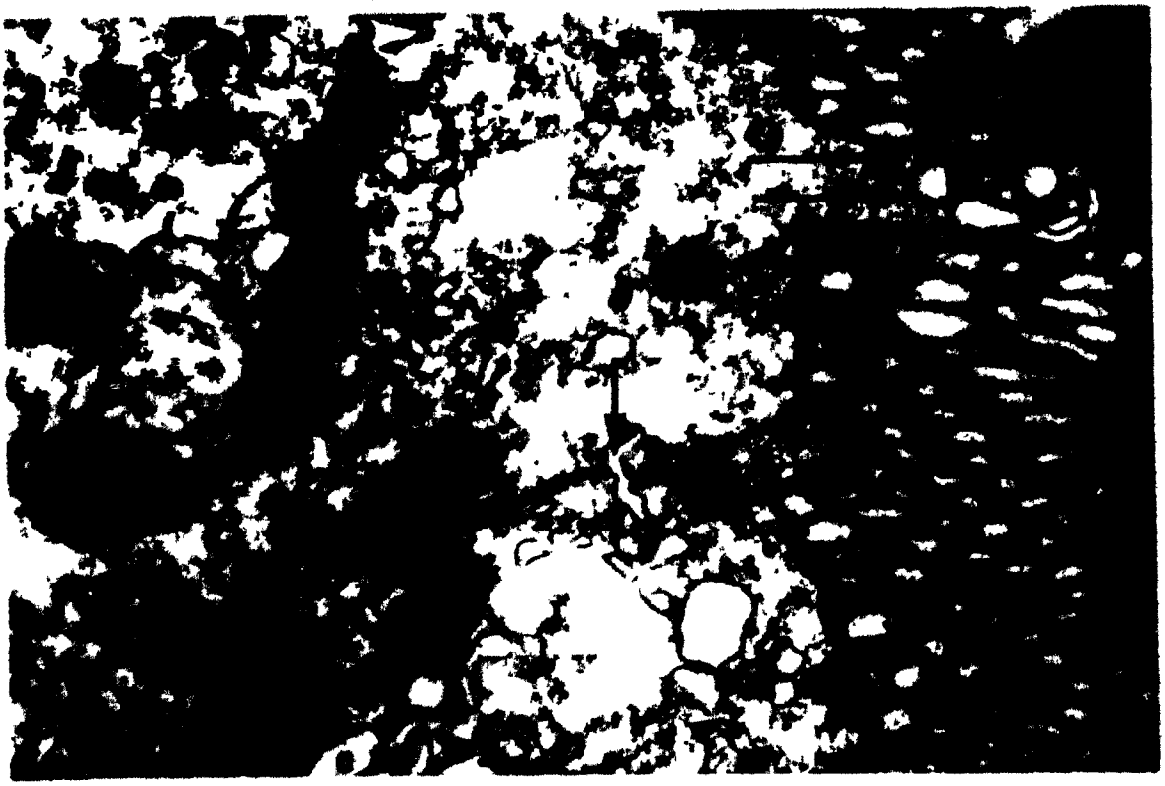


Figure 4.43

Electron micrograph of the mid regions of digestive cells of L. saxatilis parasitized by M. similis sporocysts. Note the relatively empty-looking irregularly shaped vacuoles. Note too, that in some instances these structures contain a granularity of light-medium electron density, similar to that observed in T4 vacuoles. Phagolysosomes can also be observed at this time. At such time too, some mitochondria appear swollen and may also show signs of cristolysis (arrow).

m - mitochondrion
pl - phagolysosome
v - vacuoles similar to the T4 vacuoles

x26,700

Figure 4.44

Electron micrograph of the mid region of a digestive cell of L. saxatilis parasitized by an M. similis sporocyst. Note the irregular folded Golgi body (arrow) and the presence of clear empty-looking vesicles. These contrast sharply with the striated-like Golgi vesicles characteristic of uninfected cells (Chapter II - Figure 2.15).

Gb - Golgi body
Gv - Golgi vesicle

x31,150

Figure 4.45

Electron micrograph of the most basal regions of digestive and secretory cells parasitized by M. similis sporocysts. Note how portions of digestive cells are now enclosed or surrounded by secretory cells. Basal borders are no longer definable for digestive cells due to the extensive rupturing of cellular membranes (arrow). Note the large number of phagolysosomes - a dominant feature of cells displaying pathological State 3.

DC - digestive cell
l - lipid
n - nucleus
pl - phagolysosome

x5,400

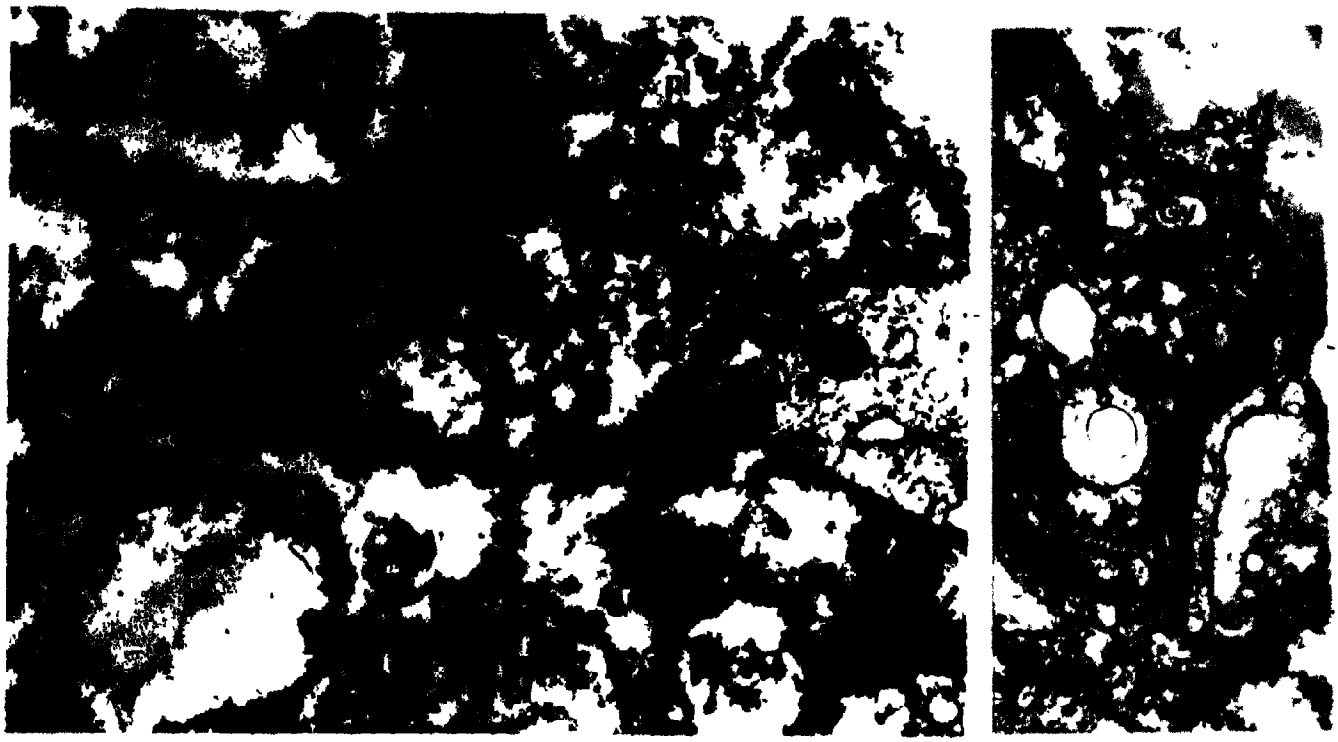


Figure 4.46

Electron micrograph of the apical regions of digestive and secretory cells parasitized by M. similis sporocysts. As in Figure 4.45, portions of digestive cells are surrounded by secretory cells and can no longer be said to possess apical borders. (Arrow points to apical most region of digestive cell). The microvilli are absent and the distal most regions of the digestive cell does not open into a lumen. As in Figure 4.45, phagolysosomes are very common.

DC - digestive cell
l - lipid
m - microvilli
pl - phagolysosome
SC - secretory cell

x5,600

Figure 4.47

Electron micrograph showing part of a syncytium composed of fused portions of fragmented digestive cells which are parasitized by M. similis sporocysts. Note the irregularly shaped nuclei which in general appear very much reduced in size.

n - nucleus

x5,400

T4

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APPENDIX I

APPENDIX I

HISTOLOGICAL AND ULTRASTRUCTURAL METHODS

1. Treatment of tissues to be examined with the light microscope

A. Fixatives

a. Bouin's fluid (Foot, 1933): This fixative was used prior to staining tissues with Mallory's Triple Stain (Humason, 1962), aldehyde-fuchsin (Cameron and Steele, 1959), Heidenhain's iron haematoxylin (Pantin, 1964) and Mercury-bromophenol blue (Bonhag, 1955) for proteins. While cytoplasmic inclusions may become slightly distorted when fixed by this method, it is good for nuclei and in general excellent for the preservation of marine Invertebrata. Materials fixed by this method may be left in Bouin's indefinitely, and it therefore serves as a very suitable fixative for use on collecting expeditions (Pantin, 1964).

b. Baker's Formaldehyde Calcium (Baker, 1944 as cited by Pantin, 1964): This fixative was used for the study of general carbohydrates and glycogen by the PAS technique and for bound lipids according to the acetone-Sudan black method. It serves as a good cytoplasmic fixative which is known to preserve lipids. Formaldehyde acts as an additive non-coagulant fixative which hardens proteins to some extent without separating the water from within them. Formaldehyde also has the property of fixing proteins in such

a way that the glycogen with which it is usually in intimate association, is not easily removed by water (Baker, 1969). $CaCl_2$ was added to the fixative (as recommended by Pearse, 1964) in order to prevent osmotic distortion.

Variations of this method were used in connection with several other techniques. Since the purpose of the calcium salts in Baker's formaldehyde calcium are primarily for the preservation of phospholipids (Pearse, 1968), in order to study neutral fats, only formalin in sea water was required. Moreover, to ensure minimal distortion of tissue for the phosphatase tests, Monosodium phosphate and sodium hydroxide provided a buffer solution (pH 7.3 - 7.6) for tissues fixed in cold formalin sea water (Pearse, 1968).

c. Ethanol (100%): Ethanol acts as a non-additive denaturing coagulant of many proteins. It does not attack the side groups of protein and therefore cannot render the tissues either more or less basic or acidic (Baker, 1969). It is believed, however, that it acts in such a way as to block or mask certain molecules making them more inaccessible to various reagents. While there are clear disadvantages to using alcohol, e.g. only moderate penetration rate, excessive shrinkage or swelling, hardening, etc., it can be considered one of the most effective agents for the study of glycogen (Pearse, 1968). The results of two studies by Trott (1961)

and Kugler (1965) as was quoted by Pearse (1968) on the preservation of glycogen have both confirmed the superiority of absolute alcohol in comparison to other simple fixatives or fixative mixtures.

Materials to be stained by the PAS and Best Carmine methods were fixed in absolute alcohol for about 18 - 20 hours at -73°C in a closed chamber containing evaporating carbon dioxide. The low temperature is significant in reducing the degree of granular polarization characteristic in alcohol-fixed cells (Pearse, 1968).

d. Methanol saturated with Barium hydroxide: In preparation for the detection of glucose by the Okamoto method (Pearse, 1968), small pieces of tissue were fixed in methyl alcohol saturated with BaOH for about 24 hours at 3 - 4°C. The resulting barium glucose molecules which form are insoluble in methanol and become transformed into silver precipitates after exposure to the appropriate silver salts.

e. Acetic-alcohol-formalin (Lillie) Pearse (1968): While opinions vary regarding the choice of fixatives in preparation for the staining of nucleic acids, Lillie"s acetic-alcohol-formalin is a preservative whose advantages outweigh the disadvantages. In contrast to the Carnoy fixative, which Pearse (1968) believes causes some extraction of RNA and DNA from the cell as well as possible hardening of tissues, the acetic-alcohol-formalin fixative

causes less hardening of tissues and fewer cellular losses.

Prior to staining by the pyronin methyl green technique (Jordan and Baker, 1955), tissues were fixed in Lillie's acetic-alcohol-formalin for 24 hours at 4°C (Pearse , 1968).

B. Stains

a. Mallory Triple Stain: Mallory's proved to be the most important general stain in this study because of its capacity to differentiate tissues by their coloring properties. The technique employed was the one proposed by Pantin (1964). By this method, nuclei stained red, connective tissue - light blue, cellular digestive vacuoles - varying shades of blue and RNA - intense red.

b. Aldehyde-Fuchsin: An alternate method for the study of uninfected tissue was the aldehyde-fuchsin staining technique of Cameron and Steele (1959). By this procedure, tissues were stained with Gomori's aldehyde fuchsin and counter-stained in Halmi's trichrome.

While this stain was initially used for the examination of neurosecretory cells, especially in invertebrates, it had also proven most useful in a related study on the digestive gland cells of Littorina littorea (Nerdsoy and Farley, 1972). In the present work, it served as an important general stain for uninfected digestive gland

tissue. Nuclei stained varying shades of yellowish orange and digestive vacuoles, purple.

c. Heidenhain's Iron Haematoxylin (Pantin, 1964): This method was previously applied in a preliminary study on the digestive gland of L. saxatilis (Davis, 1972). In the present work, this technique was not as extensively used as were the above methods, mainly because it gave rise to an irregular granularity in the cells, and also because of its inability to differentiate tissues properly.

d. Mercury-bromophenol blue: This is a method whereby proteins can be demonstrated in prepared section. The stain was introduced by Durrum (1950, as cited by Pearse, 1968) and was originally used for development of protein spots on filter paper. It was adapted as a cytological technique by Mazia et al. (1953) who reported that the sharp and intense staining of proteins permitted good differentiation of structures often difficult to observe by other methods, (e.g. cilia, spindle elements, chromosomes).

While the dye used in this technique is regarded by some as an unreliable reagent for the histochemical recognition of proteins, Mazia et al. (1953) and Baker (1958) considered it to be a strong acidic dye capable of combining with the basic groups by coupling with mercury.

In the present work, the staining procedure used was the one recommended by Pearse (1968) after Bonhag (1955). The solution is composed of 1 percent mercuric chloride and 0.05 percent bromophenol blue in 2 percent acetic acid. Proteins stain deep blue and certain tissues which display a red color are believed to represent the sites for RNA.

e. Period Acid Schiff (McManus): In order to determine the distribution of carbohydrates in the digestive gland cells, the Periodic Acid-Schiff technique was employed. The procedure for the use of this technique is reported by Pearse (1968). The results obtained with the PAS method are based on two reactions:

1. The oxidation of glycols or glycol-like groups into dialdehydes.
2. The combination of the dialdehyde with Schiff's reagent to form a reddish-purple product.

Pearse (1968) indicates that the PAS-positive substances are polysaccharides (glycoproteins, unsaturated lipids and phospholipids. Glycogen alone is affected by treatment with malt diastase or saliva prior to staining, and therefore, the latter substances were used for control purposes.

f. Best's Carmine (Pearse, 1968): Besides the PAS technique, the Best's Carmine method in conjunction with

the appropriate controls was used for the detection of glycogen. While the latter technique is basically empirical, it has proven to be highly specific for glycogen. According to Baker (1945) as cited by Pearse (1968), the active agent of the natural dye carmine is carminic acid which, on the alkaline side of its isoelectric point (4.0 - 4.5) is negatively charged, thus behaving like an acid dye. The presence of ammonia in the staining solution helps maintain the pH at a constant alkaline level.

g. The Okamoto method for glucose: For a further elaboration of the nature of carbohydrates in the digestive gland cells, the Okamoto method for glucose was attempted. As indicated in Part A (fixatives - methanol sat. with BaOH) the technique depends on the resulting barium glucose molecules which form insoluble precipitates after retention in an alcoholic silver nitrate solution for 30 minutes.

h. Oil Red O Method for neutral fats: This method was used in the study of neutral fats. Pearse (1968) indicates that the method has several distinct advantages over the more common dyes such as Sudan III and Sudan IV:

1. Deeper color and thus discerning of smaller droplets is possible.
2. Less tendency to form dye precipitates.

i. Acetone-Sudan Black Method for bound lipids: This technique was carried out in order to identify bound lipid content. Tissues were stained in 2 percent Sudan Black in acetone for 3 hours. (Pearse, 1968)

j. Pyronin/Methyl Green Technique: The RNA/DNA composition of the digestive gland cells was studied by the pyronin-methyl green technique. The staining solution was composed of 5 percent pyronin, 5 percent methyl green and acetate buffer adjusted to pH4.8, as proposed by Jordan and Baker (1955). Because materials other than RNA stain with the basic dye, pyronin G, a control was necessary to show that the substance staining with this dye was in fact RNA. Control sections were incubated for 5 hours in a 1 mg/ml solution of ribonuclease in distilled water at 37°C.

k. Phosphatases: The techniques employed for the detection of phosphotases were the calcium cobalt method for Alkaline phosphatase (Gomori, 1946) and the lead nitrate procedure for Acid Phosphotase (Gomori, 1950) as described by Pearse (1968). The tests are concerned with the phosphomonoesterases and are based on the following reaction:

$$R - O - \underset{\underset{OH}{|}}{\overset{O}{P}} - O + ROH = ROH + H - O - \underset{\underset{OH}{|}}{\overset{O}{P}} - O - R^1$$

(R is the alcohol radical)

The phosphomonoesterases are not specific in relation to the alcohol radical which is attached to the phosphoric acid group of the substrate and hence will hydrolyze a wide variety of organic phosphates.

The calcium-cobalt method for alkaline phosphatase was employed on tissues fixed in Phosphate-buffered cold formalin sea water and embedded in Cryoform. The sections were then incubated for 2 hours at 37°C in a substrate consisting of 3 percent sodium B glycerophosphate (10 ml), 2 percent sodium diethyl barbiturate (10 ml), distilled water (5 ml), 2 percent calcium chloride (20 ml), and 5 percent magnesium sulphate (1 ml) at pH 9. After incubation, the sections were rinsed in distilled water, treated for 5 minutes in 2 percent cobalt nitrate and rinsed again. Alternate slides were treated with dilute ammonium sulphide (1 - 2 min.) and the sections from these, unlike those of the untreated slides demonstrated the presence of brown-black precipitates, which were taken to be the sites of alkaline phosphatase activity.

In order to study the acid phosphatase activity, tissues were initially treated in a similar manner to above. The sections were then incubated for 2 hours at 37°C in a substrate containing 0.01 M sodium B - glycerophosphate in 0.05 M acetate buffer at pH 5, containing 0.0004 M lead nitrate. Upon completion, the sections were washed briefly

in distilled water and alternate slides were immersed for two minutes in dilute yellow ammonium sulphide. The presence of brownish-black precipitate of lead sulphide in these sections, unlike for the untreated ones, confirmed the sites of acid phosphatase activity.

2. Treatment of tissues to be examined with the electron microscope

A. Fixatives

Two types of fixatives are frequently used for the preparation of tissues to be examined with the electron microscope; these are aldehyde and osmium tetroxide.

Proteins, as wll as certain macromolecular carbohydrates, preserve well with an aldehyde fixative. However, all cellular organelles including membranes and nuclei are not well defined unless material is postfixed in osmium tetroxide (Sabatini et al., 1962; Sabatini, 1964). Furthermore, postfixation in osmium has the advantage of preventing the extraction of lipids which could ordinarily occur during the embedding process. The application of both solutions together may, however, have to be avoided if specific histochemical tests are desired since the two fixatives can contradict each other (Pease, 1964).

a. Glutaraldehyde: Sabatini and his associates examined a variety of aldehydes which could possibly be used as suitable fixatives. In the present investigation, glutaraldehyde, as proposed by Sabatini et al. (1963), with only slight modifications as recommended by Pease (1964) was used. Tissues were fixed in a 4 percent phosphate-buffered glutaraldehyde solution at 40°C for one hour at a pH of 7.3.

b. Osmium tetroxide: Even though osmium tetroxide is a widely used fixative for electron microscopy work, its exact mode of action in relation to the various cellular components is not clearly understood. It is, nevertheless, widely accepted that while saturated fats do not react with OsO_4, unsaturated fats reduce it and ultimately form black compounds containing osmium or its hydroxide (Pearse, 1968). While confusion prevails regarding the interaction of OsO_4 and proteins (Adams et al., 1967) several possibilities exist. For example, to help explain the staining of the postulated protein layer of membranous structures, it has been suggested that the negatively charged OsO_2 is able to replace the negatively charged protein groups which bind lipid to protein. The product then allows for the deposition of appropriate staining compounds which appear black in the final micrograph.

Palade (1952) recommended the use of a strong buffering solution in conjunction with the osmium tetroxide stain. Millonig (1962) demonstrated that a phosphate buffer is superior to the Veronal buffer originally proposed by Palade and depends upon the balance between the monosodium phosphate and disodium phosphate, after sodium hydroxide is introduced.

All tissues in the present work were prepared for 2 hours in Millonig's osmium tetroxide fixative adjusted to

pH 7.3. In spite of the fact that Millonig made no mention of the temperature at which he carried out his fixation, in order to ensure maximum stability of all chemicals used, the latter fixative was employed at 4°C as was the glutaraldehyde.

B. Stains

The staining of tissues to be examined under the electron microscope involves the addition of heavy atoms. Such atoms scatter the electrons and this provides the contrast for the final picture.

Watson (1958a, 1958b), in a comparative study of different heavy atom compounds, developed an alkaline lead hydroxide stain which became the basis for many of the present day staining techniques. Such a compound has the ability to enhance the contrast of sections with minimum distortion. At the same time, Watson demonstrated the effective use of uranyl acetate, containing uranium salt, as an important electron stain. Both types of stains can be used together to produce superior results.

a. Alkaline lead hydroxide stain: Reynolds (1963) discussed the general mechanisms of staining with alkaline lead salts. The divalent lead salts in alkaline solution can form compounds which give rise to certain polymeric cations.

$$Pb(OH)_2\ PbX_2 = \left[Pb(OH)_2Pb\right]^{++} + 2X^-$$

This reaction occurs at pH 12, and the polymeric cations which are formed are believed to be necessary staining agents. The technique followed in this investigation is that of Reynold (1963), and it employs citrate as a chelating agent, in this way removing the presence of lead and thus preventing its combination with carbonate to form the undesirable precipitate.

b. Uranyl acetate stain: In its use as a general stain, uranyl acetate is inferior to the lead hydroxide stain. Watson (1958) has demonstrated that while proteins stain intensely, cytomembranes do not pick up the stain as well.

In the present investigation, 5 percent uranyl acetate in 50:50 methanol:70 percent ethanol was employed. This technique was a modification of the staining method recommended by Stempak and Ward (1964). Staining, as mentioned, can be intensified by treating tissue with uranyl acetate and then with lead hydroxide. The incorporation of an organic solvent in the uranyl stain has the added advantage of serving as a "wetting agent", in this way aiding the penetration of the stains (Pease, 1964).

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