A STUDY OF THE CYTOPLASMIC INCLUSIONS OF VARIOUS CELLS IN THE ALIMENTARY TRACT OF MOLLUSCS, WITH SPECIAL REFERENCE TO CHANGES DURING SECRETORY AND DIGESTIVE PROCESSES

by

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ABSTRACT

Cytological, histochemical and electron microscopical studies were made on the digestive gland cells of certain gastropods.

The digestive gland tubule epithelium of the pulmonate Helix aspersa is made up of four types of cells: thin, digestive, calcium and excretory cells. Thin cells are narrow and undifferentiated. They divide by mitosis and are believed to develop into the other types of cells. Digestive cells are highly vacuolated. The most apical, and smallest, vacuoles have no contents which are visible with the light microscope; those further down the cell contain colourless granules. The nucleus is basal, and immediately apical to it are a number of small yellow granules of lipofuscin. Most of the cytoplasm of calcium cells is occupied by spherules which contain calcium; these spherules can be discharged from the cells. The cytoplasm contains much RNA, and there is a conspicuous Golgi apparatus; apically there are protein granules which contain much tryptophan. Excretory cells consist chiefly of a large vacuole, surrounded by a little cytoplasm. The vacuole contains one or more granules of lipofuscin; these, and the lipofuscin granules of digestive cells, are cast out of the cells and appear in the faeces; they are thus excretory. Excretory cells are degenerate, and probably derived from calcium cells.

Three other species of gastropods were studied for comparison with Helix. These were Pila ovata, a tropical prosobranch; Succinea putris, a primitive stylommatophoran pulmonate; and Testacella maugei, a carnivorous stylommatophoran pulmonate. In spite of the differences in diet and taxonomic position, the tubule epithelia of the digestive glands of these three species show a fundamental similarity to that of Helix.

Pila has protein granules in its digestive cells, as well as the inclusions normally present in such cells. Calcium is absent from the cells which correspond to the calcium cells of Helix. The excretory cells contain large brown granules which consist partly of lipofuscin, and partly of an iron-containing pigment.

In the digestive cells of Succinea there is a gradation between the colourless and the yellow granules; all these granules lie in vacuoles. Many of the spherules in the calcium cells lack calcium.

Testacella has no yellow granules in its digestive cells; these cells have large quantities of lipid in the basal half of the cell. The protein granules of the calcium cells are more numerous than in the other forms studied. Testacella shows no differences from Helix which can be attributed to a carnivorous diet.

When examined in the electron microscope Helix, Succinea and Testacella show similar fine structure in

their digestive gland cells. The structure is similar whether the fixative is osmium tetroxide or formaldehyde-sucrose. There is a good correlation between the results of light and electron microscopical studies.

The cytoplasm of thin cells contains ergastoplasm, and there is a typical lamellar-vacuolar field apical to the nucleus. The cytoplasm of digestive cells consists of a finely granular ground substance in which lie numerous small vesicles, particularly apically. These vesicles may be pinocytotic, and pinocytosis channels are sometimes visible apically. Further down the cell there are the large vacuoles visible with the light microscope. The lipofuscin granules in the more basal vacuoles have the same fine structure as mammalian lipofuscin granules. Digestive, calcium and excretory cells bear microvilli on their free surfaces.

The calcium cells contain much ergastoplasm and several lamellar-vacuolar fields, in between the calcium spherules. In Testacella secretory granules are visible apically in the cell.

Electron microscopy shows that there is an intermediate stage between calcium and excretory cells. The fine structure of these intermediate or early excretory cells and of the excretory cells shows clearly that they are degenerating calcium cells.

The digestive gland cells of the gastropods studied

are connected together by septate desmosomes.

The results of these morphological studies show that digestive cells have the characteristics of phagocytic cells, and calcium cells have the characteristics of protein secretory cells.

Experiments were carried out to study the sites of phagocytosis and lipid absorption, and to follow the fates of ingested materials, in the alimentary system of Helix. Carmine particles and lipids are taken up by the digestive cells of the digestive gland. These substances are taken up by phagocytosis, and the apical vacuoles and the vacuoles containing colourless granules are phagocytosis vacuoles. Carmine eventually becomes incorporated into the small yellow granules of digestive cells, showing that they are derived from the colourless granules. Carmine and lipids are not taken up by any other cells of the digestive gland.

Lipids are also taken up by the epithelia of the digestive gland ducts, intestine and rectum. Phagocytosis does not occur in these sites.

Since the calcium cells of Helix have the morphological characters of protein secretory cells, experiments were carried out to determine whether the granules of these cells could be discharged into the lumen of the gland. This was done by studying the effects of the drugs pilocarpine, atropine, adrenaline and 5-hydroxy-tryptamine on the protein granules of calcium cells. The

effects of these drugs on the mucous cells of the alimentary system and on the intestinal secretory cells (see page vii) were also studied.

Pilocarpine causes discharge of mucus throughout the alimentary system, discharge of protein granules from calcium cells, and slight discharge of the granules of the intestinal secretory cells. Its effects are inhibited by atropine. Adrenaline produces similar effects to pilocarpine, but its action on the intestinal secretory granules is stronger. 5-hydroxy-tryptamine causes discharge of mucus and of protein granules, but not of intestinal secretory granules. It was concluded that the protein granules of calcium cells, and the granules of intestinal secretory cells (as well as mucus) are secretory products which are discharged into the lumen of the alimentary system.

For comparison with gastropods, cytological, histochemical and electron microscopical studies were made on the digestive gland cells of certain lamellibranchs. The species studied by light microscopy were Anodonta anatina, Sphaerium corneum and Unio tumidus. The digestive gland tubule epithelium of these species consists mainly of two types of cells: digestive and basiphil cells. Digestive cells are highly vacuolated, and closely resemble the digestive cells of gastropods. Basiphil cells have a high concentration of RNA in the cytoplasm, and a conspicuous Golgi apparatus. Apically they have small granules of

protein. These cells are believed to be secretory, and the apical granules to be the secretory product. Basiphil cells are the cells considered by previous workers to be undifferentiated. Sphaerium, and possibly the other forms studied, have groups of cells bearing very long flagella, among the groups of basiphil cells at the blind end of the tubules.

Electron microscopical observations on the digestive gland cells of Anodonta confirm the results of light microscopy. The fine structure of digestive cells resembles very closely that of the digestive cells of pulmonates. The basiphil cells contain much ergastoplasm and have numerous lamellar-vacuolar fields. There are membrane-bounded bodies in the apical cytoplasm which represent the protein granules seen with the light microscope. The mitochondria in the digestive gland cells of Anodonta usually have few cristae and a matrix of low electron density. Towards the apical surface of the epithelium, the cells are joined together by septate desmosomes; basally there are considerable intercellular spaces into which amoebocytes can penetrate.

Since the functions of the digestive glands of molluscs include absorption and intracellular digestion of food, and formation of secretory products, histochemical tests were carried out on certain gastropods and lamellibranchs to localize certain enzymes involved in these processes. The enzymes studied were alkaline phosphatase,

acid phosphatase, and thiamine pyrophosphatase.

Alkaline phosphatase was found to occur in the brush border and basement membrane of digestive gland cells. Acid phosphatase was found to occur mainly in association with the lipofuscin granules of the digestive cells, and occasionally in other sites; it has not been found with certainty associated with intracellular digestion. Thiamine pyrophosphatase has been found in the Golgi apparatus and plasma membranes of digestive gland cells, but the largest amounts were found in or near the phagocytic vacuoles of digestive cells in the carnivorous slug Testacella.

The intestinal epithelium of pulmonate gastropods is usually stated to consist of ciliated and mucous cells. A third type of cell, the intestinal secretory cell, has been found in the final segment of the intestine, immediately preceding the rectum. In this study, such cells have been found in many species of pulmonates. In the part of the intestinal epithelium where they occur, intestinal secretory cells are as numerous as ciliated cells, and mucous cells are scarce.

The intestinal secretory cells have much RNA in the cytoplasm, a conspicuous Golgi apparatus, and numerous secretory granules. The secretory granules consist largely of protein, with possibly a little phospholipid.

Alkaline phosphatase occurs in the free border of all the intestinal epithelial cells, and in the pigmented

granules which are found in ciliated cells. Acid phosphatase occurs in the pigmented granules of the ciliated cells, and in the immature secretory granules of the intestinal secretory cells.

CONTENTS

Introduction.

Papers:

1. The cytology and histochemistry of the digestive gland cells of *Helix*.
   (Published in Quarterly Journal of Microscopical Science, vol. 106, pp. 173-192, 1965).
2. The cytology and histochemistry of the digestive gland cells of *Pila*, *Succinea* and *Testacella*.
3. The fine structure of digestive gland cells of *Helix*, *Succinea* and *Testacella*.
   (Accepted for publication in the Journal of the Royal Microscopical Society).
4. Experiments on phagocytosis and lipid absorption in the alimentary system of *Helix*.
   (Accepted for publication in the Journal of the Royal Microscopical Society).
5. The effect of drugs on secretion in the alimentary system of *Helix*.
6. The cytology and histochemistry of the digestive gland cells of some freshwater lamellibranchs.
7. The fine structure of the digestive gland cells of *Anodonta*.
8. The distribution of phosphatases in the digestive gland cells of certain molluscs.
9. Intestinal secretory cells in pulmonate molluscs.

INTRODUCTION

Although there have been numerous studies on the cells which make up the alimentary system of molluscs, numerous features of this system are not yet fully understood. It was therefore felt that the newer techniques of histochemistry and electron microscopy, when applied to these cells, might lead to a better understanding of their functions. The digestive gland (also called the digestive diverticula or hepatopancreas) is probably the most complex and least understood part of the alimentary system of molluscs, and is thus a particularly suitable organ to which to apply these techniques.

The digestive gland of gastropods, especially pulmonates, has been studied by many workers (among them Barfurth, 1883; Frenzel, 1885; Krijgsman, 1925, 1928; Graham, 1932; Thiele, 1953; McGee-Russell, 1955; Owen, 1958; Martoja, 1964). It has been established that up to four types of cells are present in the gastropod digestive gland, although some workers have described only two. The cell types are known here as thin, digestive, calcium and excretory cells (for details of the synonymy of these cells, see table 1 in Paper 1). Different functions have been assigned to these cells by different workers, and there has been considerable disagreement about the number of cell types. As a first step towards elucidating these problems, a detailed light microscopical study, including

histochemistry, was made on the Stylommatophoran Pulmonate Helix aspersa (Paper 1). This study confirmed the presence of the four cell types mentioned above. Thin cells appear to be undifferentiated, a view shared by the previous workers who have found these cells (MacMunn, 1900; Thiele, 1953; Nakazima, 1956). Digestive cells, which have frequently been thought to be secretory (Barfurth, 1883; Krijgsman, 1925, 1928; van Weel, 1950), are found not to have the typical features of protein secretory cells, that is, a high content of RNA, a conspicuous Golgi apparatus, and granules of protein. The calcium cells, on the other hand, do possess these features, as well as having stores of calcium compounds. The evidence obtained in this study suggested that excretory cells are derived from calcium cells, a view originally put forward by Thiele (1953).

There is no reason to suppose that Helix is a typical gastropod, and a comparative light microscopical study was therefore made on the digestive glands of three other members of the group: Pila ovata, a tropical prosobranch; Succinea putris, a primitive stylommatophoran pulmonate; and Testacella maugei, a carnivorous pulmonate. This study (Paper 2) showed that in spite of differences in diet and taxonomic position, there is a fundamental similarity between the digestive gland cells of all the forms studied.

Electron microscopical studies on the pulmonates

Helix, Succinea and Testacella (Paper 3) confirm and extend the findings of light microscopical studies. The only previous work of this kind, that of David & Götze (1963), was found to be inadequate in some respects. The digestive cells have a fine structure similar to that of phagocytic cells in other groups of animals (Gauthier, 1963; Afzelius & Rosen, 1965). Electron microscopy also shows that there is an intermediate stage between calcium and excretory cells.

The morphological studies (Papers 1, 2 & 3) showed that the structure of digestive cells is consistent with a phagocytic function, and that of calcium cells is consistent with a secretory function. It has long been thought that the digestive gland of gastropods has a phagocytic and absorptive function (Cuénot, 1892, 1899; Jordan, 1918; Peczenik, 1925; Rosen, 1941, 1952; Carriker, 1946), but some workers have failed to find any evidence for it (Krijgsman, 1928; Horstadius, 1933; Horstadius & Horstadius, 1940). Two points in particular needed further study. One was the relationship of the granules and vacuoles of digestive cells to phagocytic processes, and the other was the sites of lipid absorption. Guardabassi & Ferreri (1953) found that lipid absorption took place in the intestine of Helix, but did not find that it occurred in the digestive gland. However, Millott (1937) had found that lipid absorption took place in the digestive gland of the nudibranch

Jorunna. The results of the studies made on phagocytosis and lipid absorption in Helix are given in Paper 4; it was found that both these processes occurred in the digestive cells, but not elsewhere in the digestive gland.

To find out whether the calcium cells were secretory, and discharged their products into the lumen of the digestive gland, it was decided to attempt to stimulate these processes by injections of drugs. Although the effects of drugs on secretion in vertebrates are well known, very few such studies have been made on invertebrates. Lebedeff (1899) and Oka (1930) showed that pilocarpine would cause discharge of salivary gland secretions in insects, and Monti (1899) and Pacaut & Vigier (1906) found that pilocarpine had a stimulatory effect on the salivary glands of Helix. Yonge (1926b) showed that pilocarpine caused an increase in the number of colourless granules in epithelial cells of the style sac in Pecten. Because of the small amount of work done on this subject, a study was undertaken of the effect of a number of drugs on various secretory cells of the alimentary system of Helix aspersa (Paper 5). The secretory cells studied were the calcium cells of the digestive gland, the mucous cells of the crop, stomach and intestine, and the intestinal secretory cells (Paper 9). It was found possible to stimulate the discharge of secretory products from all these cells.

The digestive gland of lamellibranchs is widely

believed to consist of only one type of cell. The crypts of the digestive gland tubules contain darkly staining cells, which are believed to be undifferentiated; these cells are presumed to develop into mature digestive cells (Gutheil, 1912; Yonge, 1926a & b; Mansour, 1946b; Morton, 1956). If this were so, the digestive glands of lamellibranchs would differ fundamentally from those of most other molluscs, which have a type of cell corresponding to calcium cells (Cuénot, 1907; Graham, 1932, 1938; Fretter, 1937, 1939; Gabe & Prenant, 1949; Pugh, 1963; Martoja, 1964). The digestive cells of lamellibranchs have been shown clearly to be phagocytic (Yonge, 1926a & b; Owen, 1955, 1959; Morton, 1956), but there is nevertheless some evidence that the lamellibranch digestive gland is also secretory (Gutheil, 1912; Mansour, 1946a & b; Mansour-Bek, 1946; Ballantine & Morton, 1956). The techniques of histochemistry and electron microscopy were therefore applied to the digestive gland cells of some freshwater lamellibranchs, to find out to what extent they resembled gastropods, and in particular to determine whether secretory cells were in fact present (Papers 6 & 7). Both these papers show that while the so-called mature digestive cells resemble the digestive cells of gastropods histochemically and in their fine structure, the cells which have been thought to be immature digestive cells have all the features of protein secretory cells. The latter are called here basiphil cells.

The distribution of enzymes in a digestive organ is clearly of great importance. Since absorption, phagocytosis and intracellular digestion (Rosen, 1930, 1934) occur in the digestive cells, and the calcium and basiphil cells secrete granules of protein, it was desirable to study those enzymes involved in these processes. Alkaline phosphatase is typically associated with transport of materials across cell membranes, and is frequently found in the brush borders of absorptive cells (Danielli, 1952). Acid phosphatase occurs in lysosomes (de Duve, 1963), and has been used as a 'marker' for these bodies (Goldfischer et al., 1964). It has been shown that lysosomes are involved in intracellular digestion (Gordon et al., 1963, 1965). Thiamine pyrophosphatase (TPPase) has been used as a 'marker' for the Golgi apparatus (Novikoff & Goldfischer, 1961) and is thus of interest because of the association of this organelle with secretory processes. The results of the studies made on the distribution of acid and alkaline phosphatases and of TPPase in the digestive glands of various molluscs are described in Paper 8. Although alkaline phosphatase is found in the brush borders of digestive gland cells, the other enzymes do not always occur in the expected sites. Acid phosphatase has not been found with certainty associated with intracellular digestion. TPPase has been found in the Golgi apparatus of some cells, but was found in the largest amounts elsewhere in the

digestive cells of Testacella. This tends to confirm the view that TPPase is not a reliable marker for the Golgi apparatus in invertebrates (Lane, 1963; Lee, 1963).

The intestinal epithelium of pulmonates is generally described as consisting only of ciliated and mucous cells (Argaud & Bounoure, 1910; Spiro, 1911; Morton, 1955). However, in the course of the studies on the digestive glands of pulmonates, it was noticed that part of the intestinal epithelium contained a third type of cell, which possessed numerous granules of protein. These are the intestinal secretory cells, which are described in Paper 9; their protein granules are discharged into the lumen of the intestine under the influence of drugs (Paper 6), but their function has not been elucidated. Although intestinal secretory cells do not seem to have been described before in pulmonates, similar cells occur in certain other gastropods (Howells, 1942; Gabe, 1952) and probably in the Polyplacophora (Fretter, 1937).

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PAPER 1

THE CYTOLOGY AND HISTOCHEMISTRY OF THE DIGESTIVE GLAND CELLS OF *HELIX*

(Published in Quarterly Journal of Microscopical Science, vol. 106, pp. 173-192, 1965).

The cytology and histochemistry of the digestive gland cells of *Helix*

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Summary

The digestive gland tubule epithelium of *Helix aspersa* is made up of 4 cell-types: thin cells, digestive cells, calcium cells, and excretory cells. Thin cells are narrow and undifferentiated. They divide by mitosis and are believed to develop into other cell-types. Digestive cells are highly vacuolated phagocytic and absorptive cells. Food materials are taken in by phagocytosis and are concentrated and digested in the vacuoles in the cell. When digestion is complete, the residual indigestible material in the vacuoles, and excretory material in the form of small granules of lipofuscin, are cast out of the cell surrounded by a portion of cytoplasm. Calcium cells are secretory, with a prominent Golgi apparatus and a high concentration of RNA in the cytoplasm. Most of the cell is occupied by spherules which contain calcium; apically there are protein granules which contain a high concentration of tryptophane. Both these types of inclusion are extruded from the cell. Protein granules may be zymogen granules, but the function of the calcium spherules is not known. Excretory cells are degenerate, and probably derived from calcium cells. They consist chiefly of a large vacuole, surrounded by a little cytoplasm. The vacuole contains one or more granules of lipofuscin. Similar granules can be found in the faeces, and thus they are excretory material.

Introduction

THE digestive gland of the snail *Helix aspersa* consists of much-branched blindly ending tubules (Barfurth 1883; Billett and McGee-Russell, 1955; Krijgsman, 1925), bound together by connective tissue containing blood lacunae (Billett and McGee-Russell 1955). This paper is concerned only with the cells of the epithelium lining the tubules. These cells have frequently been studied in the past (e.g. Barfurth, 1883; Frenzel, 1885; Krijgsman, 1925, 1928; McGee-Russell, 1955; Thiele, 1953), but the descriptions are inadequate, the relationships and functions of the cells are not certainly known, and no histochemical studies have been done on them.

The present paper aims to give a more complete account of the cytology of the cells of the digestive gland tubule epithelium, and describes the histochemistry of these cells.

Material and methods

The digestive gland of the snail *H. aspersa* was used throughout the present study. Before fixation, the snails were either allowed to feed at will, or starved for various periods up to 132 days. Snails starved for 20 days or less are called short-starved; those starved for longer periods are called long-starved. Hibernating snails were also studied.

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For routine cytological study, small portions of digestive gland were fixed in Zenker or Champy, and embedded in paraffin. Bouin, Helly, formalin-alcohol-chloroform (Krijgsman, 1928), Altmann, Regaud, formalin-saline, Lewitsky-saline, Mann, and NH_4-Altmann (Baker, 1960) were also used, but were not found to be better than Zenker or Champy. Sections were cut at 6 to 8 μ, and stained with Heidenhain's iron haematoxylin, or with Ehrlich's haematoxylin counterstained with either eosin or light green.

Mitochondria were stained by Metzner's method (Meves, 1911), generally after fixation with Champy's fluid and sectioning at 4 μ. Altmann, NH_4-Altmann, Regaud, Mann, and Helly (post-chromed for 48 h) were also tried as mitochrondrial fixatives.

The Mann-Kopsch method (Baker, 1933) was used to show the Golgi apparatus. The methods of Kolatchev and of Aoyama were also tried unsuccessfully.

For the study of cell division, staining with crystal violet and iodine (Baker and Jordan 1953) was used, on tissue fixed in Sanfelice's fluid.

The following histochemical methods were used:

For DNA: Feulgen's method (Feulgen and Rossenbeck, 1924), on Zenker-fixed tissue.

For RNA: the P/MG test (Jordan and Baker, 1955) with salivary ribonuclease as a control (Bradbury, 1956).

For carbohydrates: the PA/S test (McManus, 1948), with diastase digestion as a control for the presence of glycogen (Lillie and Greco, 1947), on Zenker-fixed tissue.

For acid muco-substances: metachromasy with toluidine blue, on Zenker-fixed tissue.

For neutral muco-substances: metachromasy with toluidine blue after sulphation with concentrated sulphuric acid for one minute (Lison, 1953), on Zenker-fixed tissue.

For arginine: Sakaguchi's test (Baker, 1947), on Zenker-fixed tissue.

For tryptophane: DMAB/nitrite test (Pearse, 1960), on tissue fixed in formalin-saline.

For lipids: Sudan black, by the method of Baker (1956).

For phospholipids: the acid haematein test, with pyridine extraction as a control (Baker, 1946).

For calcium: the cobalt substitution method; and the calcium red method (McGee-Russell, 1955). Tissue was fixed in formalin-ethanol (1:1) (McGee-Russell, 1955), or formalin-methanol-pyridine (Baker, personal communication).

The following tests for pigments (Pearse, 1960) were used, with tissue fixed in formalin-saline:

Schmorl's test (for lipofuscin, melanin, and reducing substances generally).

Chrome-alum haematoxylin (for lipofuscin).

Long Ziehl-Neelsen, with 24 h differentiation (for lipofuscin).

Bleaching with 10% hydrogen peroxide for 48 h (for distinguishing between melanin and lipofuscin).

The methods of Lillie (1956) and of Pearse (1954) for distinguishing between melanin and lipofuscin, with Nile blue, were tried, but did not give satisfactory results.

Results

Four cell-types can be recognized in the digestive gland tubule epithelium: thin cells, digestive cells, calcium cells, and excretory cells. Table 1 gives a list of synonyms found in the literature (see Appendix, p. 189).

The short-starved state. The results of histochemical tests are summarized in table 2, and will only be referred to here when they are of particular interest (see Appendix, p. 190).

Thin cells (fig. 1) are narrow from side to side (2μ or less in width) and extend to the full height of the epithelium. The nucleus, averaging 10 by 1·5 μ is situated in the basal half of the cell. One or two nucleoli, less than 1 μ in diameter, can usually be seen in it. The cytoplasm is homogeneous and does not contain any special inclusions. Mitochondria are small, averaging 0·7 by 0·5 μ in Champy-fixed tissue, and are sparsely distributed along the cell. The Golgi apparatus consists of an irregular body, 1 to 1·5 μ across, which appears as a ring in optical section. It is immediately apical to the nucleus.

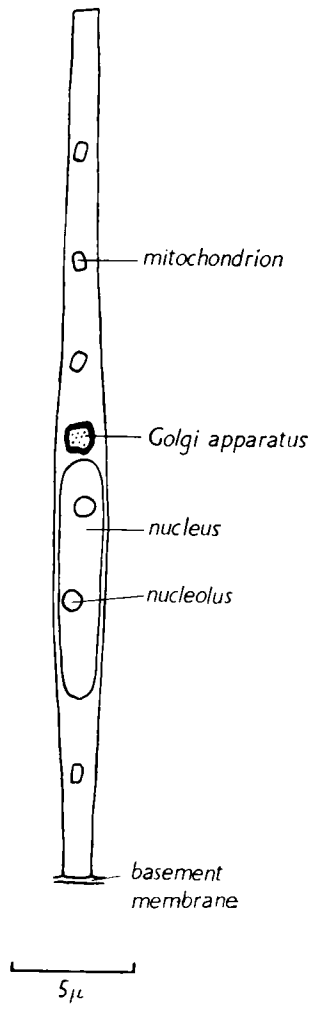


FIG. 1. Diagram of a thin cell.

Thin cells are distributed, apparently at random, between the other cells of the epithelium.

Digestive cells (fig. 2) are the most numerous type in the digestive gland epithelium, and are characterized by their highly vacuolated cytoplasm. The nucleus is basal and usually rounded, averaging 8 by 6 μ. There are usually two nucleoli, which average about 1·5 μ in diameter, but up to 5 have been seen.

In the cytoplasm just apical to the nucleus there is usually a variable number of small yellow granules, about 2 μ across, often rather irregular in shape, and sometimes appearing as clumps of smaller granules. These small yellow granules normally appear to lie directly in the cytoplasm. The histochemical tests show that their pigment is mainly, if not entirely, lipofuscin.

Most of the rest of the cytoplasm contains vacuoles, 5 to 6 μ in diameter, which contain the so-called 'green' granules (Krijgsman, 1928) (which stain readily with light green). The green granules average 3·5 by 3 μ and are usually rather irregularly shaped, often with a diffuse outline. The cytoplasm at the apex of the cell contains smaller apical vacuoles, mostly 1·5 μ or less

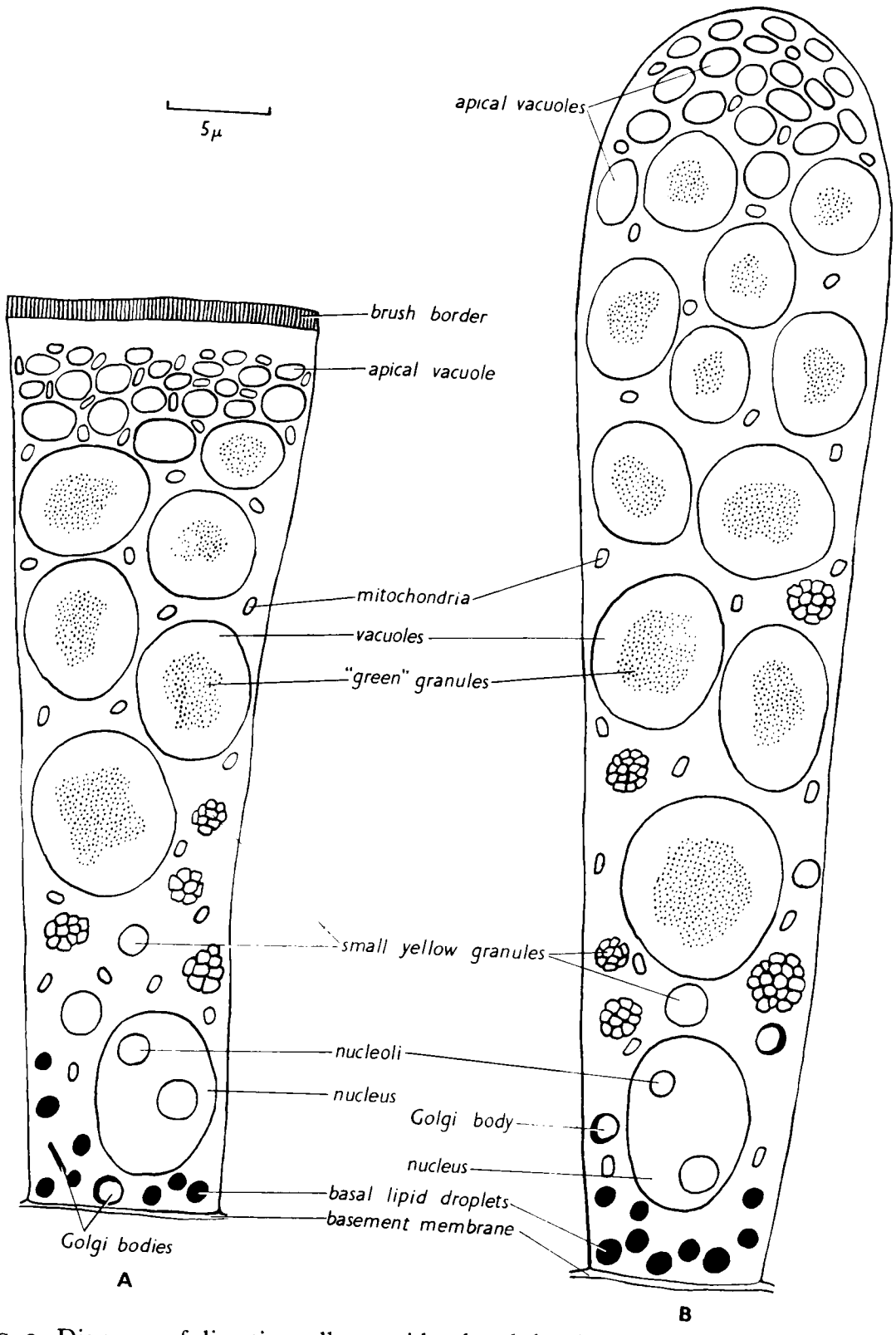


FIG. 2. Diagrams of digestive cells; A, with a brush border; B, without a brush border.

in diameter, although those next to the green granule vacuoles may be 3 by 2 μ or slightly larger. These vacuoles have no visible contents.

The free surface of a digestive cell is either straight and level with the apices of the other cells of the epithelium, in which case it bears a brush border; or it is hemispherical, protrudes into the lumen of the tubule, and has

no brush border. When a brush border is present, there is a very thin layer of non-vacuolated cytoplasm immediately below it. Cells with a brush border average 44 μ in height, and those without a brush border 61 μ in height. The average maximum width of digestive cells is about 15 μ.

Mitochondria occur throughout the cytoplasm of digestive cells between the other inclusions. They are evenly distributed except when a brush border is present, when they are concentrated apically. They average 0·8 by 0·6 μ in Champy-fixed tissue. The Golgi apparatus consists of two types of bodies, occurring basally in the cell in very small numbers. Most of the Golgi bodies are ring-shaped in optical section, and 1 to 1.5 μ in diameter. Other Golgi bodies appear as rods, about 1.5 μ long and 0.5 μ wide. Lipid droplets are found throughout the cytoplasm of digestive cells in very variable numbers. They are usually 0.5 to 1.5 μ in diameter, or up to 3 μ basally, where they are often concentrated.

The apical parts of digestive cells with no brush border can often be seen apparently constricting off and being released into the lumen of the tubule. These constricted tips may also be found in the digestive gland ducts.

In two snails only, protein granules have been found in digestive cells. For a description of these granules, see the description of protein granules in calcium cells. The protein granules of digestive cells differ from those of calcium cells in being rather larger, and distributed throughout the cytoplasm. They occur only in very small numbers.

Calcium cells (fig. 3) are characterized by calcium spherules, which occur throughout the cytoplasm except at the apex of the cell. These cells usually occur at the corners of the tubules, normally singly but sometimes in groups of two or three. They are normally triangular in vertical section. The average height of the calcium cells is 37.5 μ, and the average maximum width (at the base) is 33.5 μ.

The nucleus is in the basal half of the cell, and is ovoid, averaging 14.5 by 12 μ. There are usually two nucleoli averaging about 3 μ in diameter, although three have been seen.

The calcium spherules are almost always spherical, and give a positive reaction with tests for calcium. Their average diameter is 3 μ. Different cells have spherules of different sizes, but in any one cell all the spherules are of about the same size. A cell was chosen with spherules of large size; the average diameter of the spherules was 4·8 μ. Another cell was chosen with spherules of small size; the average diameter of the spherules was 1·25 μ. A few calcium spherules show internal structure, although most do not. Some have an inner concentric ring; others contain a small sphere, 0·4 to 1·2 μ in diameter, and with a natural pink colour, in the centre. Free calcium spherules are frequently found in the lumen of the tubules and ducts of the digestive gland.

The apical cytoplasm of calcium cells is free from calcium spherules, and stains strongly, particularly with basic dyes. Usually there is a small number of protein granules, about 4 μ in diameter, lying in the apical cytoplasm;

they stain very strongly with iron haematoxylin, or with acid fuchsin in Metzner's method; they also contain much tryptophane. The free surface of a calcium cell usually has a brush border, with a thin layer of clear cytoplasm below it.

Many calcium cells have a large vacuole in the apical half of the cell, below

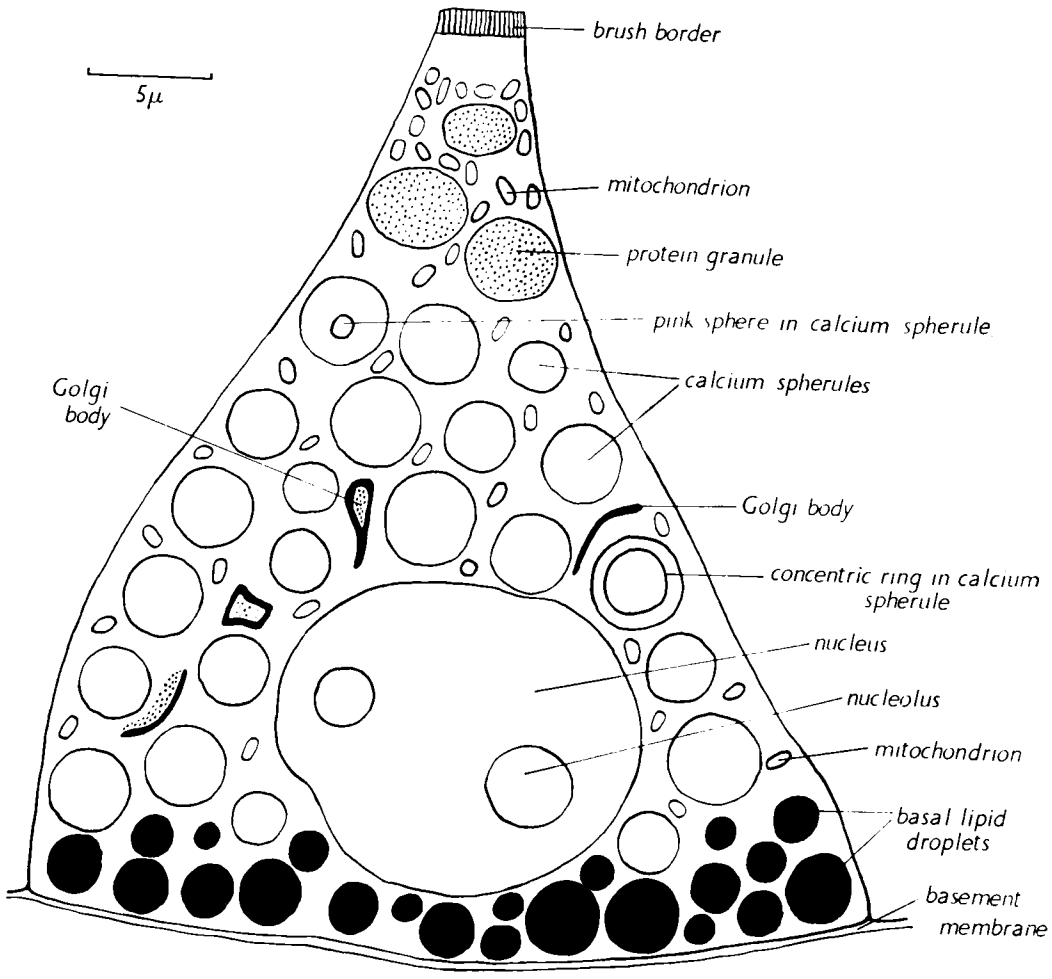


FIG. 3. Diagram of a calcium cell.

the apical cytoplasm. This replaces cytoplasm containing calcium spherules, and one cell has been seen where all the cytoplasm containing calcium spherules has been replaced by a vacuole. The vacuoles contain large yellow granules, which react positively to tests for lipofuscins.

Mitochondria occur throughout calcium cells, in between the calcium spherules and the protein granules, and also concentrated apically. They average 1·0 by 0·8 μ. The Golgi apparatus consists of two types of bodies, lying between the calcium spherules in the basal half of the cell (fig. 4). One type of body appears as a black line, slightly bent, and averaging 2·8 by 0·7 μ. The other type appears as a grey component, wholly or partly enclosed by a black line, and averaging 2·2 by 1·5 μ. Basally there are lipid droplets, ranging in diameter from about 1·5 to 5 μ. There may be few droplets, in which case they are all small; or there may be many, even extending into the apical half

of the cell, in which case the droplets are large. The cytoplasm of calcium cells has a high RNA content.

Excretory cells (fig. 5) consist of one to three large vacuoles, surrounded by a thin layer of cytoplasm. There is usually only one vacuole, which averages 27 by 21 μ, and contains one or more large yellow granules, averaging 5 μ in diameter. Apparently identical granules can be found in the lumen of the intestine and in the faeces. Often very small yellow granules are seen at the periphery of the vacuole. All these yellow granules give positive reactions to tests for lipofuscins. The average height of excretory cells is 38·5 μ, and their

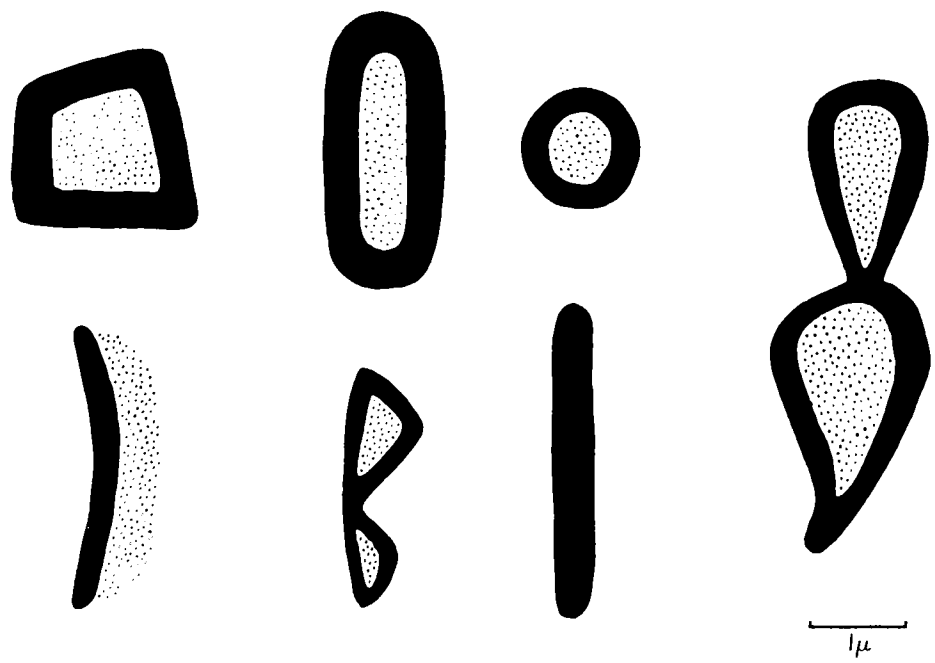


FIG. 4. Drawings of Golgi bodies in calcium cells, showing the different forms which occur. The scale represents one micron.

average maximum width 23·5 μ. The nucleus is basal, and averages 8 by 6 μ. Up to two nucleoli, 1·5 μ in diameter, can be found, but often none is visible. Feulgen's test indicates that excretory cell nuclei often have a very low DNA content. There is a layer of cytoplasm apically, which stains strongly, especially with iron haematoxylin. The apical cytoplasm often seems to have a granular or filamentous structure.

Mitochondria are scarce in excretory cells, but they occur apically in at least some cells. A few lipid globules, about 0·8 μ in diameter, can be seen apically; also, when basal lipid droplets are numerous in other cells, they appear to occur in excretory cells. No Golgi apparatus has been found in excretory cells.

The feeding state. No changes have been found in thin cells of feeding snails. However, mitosis of thin cell nuclei occurs very rarely in feeding snails, but has not been seen in starved snails.

The only change from the short-starved state in digestive cells is an increase in the amount of RNA in the basal cytoplasm.

In calcium cells two changes have been found: a decrease in the number of protein granules, and the appearance throughout the cell of small numbers of glycogen granules, about 1 μ in diameter. In one feeding snail a few calcium cells, smaller than normal, have been found in the tubule epithelium. They average 33.5 μ in height and 7 to 8 μ in width, and are columnar, not triangular, in vertical section. Their nuclei average 7 by 5.5 μ. Apart from

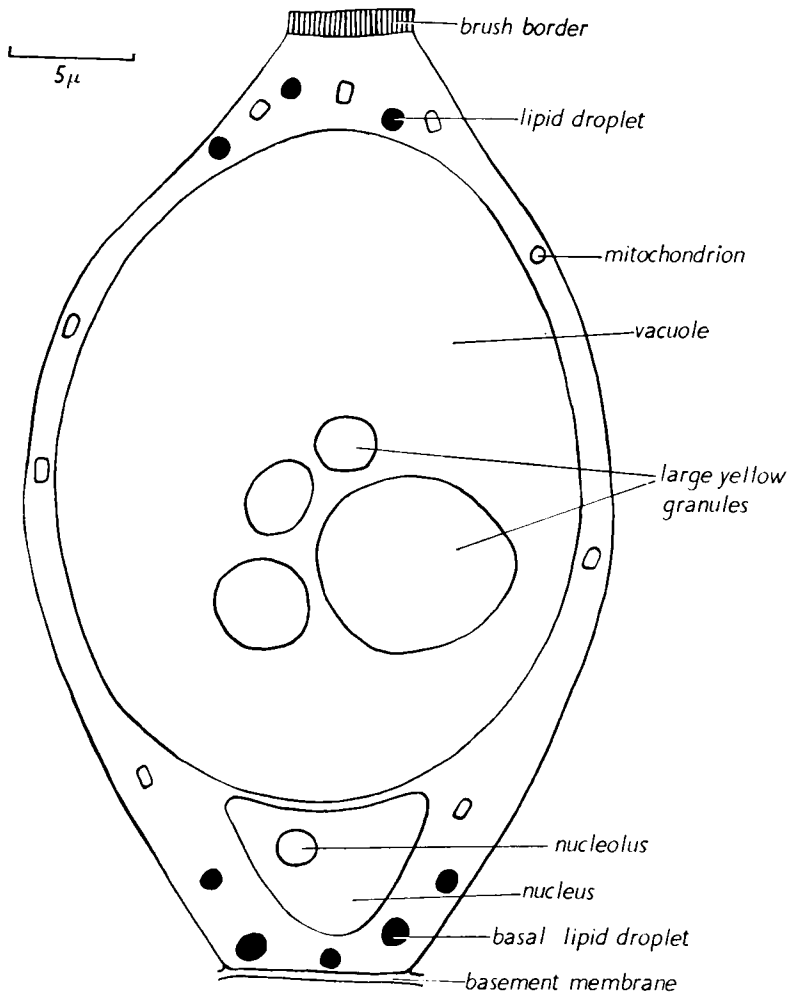


FIG. 5. Diagram of an excretory cell.

the sizes of the cell and nucleus, which are nearer the sizes of digestive cells, these small cells are typical calcium cells.

No changes have been found in excretory cells of feeding snails.

The long-starved state. Many changes occur when snails are starved for longer than 20 days. After 34 days' starvation, the 'green' granules and their vacuoles become larger (table 3 (see Appendix, p. 192) and figs. 6 and 7), a yellow pigment (which reacts negatively to tests for lipofuscins) develops in the 'green' granules, and calcium cell mitochondria become larger (table 3 and fig. 8). After 49 days' starvation the apical surfaces of all cells have become level, none protruding into the lumen of the tubule; also the DNA in the nuclei of all cells has become homogeneously distributed, instead of being in granules throughout the nucleus. After 65 days' starvation, the distinction

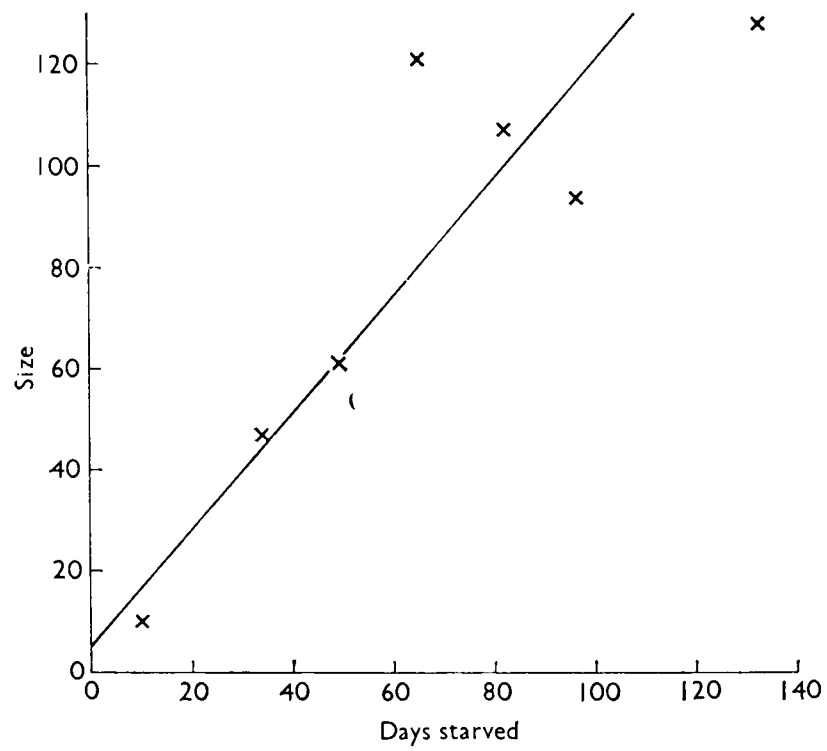


FIG. 6. Graph showing increase of size of 'green' granules with length of starvation. The sizes plotted on this graph are the products of the measurements in table 3.

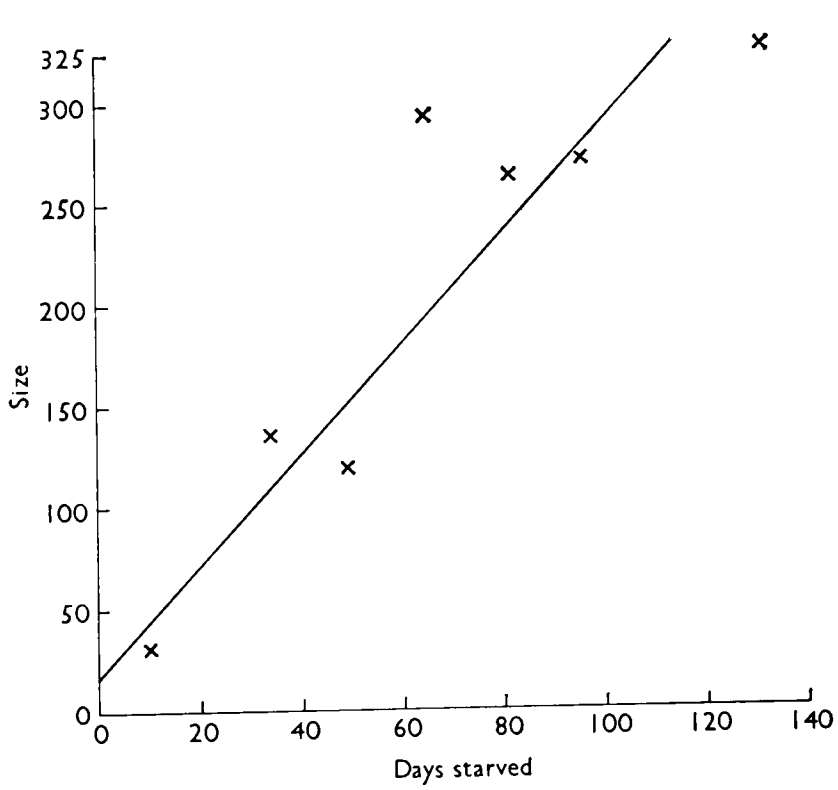


FIG. 7. Graph showing increase of size of 'green' granule vacuoles with length of starvation. The sizes plotted on this graph are the products of the measurements in table 3.

between digestive and excretory cells has been lost, and small yellow granules are no longer found in digestive cells. After 82 days' starvation, protei granules have disappeared, and the amount of RNA in calcium cells is greatly reduced.

Basal lipid droplets decrease in size and number as starvation proceeds, and after 82 days' starvation the only remaining lipid is in small droplets throughout the cells, not concentrated basally. Up to 65 days' starvation the apical

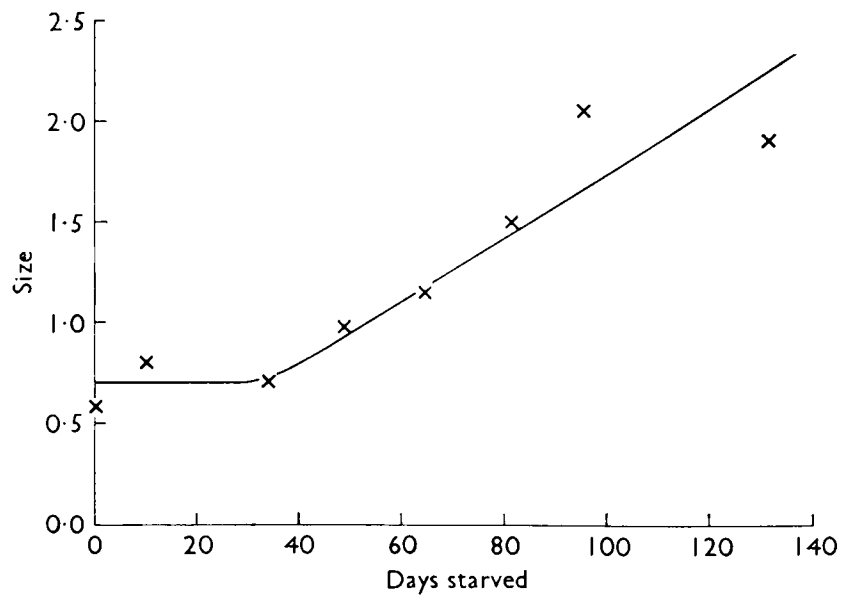


FIG. 8. Graph showing increase in size of calcium cell mitochondria with length of starvation. The sizes plotted on this graph are the products of the measurements in table 3.

cytoplasm of digestive and calcium cells may show a positive reaction for phospholipids, but this has disappeared after 82 days' starvation. During prolonged starvation, the Golgi apparatus of calcium cells decreases in size and appears as rings or crescents, 1 to 1·5 μ across, in the basal part of the cell.

The hibernating state. The digestive gland of hibernating snails resembles that of short-starved snails: there are no histochemical differences. There are the following differences in structure, however. In digestive cells, small yellow granules extend almost throughout the cytoplasm, leaving only a small area apically free of yellow granules. Similar yellow granules form a large part of the contents of the intestine of hibernating snails, and appear in the faeces shortly after the snails come out of hibernation. 'Green' granule vacuoles become smaller than in short-starved snails, averaging about 4·5 μ in diameter, although the 'green' granules themselves do not change in size. Calcium cell mitochondria become longer and thinner, averaging 2·0 by 0·5 μ in Champy-fixed tissue; digestive cell mitochondria also appear to be longer and thinner, averaging 1·6 by 0·6 μ in Champy-fixed tissue. The Golgi apparatus of calcium cells appears as small rings, usually 1 to 1·5 μ in diameter, in optical section; there are several of these bodies in each cell. The Golgi

apparatus of digestive cells is similar to that of short-starved snails. There are very few basal lipid droplets in digestive gland cells in hibernating snails.

The calcium cells of hibernating snails characteristically, but not invariably, contain objects here termed rings (fig. 9). They consist of a cortex, about 1·5 μ thick and rich in RNA, surrounding a calcium spherule. The outside diameter of rings averages between 6 and 7 μ.

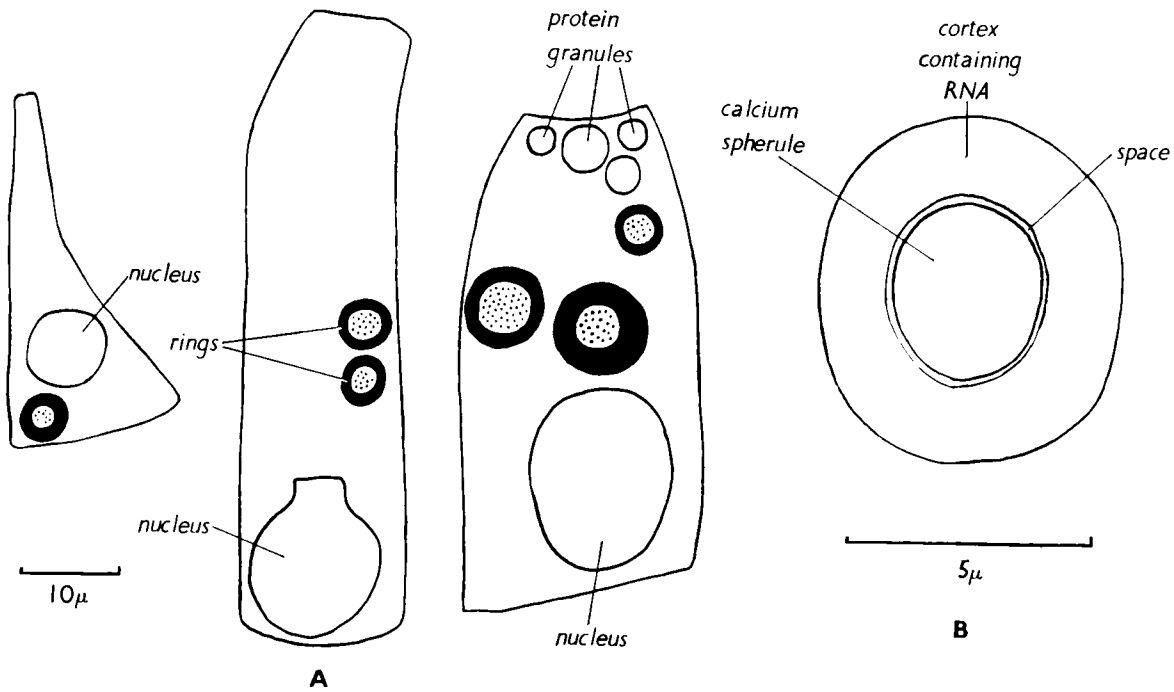


FIG. 9. Rings in calcium cells of hibernating snails. A, drawings of calcium cells showing distribution of rings within them; B, diagram showing the structure of a ring.

Discussion

Relationships of the cell-types. Barfurth (1883) described three cell-types in the digestive gland of *Helix* and *Arion*. These were Kalkzellen, Leberzellen, and Fermentzellen, equivalent respectively to calcium, digestive, and excretory cells as defined here. Calcium cells have since been found in many other species of pulmonates (Cuénot 1892; MacMunn, 1900; Fretter, 1952; van Weel, 1950; Thiele, 1953; Nakazima, 1956).

Early authors distinguished clearly between digestive and excretory cells. However, beginning with Krijgsman (1928), excretory cells have been widely considered as a variety of digestive cell (e.g. Fretter, 1952; Billett and McGee-Russell, 1955). Thiele (1953) regards excretory cells (which he calls Kalkzellen Stadium III) as degenerating calcium cells (Kalkzellen Stadium II). This is my own view. The relationship with calcium cells is indicated by the strongly staining cytoplasm, especially apically, and by the fact that many calcium cells, as well as excretory cells, possess a large vacuole containing yellow granules. Particularly interesting is a cell (described on p. 178) which has the appearance of a calcium cell except that all the calcium spherules have been lost and replaced by a large vacuole. Abolins-Krogis (1961) describes excretory cells which have protein granules apically; these must be an inter-

mediate stage in the formation of excretory cells from calcium cells. Against these points may be set the fact that excretory cell nuclei are of the same size as digestive cell nuclei, and smaller than calcium cell nuclei. This is probably coincidental, as many excretory cell nuclei have very small quantities of DNA, and Thiele (1953) describes them as strongly shrunken. There is much evidence that they are degenerating (see below). In long-starved snails digestive cells resemble excretory cells as a result of the enlargement of 'green' granule vacuoles. This cannot be taken as an indication of a relationship between the two cell-types. No cells have been found which are intermediate between digestive and excretory cells.

Billett and McGee-Russell (1955) describe two types of digestive cell, one with 'green' granules throughout the cytoplasm and the other with yellow granules basally and 'green' granules apically. There does not appear to be any fundamental difference between these two types of digestive cell; one type has merely accumulated yellow granules while the other has not. In fact, all intermediates can be found between digestive cells with almost no yellow granules and those with very large numbers of yellow granules, as found in hibernating snails.

Thin cells were first described by MacMunn (1900); he called them young cells, presumably implying that they were undifferentiated. Thiele (1953) also believed them to be undifferentiated; he found them between digestive and calcium cells, and believed they could develop into either type. I have not found thin cells restricted to positions between digestive and calcium cells, and they may occur anywhere. I have found no direct evidence that they develop into any other cell-type, although I believe they must do so. The occurrence of mitosis in thin cells indicates, first, that they are undifferentiated (since differentiated cells do not normally divide), and secondly, that they do develop into other cell-types (since they do not appear to increase in number with age). Thiele (1953) claimed that mitosis only occurred in young snails, and that in older snails amitosis occurred. I have not found amitosis in snails of any age. No doubt Thiele overlooked mitotic nuclei in older snails, as they are very rare; I have only found them in feeding snails. Nakazima (1956), working on Japanese snails, found alkaline phosphatase in thin cells. I have found alkaline phosphatase in calcium cells (Sumner, unpublished), which suggests that thin cells develop into calcium cells rather than digestive cells, which only have a little alkaline phosphatase apically, if any.

Functions of the cell-types. It has been stated that the function of calcium cells is to store calcium salts (Fretter 1952), although Filhol (1937) believed this to be only one aspect of a general property of storage. Grünbaum (1913) and Nakazima (1956) both found lipid stored in calcium cells, and the latter found that the amount of lipid varied with starvation. I find that the amount of lipid in calcium cells decreases as starvation proceeds, and thus it is clearly a reserve food substance.

There is less certainty about the function of the calcium spherules. Wagge (1951), McGee-Russell (1955), and Abolins-Krogis (1961, 1963*b*) believed

that the calcium was used for shell repair, whereas Krijgsman (1928) believed it was secreted into the crop-juice and acted as a buffer against changes in pH. I have found that calcium spherules are secreted into the lumen of the digestive gland tubules, and pass down the ducts of the gland. However, there is much evidence that the spherules are not simply balls of calcium salts, but have a much more complicated structure and composition (Grünbaum, 1913; Wagge, 1951; McGee-Russell, 1957; Abolins-Krogis, 1963*a*, 1963*c*; Sumner, unpublished); thus their function cannot be considered merely in terms of their calcium content.

The high RNA content and prominent Golgi apparatus of calcium cells indicates strongly that they must have a secretory function. Apart from the calcium spherules, the protein granules are probably passed out of the cell. Although they have not been found in the lumen of the tubules, they decrease greatly in number in feeding snails, and this suggests that they are released into the tubules during feeding. Protein granules have been observed previously in calcium cells, but no suggestions have been made as to their function (Grünbaum, 1913; Filhol, 1937; Abolins-Krogis, 1961).

Calcium cells also have an excretory function; many form vacuoles containing lipofuscin granules, but it is not certain whether the calcium cells are transformed into excretory cells before the lipofuscin granules are released.

Excretory cells were at first believed to secrete enzymes (Barfurth, 1883; Frenzel, 1885; Krijgsman, 1925); other workers believed that they were excretory, and eliminated chlorophyllous pigments taken in with the food (MacMunn, 1883, 1886, 1900; Dastre, 1899; Dhéré and Vegezzi, 1916). Cuénot (1892), Thiele (1953), and McGee-Russell (1955) also believed that excretory cells were excretory, but Krijgsman (1928) was unable to assign any function to them. Abolins-Krogis (1961) thought that the material of the yellow granules was used in shell regeneration. She believed that the main pigments present were urochrome and melanin, although later (1963*b*) she admitted that lipofuscin might be present. She also found many other substances in yellow granules (Abolins-Krogis, 1961, 1963*a*). Zacks (1955) appears to be the only previous worker who found lipofuscin in a mollusc, in this case in the amoebocytes and intestinal epithelium of the lamellibranch *Venus mercenaria*; he believed the pigment to be excretory.

My own work confirms the majority view that excretory cells have an excretory function. Lipofuscin granules similar to those in excretory cells can be found in the intestine and faeces, and thus they are being excreted.

It is clear that excretory cells are degenerating, as Thiele (1953) thought. The nucleus often has a low DNA content, sometimes almost none; the amount of cytoplasm is very small; mitochondria are few; and I have found no Golgi apparatus. Thiele (1953) claimed that he had found a Golgi apparatus, which, however, was degenerating.

The digestive cells are widely believed to secrete, and to absorb soluble food material (Krijgsman, 1925, 1928; Fretter, 1952; Thiele, 1953); therefore they have been named secretory-resorption (SR) cells. The presence of a

brush border, an apical concentration of mitochondria, and a high concentration of phospholipid apically all indicate that absorption occurs in digestive cells. Krijgsman (1928) described a secretory cycle, synchronous in all cells, in which small yellow granules were transformed into 'green' granules, which were passed out of the cell; Thiele (1953) supported this hypothesis. However, it is obvious that yellow granules cannot be transformed into 'green' granules, because of their histochemical differences. There appears to be no connexion between the two types of granule, except that they occur together in the same cell. The inconspicuous Golgi apparatus and low RNA content of digestive cells show that they do not secrete much. Nevertheless, it is true that both small yellow and 'green' granules are secreted in the sense that they are cast out of the cell. The small yellow granules, like the large yellow granules of excretory cells, are excreted. Barfurth (1883) noticed that yellow granules, similar to those of digestive cells, could be found in the faeces; and I have found this particularly obvious in hibernating snails. It seems probable that the secretion of 'green' granules is also excretory. Digestive cells have been shown to take up solid food particles, which accumulate in vacuoles (Jordan, 1918; Rosen, 1941). My own experiments (Sumner, unpublished) show that phagocytosed particles accumulate in vacuoles similar to those occupied by 'green' granules. Thus it seems that 'green' granules represent ingested food material and indigestible residues; this is supported by the fact that they contain muco-substances, which are mixed with the food in the gut before it enters the digestive gland (Graham, 1949; Morton, 1952). No doubt the 'green' granules which are extruded from the cell are made up of these indigestible residues.

Other functions which have been attributed to digestive cells are participation in shell regeneration (Abolins-Krogis, 1961), and storage of glycogen and fat (Fretter, 1952). The subject of shell regeneration is not considered here. I have found that digestive cells store lipid, in their basal lipid droplets, and that these decrease in number as starvation proceeds. However, I have found no storage of glycogen; if this does occur, it cannot be of great importance to the snail.

The changes occurring in digestive gland cells of feeding snails, compared with short-starved snails, are few but significant. The increase in RNA in digestive cells is no doubt connected with synthesis of enzymes for intracellular digestion, which is believed to occur (Rosen, 1941). The reduction in number of protein granules is probably caused by the passage of some of them out of the cell (see p. 185).

Most of the changes occurring during prolonged starvation have been found to occur in other organisms. In human tissue culture cells (Cohen and others, 1961) and in *Euglena* (Malkoff and Buetow, 1964), starvation is accompanied by vacuolization of the cytoplasm, the vacuoles growing larger as starvation proceeds. In *Helix* vacuoles are already present in the cytoplasm of digestive cells, but they also grow larger as starvation proceeds. In *Euglena*, starved for more than 8 days, it was found that mitochondria became swollen (Malkoff and Buetow, 1964), as occurs in the calcium cells of *Helix*. In rat

liver (Lagerstedt, 1949) and in *Euglena* (Malkoff and Buetow, 1964) starvation results in the DNA becoming diffusely distributed throughout the nucleus; and this also occurs in *Helix*. However, *Helix* differs from these other organisms in that it takes several weeks, rather than a few days, for the changes to occur. It seems, as one would expect, that *Helix* is adapted to going without food for long periods. This is a necessary attribute for an animal which frequently meets adverse conditions and has to stop feeding.

The changes already discussed appear to be typically associated with starvation. Other changes found in digestive gland cells of *Helix* are more probably connected with inactivity of the cells rather than an absence of food supply. Such changes are the reduction of the amount of RNA in calcium cells, loss of phospholipid in the apical cytoplasm, and reduction in size of the Golgi apparatus of calcium cells. Reduction of the amount of RNA and reduction in the size of the Golgi apparatus are presumably associated with the cessation of secretion, which is no longer necessary when there is no food to digest. Reduction of the amount of RNA during starvation has also been found in rat liver cells (Lagerstedt, 1949). Loss of phospholipid in the apical cytoplasm is probably because absorption and phagocytosis no longer occur.

The changes in the digestive gland cells which occur during hibernation are not similar to those occurring during prolonged starvation. This confirms the view that it is a condition which is specially prepared for (Howes and Wells, 1934), and not merely a prolonged starvation. The most obvious feature of the digestive gland of hibernating snails is the great accumulation of small yellow granules in digestive cells. This was noted by MacMunn (1886), who believed they consisted of enterochlorophyll derived from the food. He thus had difficulty in explaining this increase. It is clear that since no faeces can be passed out during hibernation, excretory material, including yellow granules, must accumulate. In fact, not only the digestive cells of hibernating snails, but also their intestines, are full of small yellow granules. The reduction in size of the Golgi apparatus of calcium cells, which parallels that found in long-starved snails, is probably due to the same cause, that is reduction or cessation of secretion. The small number of basal lipid droplets found in digestive gland cells of hibernating snails is a consequence of using up this food reserve. The elongation of mitochondria might be a result of starvation. However, in hibernation mitochondria become long and thin, while during prolonged starvation they become thicker as well as longer. The other changes in digestive gland cells of hibernating snails cannot be satisfactorily explained at present.

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Appendix

TABLE 1

Synonyms of names of cells

Reference	*Genus or species*	*Thin cell*	*Digestive cell*	*Calcium cell*	*Excretory cell*
Abolins-Krogis, 1961	*Helix*	—	Digestive cell	Calcium cell	Excretory cell
Barfurth, 1883	*Arion, Helix*	—	Leberzell	Kalkzell	Fermentzell
Billett and MacGee-Russell, 1955	*Helix pomatia*	—	Digestive cell: A & B_1	Chalk cell	Digestive cell: B_2
Cuénot, 1892	Many species	—	Cellule hépatique	—	Cellule vacuolaire
Frenzel, 1885	*Arion, Helix*	—	Körnerzell	—	Keulenzell
Fretter, 1952	*Agriolimax, Arion, Helix*	—	Digestive cell	Lime cell	Digestive cell
Krijgsman, 1925	*Helix pomatia*	—	Resorption cell	Chalk cell	Enzyme cell
Krijgsman, 1928	*Helix pomatia*	—	Granule or Enzyme cell	Chalk cell	Granule or Enzyme cell
McGee-Russell, 1955	*Helix aspersa*	—	Digestive cell	Calcium cell	Excretory cell
MacMunn, 1900	*Arion, Helix, Limax*	Young cell	Hepatic cell	Lime cell	Ferment cell
Nakazima, 1956	Many Japanese species	Narrow cell	—	Calcium or Calcareous cell	—
Thiele, 1953	Many species	Indifferent cell	S.R. cell	Kalkzell St. II	Kalkzell St. III
van Weel, 1950	*Achatina*	—	p-cell; y-cell	Calcium cell	b-cell

TABLE 2

Results of histochemical tests on digestive gland cells of short-starved Helix aspersa

	Test	*Fixative*	*Embedding medium*	*Thickness of section (μ)*	*Reference*	*All cells*			*Thin cells*	
						Nucleoli	*Small yellow granules*	*Large yellow granules*	*Nuclei*	*Cytoplasm*
1	Feulgen	Ze	P	8	Feulgen and Rossenbeck, 1924	o	o	o	. .	o
2	Pyronine/methyl green	Ze3	P	8	Jordan and Baker, 1955	+++	+++	+++		o/+++
3	RNAase+P/MG	Ze3	P	8	Bradbury, 1956	o	+	+		o
4	PA/S	Ze	P	8	MacManus, 1948	o	++	+		—
5	Diastase - PA/S	Ze	P	8	Lillie and Greco, 1947		++	+		—
6	toluidine blue (metachromasy)	Ze	P	8	—	—	o	o		—
7	sulphation + toluidine blue	Ze	P	8	Lison, 1953	o	—	o/+		—
8	Sakaguchi	Ze	P	16	Baker, 1947	—	o	o	—	—
9	DMAB/nitrite	F–Na	P	8	Pearse, 1960	—	o	o	—	—
10	Sudan black	F–Ca/PC	G	10	Baker, 1956	o	o/+	o/++	—	—
11	acid haematein (AH)	F–Ca/PC	G	10	Baker, 1946	o	o/+	o/++	—	—
12	pyridine extraction + AH	WB/PC	G	10	Baker, 1946	+++	+ +	+/++	—	—
13	cobalt method for calcium	FE:FMP	P	8	—	o	-	—	o	o
14	calcium red method	FE:FMP	P	8	McGee-Russell, 1955	o	—	—	o	o
15	Schmorl	F–Na	P	8	Pearse, 1960	o	++/+++	o/+++	—	—
16	chrome-alum haematoxylin	F–Na	P	8	Pearse, 1960	o	+	+/++	—	—
17	long Ziehl-Neelsen	F–Na	P	6	Pearse, 1960	—	+ +	++/+++	—	—
18	10% hydrogen peroxide (48 h)	F–Na	P	8	Pearse, 1960	—	*	*	—	—

	Digestive cells					*Calcium cells*						*Excretory cells*		
	Nuclei	*Ground cytoplasm*	*Apical cytoplasm*	*'Green' granules*	*Basal lipid droplets*	*Nuclei*	*Ground cytoplasm*	*Apical cytoplasm*	*Protein granules*	*Calcium spherules*	*Basal lipid droplets*	*Nuclei*	*Ground cytoplasm*	*Apical cytoplasm*
1	+++	o	o	o	–	+++	o	o	o	o	–	+/+++	o	o
2	o	+/++	++	++	–	–	++/+++	++/+++	+	–	–	–	+/++	+/++
3	o	+	+	+	–	–	o	o	+	–	–	–	+	+
4	+	+	+	+++	–	–	o/+	+	++	–	–	–	+	+
5	–	+	+	+++	–	–	+	o/+	++	–	–	–	+	+
6	–	o	o	o	–	–	o	o	o	–	–	–	+	+
7	–	o/++	++	++/+++	–	–	o	++	+/++	–	–	–	+	o/+
8	++	+	++	++	–	–	+	+	+/++	–	–	–	+	+
9	–	+	+/++	+	–	–	+	+	+++	–	–	–	+	o
10	o	o	o	o	+++	–	–	–	o	–	+++	–	o	o
11	o	o/+	o/++	o	o	–	–	+++	o	–	o	–	o	++
12	+/+++	o	o	o	–	–	–	o	–	–	–	–	–	o
13	o	o	o	o	–	o	o	o	o	+/+++	–	–	o	o
14	o	o	o	o	–	o	o	o	o	+++	–	–	o	o
15	o	+	+	+	–	–	–	–	–	–	–	–	–	–
16	o	o	o	o	–	–	–	–	–	–	–	–	–	–
17	o	–	–	–	–	–	–	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–

The following abbreviations and symbols are used:
F–Ca/PC, formaldehyde-calcium with post-chroming; FE, formalin-ethanol (1:1); FMP; formalin-methanol-pyridine; F–Na, formalin-saline; Ze, Zenker; Ze3, Zenker for 3 h; o, no reaction; –, no observation; +, weakly positive reaction; ++, moderately positive reaction; +++, strongly positive reaction; *, not bleached; /, indicates variation; e.g. o/++ indicates a reaction varying between none and moderately positive.

TABLE 3

Changes of size with starvation

Length of starvation	*'Green' granules*	*Their vacuoles*	*Calcium cell mitochondria*
feeding	—	—	1·0 × 0·6
short-starved*	3·5 × 3·0	6·1 × 5·1	1·0 × 0·8
34 days	7·5 × 6·3	13·1 × 10·2	1·0 × 0·7
49 days	9·1 × 6·7	12·5 × 9·5	1·2 × 0·8
65 days	12·6 × 9·6	19·2 × 15·3	1·3 × 0·8
82 days	11·5 × 9·3	18·0 × 14·7	1·5 × 1·0
96 days	11·2 × 8·4	17·9 15·5	1·7 × 1·2
132 days	12·6 × 10·2	20·6 × 15·7	1·7 × 1·1

All the above measurements are in μ.

* Average of measurements from several snails starved for between 7 and 20 days.

PAPER 2

FINE CYTOLOGY AND HISTOCHEMISTRY OF THE DIGESTIVE GLAND CELLS OF _ILA_, _NUCULA_ AND _TELLINELLA_

SYNOPSIS

The structure of the digestive gland cells of the following forms has been compared with that of the digestive gland cells of Helix: a prosobranch, Pila; and two stylommatophoran pulmonates, Succinea, a primitive herbivorous form, and Testacella, a carnivorous form. All the forms studied have cells corresponding to the thin, digestive, calcium and excretory cells found in Helix.

Pila has protein granules in its digestive cells. Calcium is absent from the cells corresponding to the calcium cells of Helix. The excretory cells contain large brown granules which consist partly of lipofuscin, and partly of an iron-containing pigment. The changes which occur in the digestive gland cells of Pila on aestivation are similar to those which occur in Helix when starved.

In the digestive cells of Succinea there is a gradation between the colourless and the yellow granules. Many of the spherules in the calcium cells lack calcium.

Testacella shows no differences from Helix which can be attributed to a carnivorous diet.

INTRODUCTION

Although the digestive systems of numerous species from most orders of gastropods have been studied by several authors, no detailed cytological and histochemical studies have been made on the digestive glands of most species, comparable to the study made on Helix (Paper 1). It was

therefore decided to extend that study to other forms. The forms studied were Pila, an amphibious herbivorous prosobranch; Succinea, a primitive herbivorous stylommatophoran pulmonate (Morton, 1958); and Testacella, a carnivorous pulmonate.

Species of the genus Pila occur in swamps and slow-moving water in India and Africa (Andrews, 1965); the specimens used in this study came from Uganda. They feed chiefly on aquatic plants, but will also eat animal remains (Andrews, 1965). When their habitat dries out, species of Pila will aestivate, often for several months (Meenakshi, 1964).

Succinea putris lives in damp situations, and can be found on vegetation in marshy places or by ditches (Rigby, 1965; Sumner, personal observations).

Species of Testacella live largely underground, feeding chiefly on earthworms but also on other slugs (Quick, 1960).

There have been a number of differing descriptions of the digestive glands of prosobranchs. Some authors describe arrangements similar to those in Stylommatophora, while others claim that the histological structure is different. The prosobranchs clearly needed reinvestigation.

Succinea, as a herbivorous Stylommatophora, might be expected to resemble Helix closely in the structure of its

digestive gland cells, and if so, would suggest that this structure is representative of the Stylommatophora. Testacella, as a carnivore, might be expected to show adaptations to diet. It is generally accepted that phagocytosis, which occurs in the digestive gland of Helix (Paper 4), is associated particularly with a herbivorous diet (Prosser & Brown, 1961). Thus there might be structural differences in the digestive gland cells of Testacella, related to a reduction in phagocytosis.

MATERIAL AND METHODS

The species used were Pila ovata, Succinea putris and Testacella maugei. Only the tubule epithelium of the digestive gland was studied. The digestive glands of the specimens of Pila were fixed and embedded in Uganda, and sent to me as blocks ready for sectioning by Professor L.C. Beadle.

The specimens of Pila were feeding, or had been aestivating for 4 weeks, 3 months, or 6 months. The specimens of Succinea were either feeding, or had been starved for 10 to 12 days to correspond with the short-starved state in Helix (Paper 1). The specimens of Testacella were provided with food, but no food was found in their alimentary systems. Since Testacella is only an intermittent feeder (Barnes & Stokes, 1951; Stokes, 1958), these specimens were probably in a condition corresponding to the short-starved state in Helix.

For general cytological study of the digestive glands of Pila, Succinea and Testacella, small portions were fixed in Zenker (or Helly for some specimens of Pila), Champy or formaldehyde-saline, and embedded in paraffin. Tissue fixed in Zenker, Helly or Champy was sectioned at 6μ, and tissue fixed in formaldehyde-saline at 8μ. Sections of tissue fixed in each of the fixatives were stained with Heidenhain's iron haematoxylin. Sections of tissue fixed in Zenker or Helly were stained with Ehrlich's haematoxylin and counterstained with eosin, phloxine or light green. Some sections of tissue from Testacella fixed in formaldehyde-saline were stained in aldehyde fuchsin after permanganate oxidation (Dawson, 1953). Mitochondria were stained by Metzner's method (Meves, 1911), in 4μ sections of tissue fixed in Champy. The Golgi apparatus (in Testacella alone) was shown by the Mann-Kopsch method (Baker, 1933).

The histochemical tests used are listed in table 1.

RESULTS

The tubule epithelia of the digestive glands of Pila, Succinea and Testacella contain four cell types, corresponding to and resembling the thin, digestive, calcium and excretory cells of Helix (Paper 1). The features of these cells which are common to all the species will be described briefly first, and the specific variations described in more detail afterwards.

The thin cells are very narrow and columnar, with an

elongated nucleus and no evident cytoplasmic inclusions. Digestive cells are also columnar, but wider than thin cells. The nucleus is basal and the cytoplasm vacuolated. The more apical vacuoles contain colourless granules (the "green" granules of Krijgsman (1928), so called because they stain readily with light green), and the more basal vacuoles contain yellow granules. Calcium cells are generally conical, with a large nucleus and large nucleoli, and a high concentration of RNA in the cytoplasm. The cytoplasm is usually filled with a large number of refractile spherules, and apically there may be granules which contain protein. Excretory cells consist of a thin layer of cytoplasm surrounding a large vacuole. The vacuole contains one or more yellow granules.

The dimensions of the cells in the different species are given in table 2. The results of histochemical tests are compared with those in Helix (table 4 in Paper 1), and will only be referred to where they show differences from Helix.

Pila (fig. 1A)

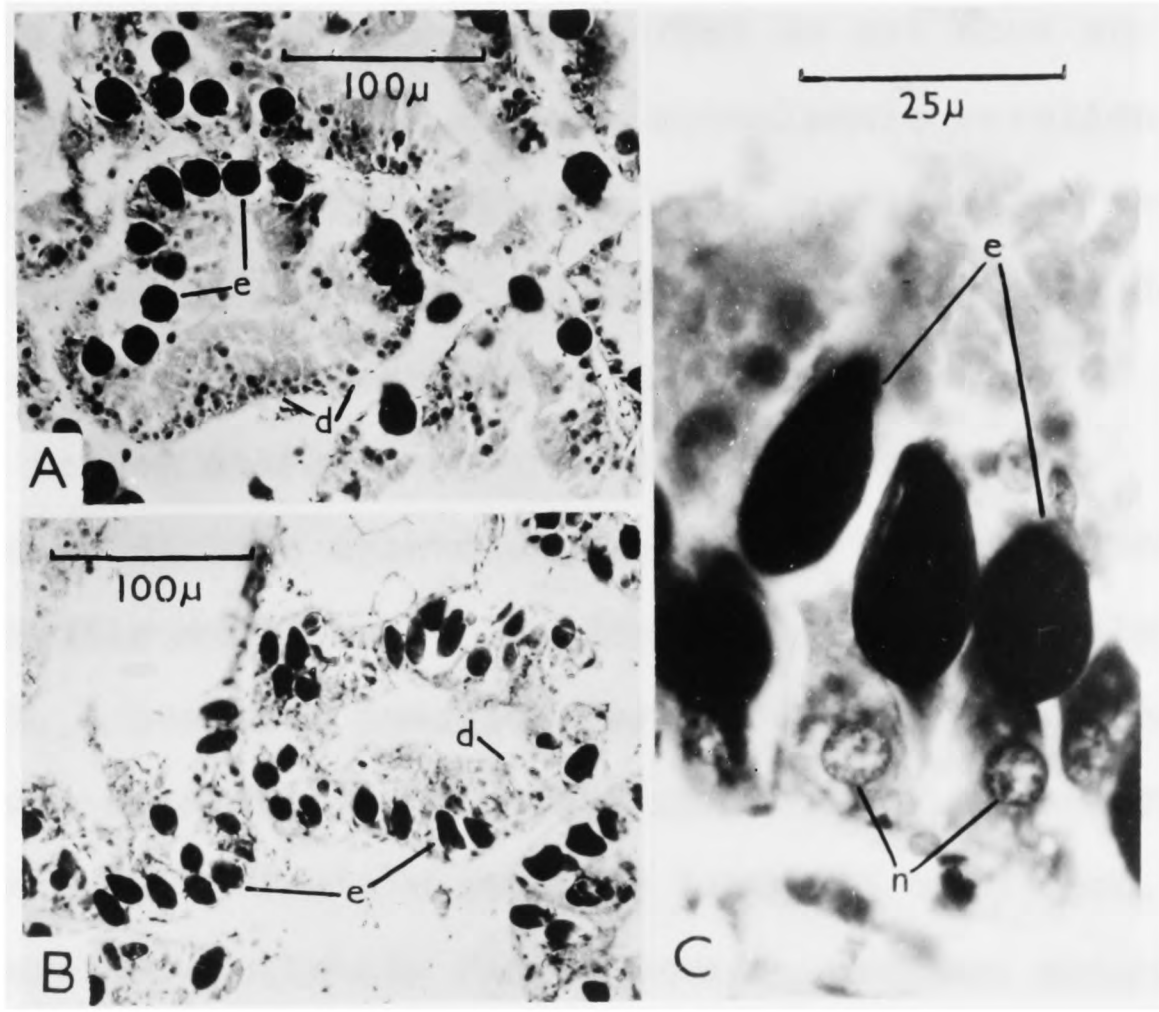
The thin cells do not differ from the normal pattern.

In the digestive and calcium cells of feeding specimens, a mass of basal lipid droplets, 2 to 3μ in diameter, extends about one third of the way up the cell. A moderate number of smaller lipid droplets occurs throughout

Fig. 1. Pila. A and B, micrographs showing the structure of the digestive gland. (Fixed in Helly, and stained with iron haematoxylin). A is from a feeding specimen (note the rounded excretory granules); B is from a specimen which had been aestivating for 6 months (note the elongated and more numerous excretory granules).

C, Micrograph showing excretory cells (from a feeding specimen). (Fixed in Helly, and stained with Ehrlich's haematoxylin and phloxine).

d, digestive cells; e, excretory granules; n, nuclei.


100μ
e
d
A
100μ
d
e
B
25μ
e
n
C

the other parts of the cells. The digestive cells usually have a brush border. As well as the inclusions usually present in digestive cells, the digestive cells of Pila contain a number of granules which are stained black by iron haematoxylin, and pink by phloxine; they are strongly positive to the DMAB/nitrite test, and moderately positive to Millon's test. Apart from this, they do not show any reaction stronger than the general cytoplasmic reaction. The small yellow granules give the same reactions to tests for lipofuscins as the small yellow granules of Helix do.

The cells corresponding to the calcium cells of Helix are rather scarce, and are conical or columnar. Instead of refractile spherules, they have numerous vacuoles, which generally appear empty in feeding snails. No calcium is present. A positive reaction for iron occurs, but this might be due to diffusion from the granules of excretory cells (see below). Protein granules have not been seen.

Excretory cells are fairly common, and are second only to digestive cells in abundance (fig. 1A). Each contains a large ovoid granule, which has its long axis along the long axis of the cell (fig. 1C). These granules are yellow, brown, or blackish; their reactions to histochemical tests are summarized in table 3. The cytoplasm of excretory cells is basiphil.

Changes in Pila during aestivation

The changes are similar to those which occur in Helix when

starved (Paper 1). The colourless granules of digestive cells, and the vacuoles in which they lie, grow larger as aestivation proceeds. Granules appear in the vacuoles of calcium cells. After 4 weeks' aestivation, they are colourless, but stain with phloxine or iron haematoxylin. After longer periods of aestivation, these granules become yellow, and react positively to tests for lipofuscins. The large coloured granules of excretory cells remain roughly constant in size, but become longer, thinner and more numerous (fig. 1B). The basal lipid droplets in all cells are reduced in number as aestivation proceeds, until after 6 months' aestivation none remain.

Succinea

Digestive cells are fewer in Succinea than in Helix, since calcium and excretory cells are more numerous. Thin cells occur almost always next to calcium cells.

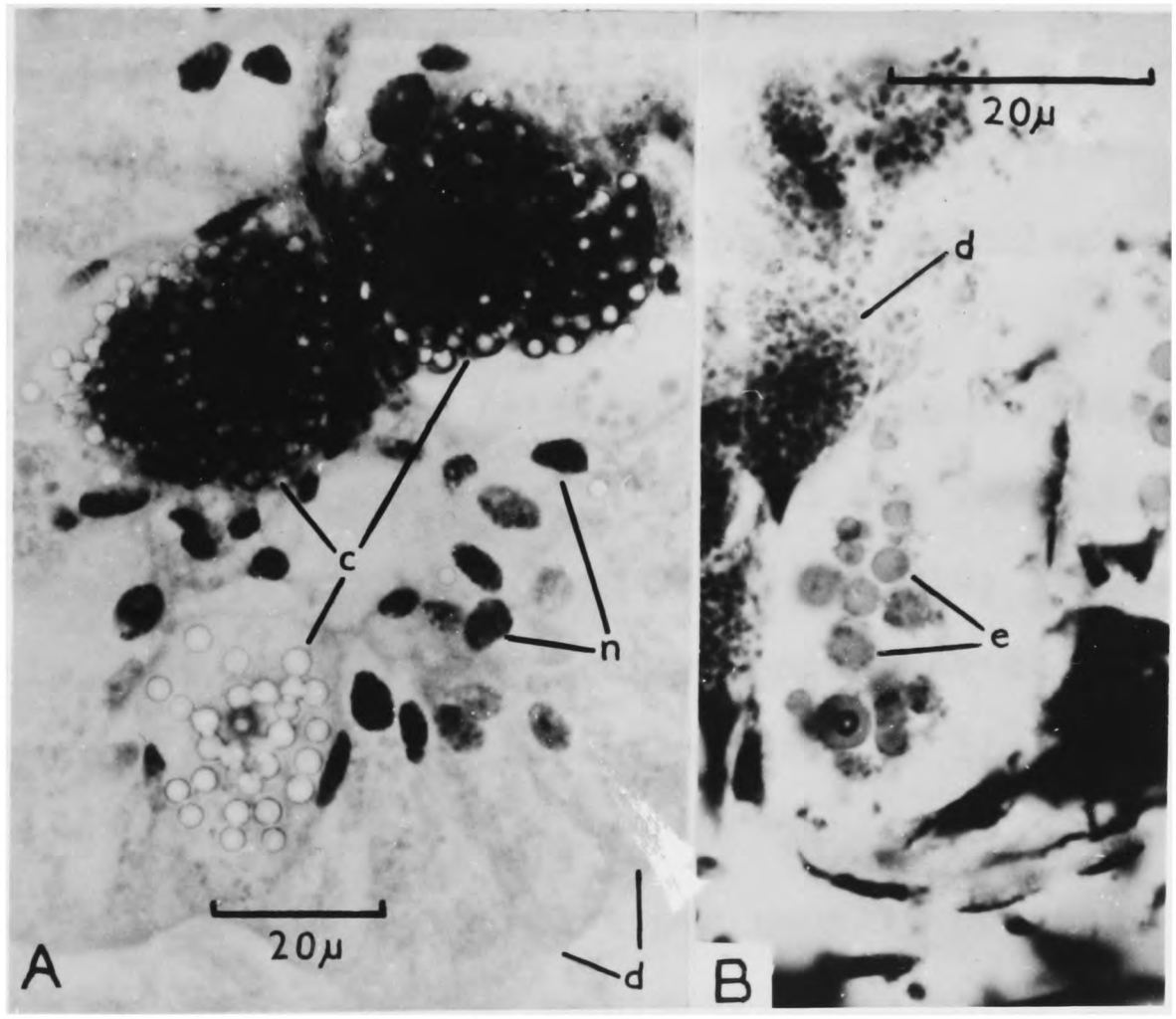
The digestive cells in Succinea conform to the usual pattern, but there is a gradation between the colourless and the yellow granules. In the apical part of the cell, the granules are colourless; in the middle part they are pale yellow, but not refractile; and the most basal granules are deeper yellow and refractile. Most of the granules lie in separate vacuoles, but some of the basal yellow granules lie together in a single larger vacuole. The increase in colour and refractility is accompanied by an increase in the degree of positivity to the long Ziehl-Neelsen test for

See over.

Fig. 2. Succinea. A. Calcium cells, two with spherules containing calcium (upper), and one with spherules containing no calcium (lower). (Fixed in formaldehyde-methanol-pyridine, and stained with calcium red).

B. An excretory cell, showing several small excretory granules. (Fixed in Zenker, and stained with iron haematoxylin).

c, calcium spherules; d, digestive cells; g, excretory granules; n, nuclei.


20μ
c
n
d
20μ
d
e
A
B

lipofuscins. The colourless granules show a moderately to strongly positive reaction to the DMAB/nitrite test for tryptophan.

The calcium cells of Succinea differ from those of Helix in that some or all of their spherules lack histochemically detectable calcium (fig. 2A). The vacuoles where the spherules normally are sometimes contain granules which either stain with eosin, or have a natural yellow colour. Protein granules have only been found in starved Succinea. As in Helix, iron occurs in small granules scattered through the cytoplasm.

The excretory cells (fig. 2B) are columnar, and have only a very thin layer of cytoplasm round the vacuole. Often no nucleus is visible. When it is visible, it stains very feebly, but there is no good evidence that it has a low DNA content. Often the nucleus appears to lie in the vacuole.

As well as the four cell types usually present, a fifth type occurs in starved Succinea. These cells are very scarce. They are about 50μ high, and 14μ wide; the nucleus is about 6.4 by 4.8μ. The characteristic feature of these cells is the presence of numerous pale yellow refractile granules, irregular in shape, and 1.6 to 3.2μ across. Apart from these granules, the cytoplasm is homogeneous. The granules are weakly positive to the hexamine-silver test for urates.

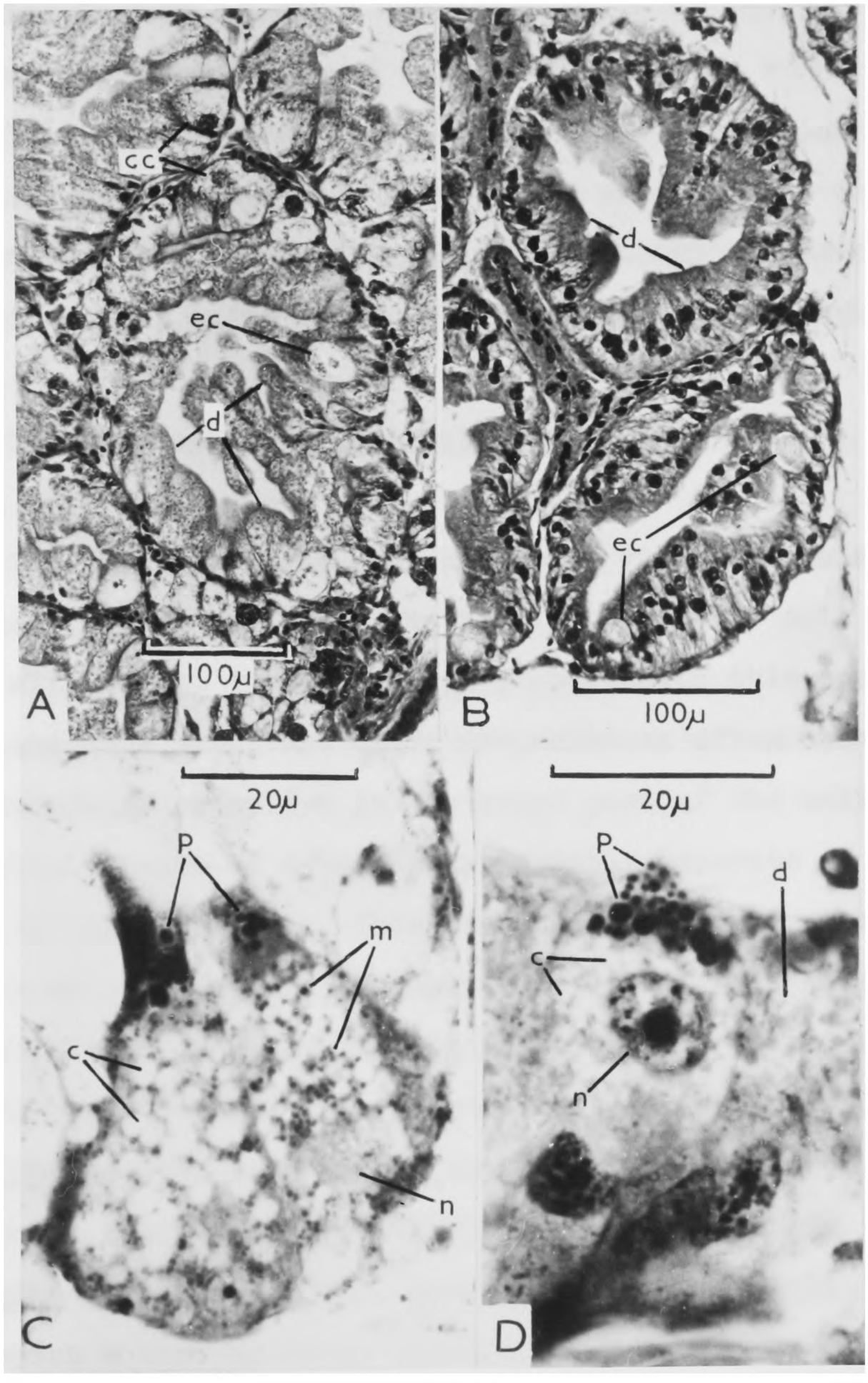
See over.

Fig. 3. Micrographs comparing Helix (A and C) and Testacella (B and D).

A and B. Micrographs showing the structure of the digestive gland tubules (Fixed in Zenker, and stained with Ehrlich's haematoxylin and eosin).

C and D. Calcium cells. (Fixed in Champy, and stained by Metzner's method for mitochondria).

c, calcium spherules; cc, calcium cells; d, digestive cells; ec, excretory cells; m, mitochondria; n, nuclei; p, protein granules.


cc
ec
d
100μ
A
d
ec
100μ
B
20μ
P
m
c
n
C
20μ
P
d
c
n
D

Testacella

A striking feature of the tubule epithelium of Testacella, when compared with Helix and other forms, is its regularity (figs. 3A & B). Whereas in most forms many of the digestive cells have a rounded apical surface and project into the lumen of the tubule, in Testacella the luminal surfaces of the digestive cells are straight and level with each other.

The thin cells of Testacella conform to the general pattern.

The digestive cells are columnar, with the nucleus about half way up the cell. The basal half of the cell is filled with lipid. In Sudan black preparations this appears as a continuous mass, but other preparations often show thin strands of cytoplasm in the basal part of the cell. These thin strands of cytoplasm presumably separate a number of lipid droplets. This basal mass of lipid extends apically to just past the nucleus. The apical half of the cell is full of vacuoles containing colourless granules. No yellow granules have been found in the digestive cells of Testacella. The colourless granules are strongly positive to the DMAB/nitrite test. Digestive cells in Testacella almost invariably bear a brush border. The Mann-Kopsch method blackens numerous objects in the apical half of the cell; they are either rings, 1.5μ in diameter, or threads, 1 to 2μ long, and 0.4μ or less wide.

The calcium cells of Testacella are columnar, and widest at the level of the nucleus; they have 20 or more protein granules apically (fig. 3D). They differ in these two respects from the calcium cells of Helix and other forms, which are conical and have few protein granules (fig. 3C). The nucleus of calcium cells in Testacella is about one third to one half of the way down the cell from the apex. There is a basal concentration of lipid, but the amount is much smaller than in digestive cells. Small granules containing iron occur in the cytoplasm, as in Helix and Succinea. The calcium cells of Testacella do not normally have large vacuoles, as often occurs in Helix.

The excretory cells of Testacella have very pale yellow granules, but they nevertheless react positively to tests for lipofuscins.

DISCUSSION

The most striking feature of the various species studied is the close resemblance in the structure of the epithelia of the digestive gland tubules. Pila, Succinea and Testacella show the same four cell types as Helix (Paper 1) in spite of their differences in taxonomic position and diet.

Most accounts of the digestive glands of prosobranchs describe two types of cells. One corresponds to the type called here digestive cells. A feature of these cells in Pila is the presence of protein-containing granules lying directly in the cytoplasm, not in vacuoles. Such granules

have not generally been found in prosobranchs (or in other gastropods), although granules which may correspond to those described here have been found in Nassarius (Martoja, 1961, 1964). The second type of cell usually described corresponds to the calcium cell, although given a variety of names. A few authors describe the second type of cell as an excretory cell (Morton, 1952; Owen, 1958; Andrews, 1965). In fact, both cell types are present. In Pila, excretory cells are particularly prominent, whereas calcium cells are not, and a similar situation in other species may have led some workers to believe that only excretory cells are present. A high content of iron appears to be characteristic of the calcium and excretory cells (Owen, 1958; Martoja, 1961, 1964). Martoja (1964) claimed to have found calcium as well as iron in these cells, but the methods she used were not in fact sufficiently specific to distinguish between calcium and iron. Apart from indicating a homology with the calcium cells of Stylommatophora, the name calcium cell is unsuitable for the conical, basiphil cells in Pila. These cells have been referred to as secretory cells in some prosobranchs (e.g. Graham, 1932), but no secretory product has been found in Pila, as it has in Helix (Papers 1 & 5).

The pigment of the granules in the excretory cells of Pila is interesting. The tests which are typically positive for lipofuscins are often only weakly positive or

negative with these granules. If no pigment other than lipofuscin were present, one would expect the reactions to be strongly positive, because of the darkness of the granules. The presence of iron, and the ease with which the granules are bleached by a variety of agents, suggest that the granules contain only a small proportion of lipofuscin, and that most of the colour is due to an iron-containing pigment.

An interesting feature in activating specimens of Pila is the formation of granules of lipofuscin in the vacuoles of calcium cells. Yellow granules have also been found in the vacuoles of calcium cells in Succinea, and provide further evidence for the suggestion that excretory cells are derived from calcium cells (Thiele, 1953; Papers 1 & 3). Many of the yellow granules of excretory cells in Succinea are the same size as the vacuoles of the calcium cells.

Since the yellow granules of digestive cells are derived from the colourless granules (Paper 4), a gradation between these granules, as occurs in Succinea, would be expected; nevertheless, in other species of molluscs studied, there is a sharp distinction between colourless and yellow granules (Papers 1 & 6).

The frequent lack of calcium in calcium cells of Succinea may be related to the low calcium content of the shell (Sumner, unpublished). Such a relationship to the

amount of calcium in the shell suggests that the calcium in the digestive gland may not be important in digestive processes, as has been suggested (Krijgsman, 1928; Yonge, 1937).

Testacella does not show any fundamental differences when compared with herbivorous forms. The differences are ones of degree rather than kind. Virtually all cells bear a brush border, instead of some only; there is far more basal lipid in the digestive cells; the calcium cells contain more protein granules; and there is very little tendency to form lipofuscin granules. These differences are not obviously related to a carnivorous diet, and at present there is no simple explanation for them. It is not clear, for example, why Testacella should need much greater stores of lipid in its digestive cells than other Stylommatophora. There is also no evidence for a reduction in phagocytosis in the digestive gland. The digestive cells have vacuoles containing colourless granules, just as herbivorous forms do; and there is no reason not to suppose that they are phagocytic in Testacella, as they are in Helix (Paper 4).

I thank Dr. J.R.Baker, F.R.S., for his help and supervision during the course of this work; and Professor J.W.S.Pringle, F.R.S., for accommodating me in his Department. The material from Pila was supplied by Professor

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TABLE 1. HISTOCHEMICAL TESTS USED

Test	Fixative	Thickness of sections (μ)	Method to show	Reference	Pila	Succinea	Testacella
Feulgen	Ze	6	DNA	Feulgen & Rossenbeck, 1924		+	+
Pyronine/methyl green (P/MG)	Ze3	6	RNA	Jordan & Baker, 1955	+	+	+
Ribonuclease + P/MG	Ze3	6	RNA	Bradbury, 1956	+	+	+
Periodic acid/ Schiff (PA/S)	Ze	6	Carbo-hydrates	MacManus, 1948	+	+	+
Diastase + PA/S	Ze	6	Glycogen	Lillie & Greco, 1947	+	+	+
Toluidine Blue (metachromasy)	Ze	6	Acid mucopoly-saccharides	———			+
Sulphation + toluidine blue	Ze	6	Neutral mucopoly-saccharides	Lison, 1953			+
DMAB/nitrite	F-Na	8	Tryptophan	Pearse, 1960	+	+	+
Millon	F-Na	8	Tyrosine	Baker, 1956a	+	+	+
Sakaguchi	Ze	8	Arginine	Baker, 1947			+
Peracetic acid /alcian blue	F-Na	8	Cystine	Pearse, 1960	+		+
Sudan black	F-Ca/PC	10	Lipids	Baker, 1956b	+	+	+
Acid haematein	F-Ca/PC	10	Phospho-lipids	Baker, 1946	+	+	+

Table 1. (continued).

Test	Fixative	Thickness of sections (μ)	Method to show	Reference	Pila	Succinea	Testacella
Cobalt substitution	FMP	10	Calcium	———	+	+	+
Calcium red	FMP	10	Calcium	McGee-Russell, 1955	+	+	
Perls' test	F-Na	8	Iron	Pearse, 1960	+	+	+
Hexamine-silver	Ze3	8	Urates	Pearse, 1960		+	
Long Ziehl-Neelsen (24h differentiation)	F-Na	8	Lipofuscins	Pearse, 1960	+	+	+
Peracetic acid/ Schiff	F-Na	8	Oxidised lipid	Pearse, 1960	+		
Bleaching with 10% hydrogen peroxide (48h)	F-Na	8	Lipofuscins	Pearse, 1960	+		

The following abbreviations are used: F-Ca/PC, formaldehyde-calcium with postchroming; FMP, formaldehyde-methanol-pyridine; F-Na, formaldehyde-saline; Ze, Zenker; Ze3, Zenker for 3h.

TABLE 2. AVERAGE DIMENSIONS OF CELLS

	Pila	Succinea	Testacella
Thin cells			
Width		2.8	
Nucleus		8.8 x 2.6	
Mitochondria		1.2 x 0.4	
Digestive cells			
Height	50.0	63.2	44.6
Width	15.9	12.0	7.3
Nucleus	8.0 x 6.6	8.0 x 6.4	7.1 x 5.5
Nucleolus	2.0 x 1.8	1.2 x 1.2	1.7 x 1.6
Vacuoles	3.7 x 3.2		2.5 x 2.3
"Green" granules	2.6 x 2.0	2.0 x 2.0	1.5 x 1.2
Small yellow granules	6.9 x 5.8		Not present
Protein granules	1.9 x 1.8	Not present	Not present
Mitochondria		1.6 x 1.0	1.0 x 0.5
Calcium cells			
Height	44.2	56.0	41.2
Width	15.9	36.0	12.2
Nucleus	8.0 x 6.6	20.8 x 20.8	8.8 x 7.4
Nucleolus	2.0 x 1.8	3.6 x 3.6	3.1 x 2.7
Spherules (diameter)	2.0	4.4	2.0

Table 2. (continued).

	Pila	Succinea	Testacella
Calcium cells (continued)			
Protein granules		4.6 x 4.6	1.4 x 1.2
Mitochondria		1.4 x 1.0	1.0 x 0.3
Excretory cells			
Height	38.9	48.0	40.4
Width	24.4	22.4	18.8
Nucleus	7.1 x 4.4		8.4 x 4.6
Nucleolus	1.8 x 1.5		1.5 x 1.4
Vacuole			19.4 x 15.9
Yellow granules	23.3 x 20.3	5.4 x 5.4	8.2 x 7.6
Mitochondria		0.6 x 0.6	1.2 x 0.4

All measurements are in μ.

The figures for Pila refer to feeding snails.

TABLE 3. RESULTS OF HISTOCHEMICAL TESTS ON THE LARGE GRANULES OF EXCRETORY CELLS IN PILA

Test	Result
Sudan black	Negative
Acid haematein	Negative in feeding snails, increasing to moderate positivity after 3 months' aestivation.
Periodic acid /Schiff	Negative or weakly positive. Unaffected by diastase digestion.
DMAB/nitrite	Negative. Pigment largely decolorized (whereas small yellow granules are not).
Millon	Negative or very weakly positive.
Peracetic acid /alcian blue	Moderately positive. Pigment of granules completely decolorized.
Basiphilia (pyronine at pH4.8)	Strongly basiphil.
Long Ziehl-Neelsen	Usually strongly positive, but sometimes only weak reaction.
Bleaching with 10% hydrogen peroxide	Pigment largely or wholly bleached, sometimes leaving groups of tiny black granules.
Peracetic acid /Schiff	Pigment completely decolorized, leaving mass of tiny black granules. Reaction usually negative, but sometimes weakly or moderately positive.
Perls' test for iron	Positive reaction, but positivity often restricted to outer layers of granule.

PAPER 3

THE FINE STRUCTURE OF DIGESTIVE GLAND CELLS OF HELIX, SUCCINEA AND TESTACELLA

(Accepted for publication in the Journal of the Royal Microscopical Society).

SYNOPSIS

Helix, Succinea, and Testacella show similar fine structure in their digestive gland cells. The fine structure found in Helix is similar whether the fixative is osmium tetroxide or formaldehyde-sucrose.

There is a good correlation between the results of light and electron microscopy. The cytoplasm of thin and calcium cells, which contains RNA, is found with the electron microscope to contain much ergastoplasm; in the same cells, the objects blackened by the Kaiserling-Lepehne method are typical lamellar-vacuolar fields. The brush borders of the digestive gland cells consist of microvilli.

The cytoplasm of digestive cells consists of a finely granular ground substance in which lie numerous small vesicles, which may be pinocytotic vesicles; pinocytosis channels are sometimes visible apically. Further down the cell there are the large vacuoles visible with the light microscope. The lipofuscin granules in the more basal vacuoles have the same fine structure as mammalian lipofuscin granules.

Electron microscopy shows that there is an intermediate stage between calcium and excretory cells, called early excretory cells. The fine structure of early excretory and excretory cells shows clearly that they are degenerating calcium cells.

INTRODUCTION

There have been numerous light microscopical studies of the digestive gland cells of pulmonate gastropods (e.g. Barfurth, 1883; Fremsel, 1885; Krijgsman, 1925, 1928; Thiele, 1953; McGee-Russell, 1955; Sumner, 1965a), and the structure of the cells at this level is now well known. However, there has been only one electron microscopical study of digestive gland cells of pulmonates (David & Götze, 1963). Since some of the results and conclusions of that study were at variance with the results of light microscopical studies, it was clearly necessary to carry out a more detailed electron microscopical study of the digestive gland cells. This paper describes the fine structure of digestive gland cells of three Stylommatophora: Helix, a typical herbivorous form; Succinea, generally considered to be a primitive herbivorous form (Morton, 1958); and Testacella, a carnivorous form.

MATERIAL AND METHODS

The digestive glands of the snails Helix aspersa and Succinea putris, and of the slug Testacella maugei, were used for this study. The specimens of Helix used were either feeding, or had been starved for 20 days; the specimens of Succinea were feeding. The specimens of Testacella were supplied with food, but no food was found in any part of their alimentary systems when they were dissected; thus it seems most likely that they were in a state corresponding

to the short-starved state in Helix (Sumner, 1965a).

Small pieces of digestive gland tissue were fixed in cold 1 per cent osmium tetroxide in distilled water for 1h, dehydrated in ethanol without any washing in water, and embedded in araldite. Some pieces of digestive gland tissue from a feeding specimen of Helix were fixed in cold formaldehyde-sucrose for 3h (Baker, 1965), rinsed with distilled water, and post-fixed in cold 2 per cent osmium tetroxide for 2h. The tissue was then dehydrated and embedded as before. Silver or gold sections were cut and mounted on grids for examination with an Akashi TRS 50 electron microscope.

Sections were stained before examination, usually with a saturated solution of uranyl acetate in 50 per cent ethanol (Gibbons & Grimstone, 1960). Some sections were stained with a solution of uranyl acetate in methanol, and some blocks of tissue were stained in bulk with 1 per cent uranyl nitrate in 96 per cent ethanol before embedding; neither of these methods gave such good results as the method of Gibbons & Grimstone.

RESULTS

The terms used here for digestive gland cells and their inclusions are those used in an earlier paper (Sumner, 1965a).

Helix aspersa

The results obtained with the two fixatives used were substantially similar; the minor points of difference will be mentioned

in due course. No differences have been found in the fine structure between feeding snails and those starved for 20 days.

The cytoplasm of thin cells is full of ergastoplasm (the term ergastoplasm is used here in the sense defined by Haguenau (1958)). Immediately apical to the nucleus is the Golgi apparatus; this consists of a single lamellar-vacuolar field (Hirsch, 1961) containing the usual three components (Sjöstrand & Hanzon, 1954; Sjöstrand, 1956; Dalton and Felix, 1957). Mitochondria are distributed along the cell, and have the typical fine structure.

The free surface of a digestive cell usually bears microvilli, which are separated from each other by half to twice their own thickness. Occasionally microvilli are much scarcer, or are absent altogether, in which case the free surface of the cell is straight or bulges outwards slightly. Since calcium and excretory cells also usually bear microvilli, a full description is given here. No differences in structure have been found between the microvilli of different cell types. Microvilli in tissue fixed in osmium tetroxide average 84mμ in diameter and 0.93μ in length, while those fixed in formaldehyde-sucrose average 58mμ in diameter and 0.99μ in length; and are thus considerably thinner. Microvilli (Fig.1A) are covered by an extension of the plasma membrane, which is a unit membrane, 11 to 17 mμ thick. The structure of the plasma membranes, and the membranes of the small vesicles in the apical cytoplasm of digestive cells (see below) is unusually clear (Figs. 4A & B) and the membranes

See over.

Fig. 1. A. Apical cytoplasm of a digestive cell (*Testacella*).

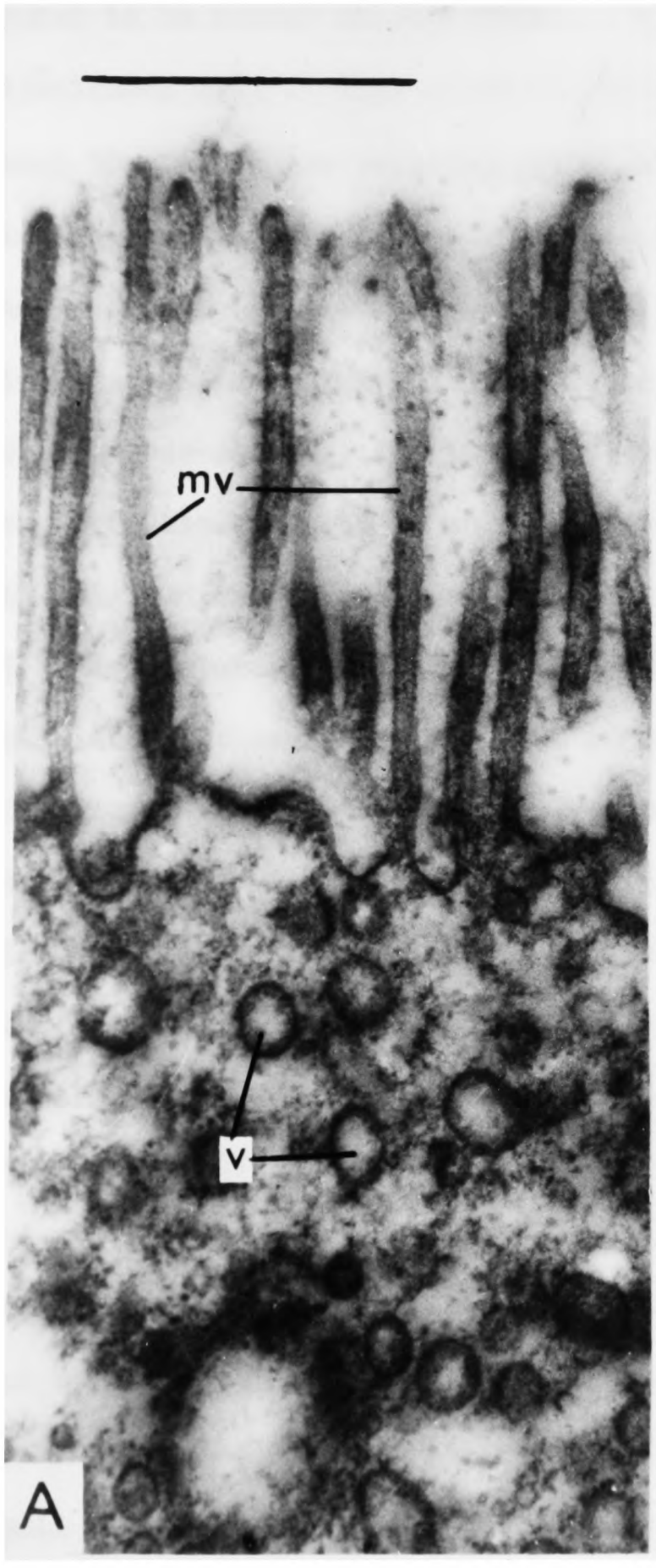
B. Vacuolated cytoplasm of a digestive cell, showing 'green' and yellow granules (*Succinea*).

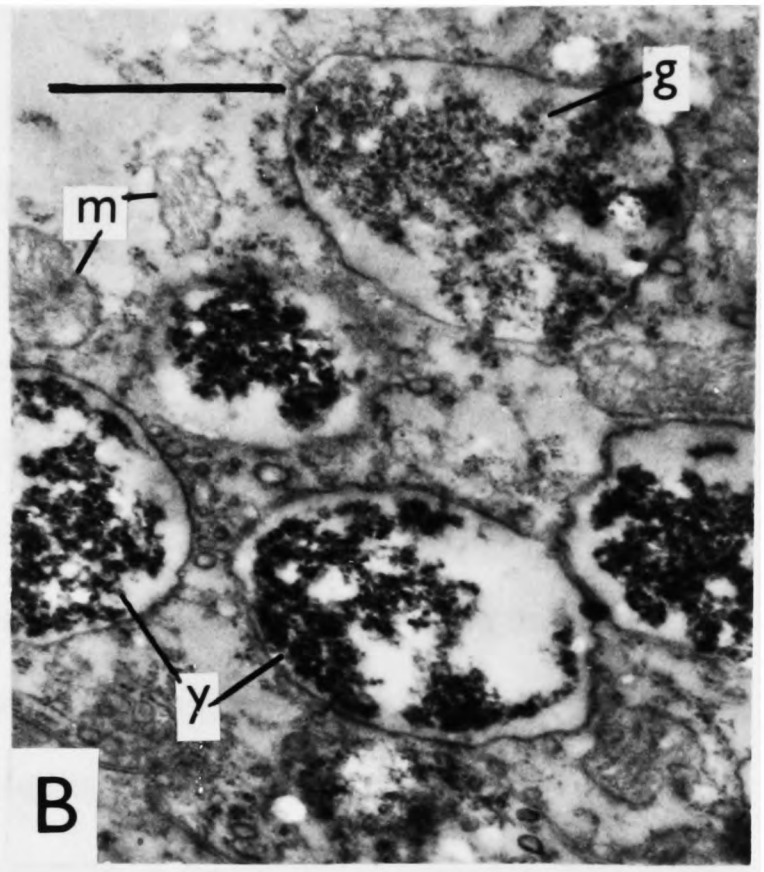
C. A yellow granule of a digestive cell (*Helix*).

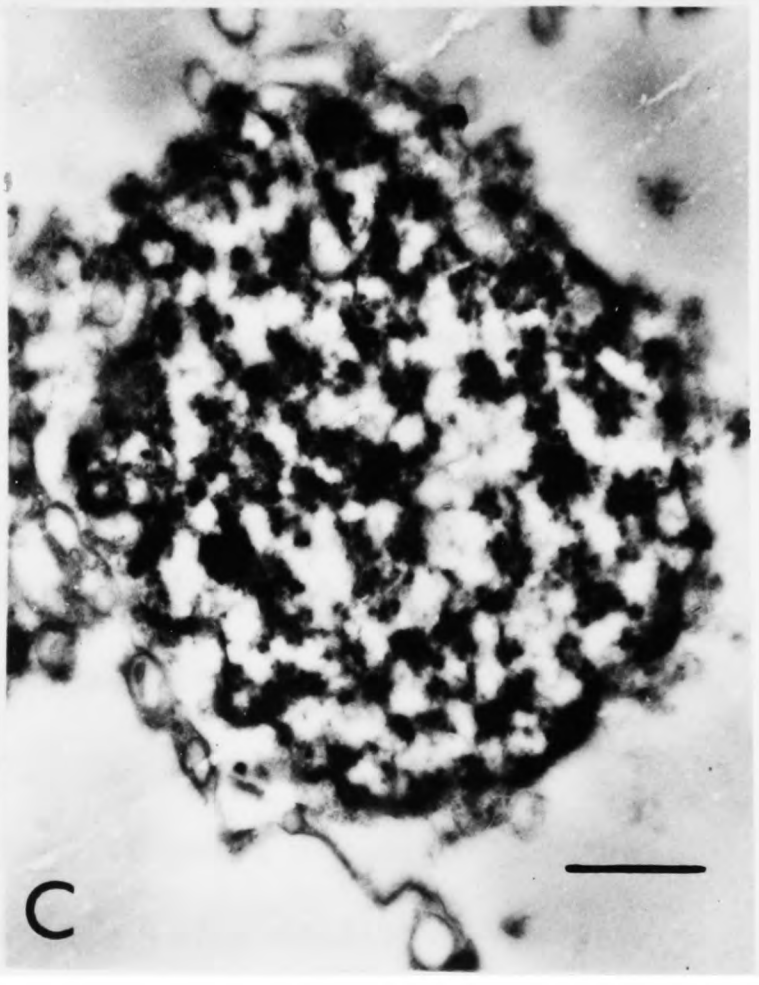
All micrographs are of tissue fixed for 1h in 1% osmium tetroxide in distilled water, and stained with uranyl acetate.

g, 'green' granule; *m*, mitochondrion; *mv*, microvilli; *v*, vesicles; *y*, yellow granules.

The scale on each micrograph represents 1μ.


mv
v
A


g
m
y
B


C

appear to be rather thicker than has been described previously (Sjöstrand, 1963). When fixed in osmium tetroxide, central filaments about 10mμ thick are visible; often only one or two filaments are visible, but up to 4 or 5 can sometimes be seen, grouped together in the centre of the microvillus. Occasionally the central filaments give the appearance of a ring in cross-section. When fixed in formaldehyde-sucrose, the internal structure of the microvilli is more variable. Sometimes no axial structures are visible. Sometimes (in longitudinal section) a pair of lines, each about 5mμ thick, and 17 to 44mμ apart, run along the length of the microvillus; it is not clear whether this appearance represents a pair of filaments or a tubule. A third variation in appearance is the presence of a pale core, 33 to 67mμ wide, around which is a darker region, 22 to 33mμ wide; the two regions are not separated by a membrane. Occasionally there is a layer of amorphous material, sometimes containing small granules, and sometimes appearing as a meshwork, along the tips of the microvilli.

Microvilli sometimes appear to be branched. In cross-section they sometimes appear to be dumb-bell shaped, or are connected by a thin curved line, 20mμ thick.

The apical cytoplasm of digestive cells contains numerous small vesicles, 100 to 450mμ in diameter (Fig.1A), delimited by a membrane, and usually having no visible contents. Occasionally, however, there is a layer of amorphous material, about 40mμ thick, on the inner

surface of the membrane of the vesicles. When the tissue is fixed in osmium tetroxide, the spaces between the vesicles appear largely empty, only a few scattered granules being present; when the tissue is fixed in formaldehyde-sucrose, the spaces are full of granular material. Occasionally there are what are believed to be pinocytosis channels extending into the apical cytoplasm, from between the bases of the microvilli. Below this region (in which there are no vacuoles visible with the light microscope) there are the apical vacuoles, and below them, the 'green' granule vacuoles. (The 'green' granules are so called because they stain readily with the dye light green (Krijgsman, 1928). All these vacuoles contain some material, although often very little in the apical vacuoles. This material appears as an irregular, homogeneous grey mass. Further down the cell, near the base, are vacuoles containing the yellow granules. The yellow granules are easily distinguished from the 'green' granules by their high electron density, and by their structure (Fig.1C); each yellow granule is made up of smaller granules, 0.4 to 0.6μ across, which are themselves made up of smaller granules.

In between the vacuoles containing the 'green' and the yellow granules, the cytoplasm contains small vesicles, like those in the apical cytoplasm, but not so closely packed; a few cisternae of ergastoplasm, mitochondria, lipid droplets, and paired membranes, sometimes single and sometimes grouped together. In tissue fixed in osmium tetroxide there is also a fair amount of empty space, but in

tissue fixed in formaldehyde-sucrose this space is filled with granular material, as in the apical cytoplasm. The mitochondria may have transverse, oblique or longitudinal cristae, but all the cristae in any one mitochondrion are roughly parallel. All or most of the cristae extend right across the mitochondrion.

The nucleus is near the base of the cell, and is bounded by a double membrane containing a few pores. The nucleoplasm contains large and small electron-dense bodies, which probably represent nucleoli and chromatin.

Most of the cytoplasm of calcium cells is filled with ergastoplasm (Fig2), among which are the calcium spherules and numerous mitochondria. The ergastoplasm consists of cisternae which are usually flattened, and about 20 to 40 mμ thick, although they sometimes appear swollen. The cisternae usually run approximately parallel to each other for variable distances, which are usually rather greater in tissue fixed in formaldehyde-sucrose than in tissue fixed in osmium tetroxide. The outer surfaces of the cisternae bear numerous ribosomes, about 15mμ in diameter.

The calcium spherules are bounded by a single membrane, which is often rather sinuous; immediately within this is a space of low electron density, varying in width from 80 to 575mμ. The central part of the spherule normally contains highly electron-dense material, usually with longitudinal striations (which are probably chatter marks produced in sectioning), surrounded by two rings of electron dense

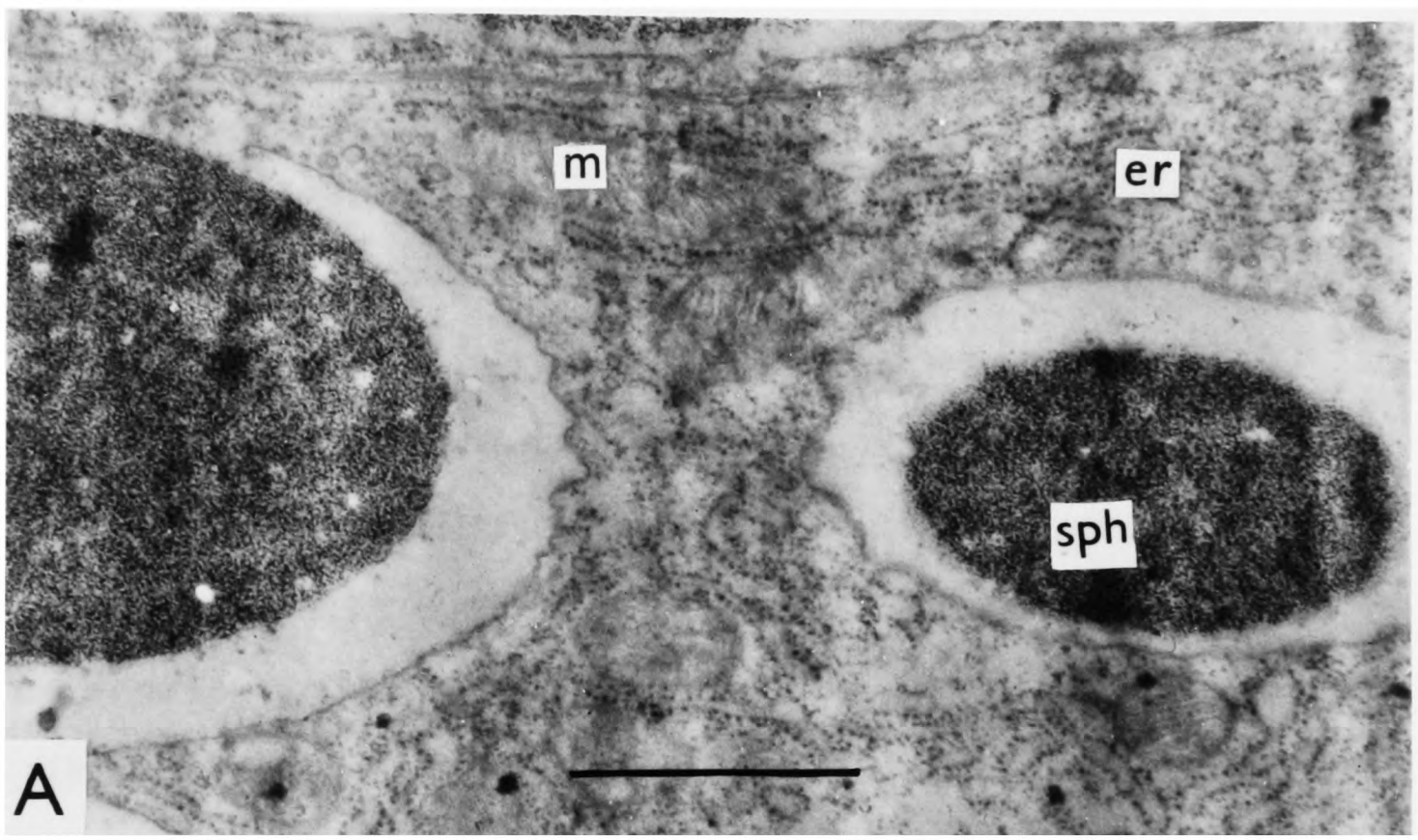
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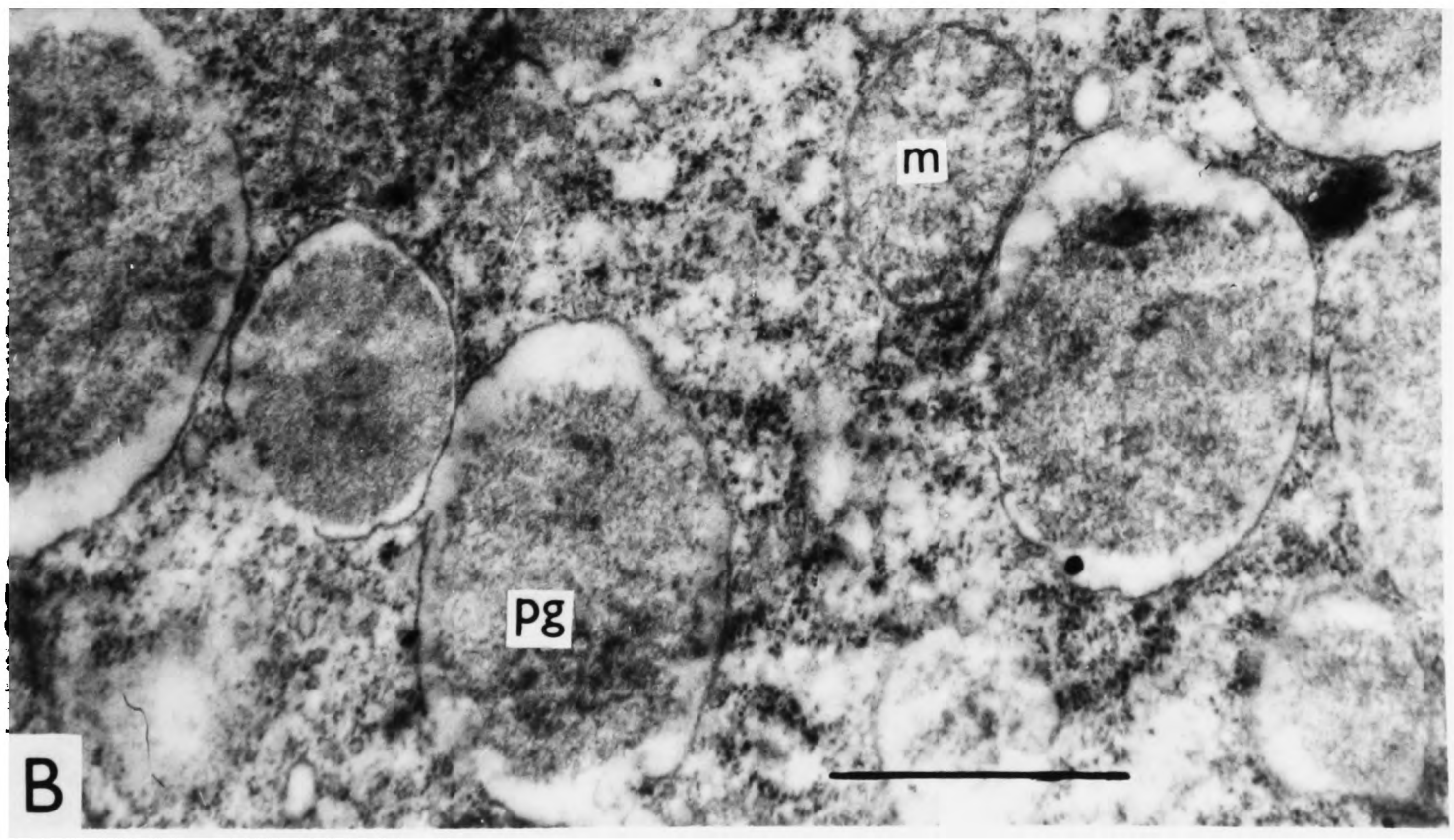
Fig. 2. A. Cytoplasm of a calcium cell (Succinea).
B. Apical cytoplasm of a calcium cell (Testacella).

All micrographs are of tissue fixed for 1h in 1% osmium tetroxide in distilled water, and stained with uranyl acetate.

er, ergastoplasm; m, mitochondrion; pg, protein granule; sph, spherule.

The scale on each micrograph represents 1 μ.


m
er
sph
A


m
pg
B

particles, 40 to 160 mμ wide, separated by a pale space, 10 to 100 mμ wide. Sometimes the electron dense material is missing, probably having been torn out during sectioning.

Mitochondria are numerous throughout the cytoplasm. The cristae may be transverse, oblique, or occasionally longitudinal, and almost always extend right across the mitochondrion.

The apical surface of calcium cells has a domed outline, and usually bears microvilli, but they may be few and rather small, or occasionally absent altogether. The apical cytoplasm has rather little ergastoplasm, and the cisternae tend to be swollen; sometimes the ribosomes appear to be free. The apical cytoplasm may also contain small vesicles about 150mμ in diameter, and one or two multivesicular bodies, 200 to 450 mμ in diameter.

Towards the base of the cell, elements of the Golgi apparatus can be found. They each consist of 5 to 6 parallel, slightly curved cisternae, each about 40 mμ thick and separated from each other by a space about 15 mμ wide; they are up to 1.86 μ long. On the concave side of the cisternae are bodies 240 to 400 mμ in diameter, with granular contents and a limiting membrane. Small vesicles, 40 to 160 mμ in diameter, occur mostly on the convex side of the cisternae, but also on the concave side, and at the ends of the cisternae.

The nucleus of calcium cells is towards the base, and closely resembles that of digestive cells (see p.); the outer nuclear membrane sometimes bears ribosomes. In the cytoplasm around the nucleus there are bodies with granular contents of moderate electron

density, and with a single limiting membrane; they average 740 by 500 mμ.

At the base of the cell, the plasma membrane is invaginated to form β-cytomembranes (Björstrand, 1956), which are usually highly sinuous.

As well as the typical excretory cells of the pulmonate digestive gland, which contain one or more large yellow granules in a single large vacuole, electron microscopy reveals cells in which there are round electron dense granules each lying in a separate vacuole. These are called here early excretory cells.

The apical surface of early excretory cells usually bears microvilli. Immediately below the brush border is a layer of dense granular cytoplasm, 80 to 100 mμ wide. The rest of the cytoplasm contains rather empty, consisting of short tubules, 80 to 240 mμ long, and 20 mμ in diameter; the tubules are usually bent (Fig. 3A). The cytoplasm contains mitochondria with transverse or oblique cristae, multivesicular bodies, and large vacuoles, about 2.5 mμ in diameter, and containing round dense homogeneous bodies, 1 to 1.5 μ in diameter.

The cytoplasm of excretory cells (Fig.3B) consists of a layer 0.6 to 0.7 μ thick surrounding the central vacuole; however, the layer of cytoplasm is sometimes thicker apically and basally. The cytoplasm contains small vesicles, about 100 mμ in diameter, and larger vacuoles, occupying nearly the whole width of the cytoplasm and often rather irregular in shape. The cytoplasm in between the vacuoles and and vesicles is filled with finely granular material. Occasionally,

See over.

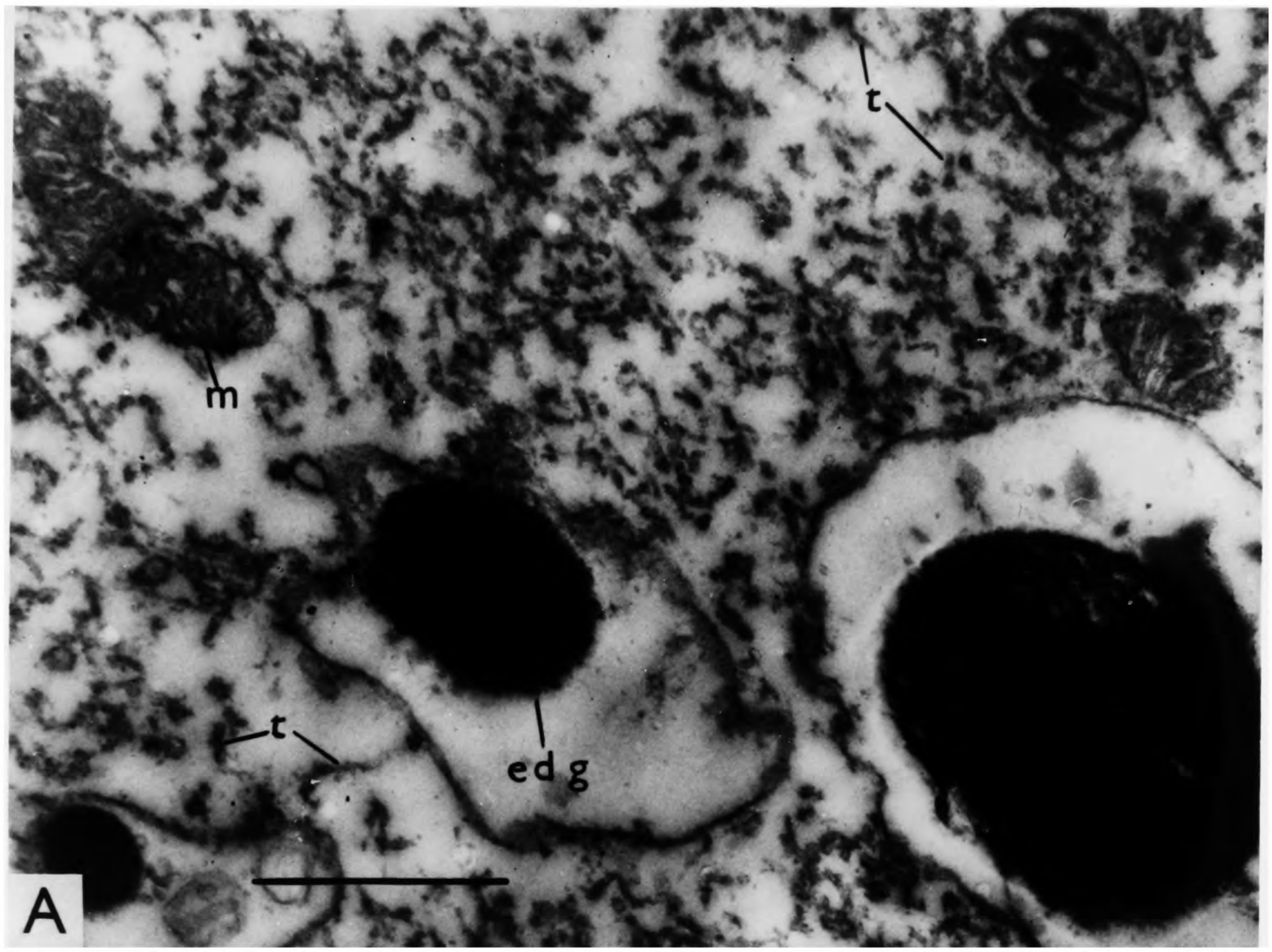
Fig. 3. A. Early excretory cell cytoplasm (Helix).

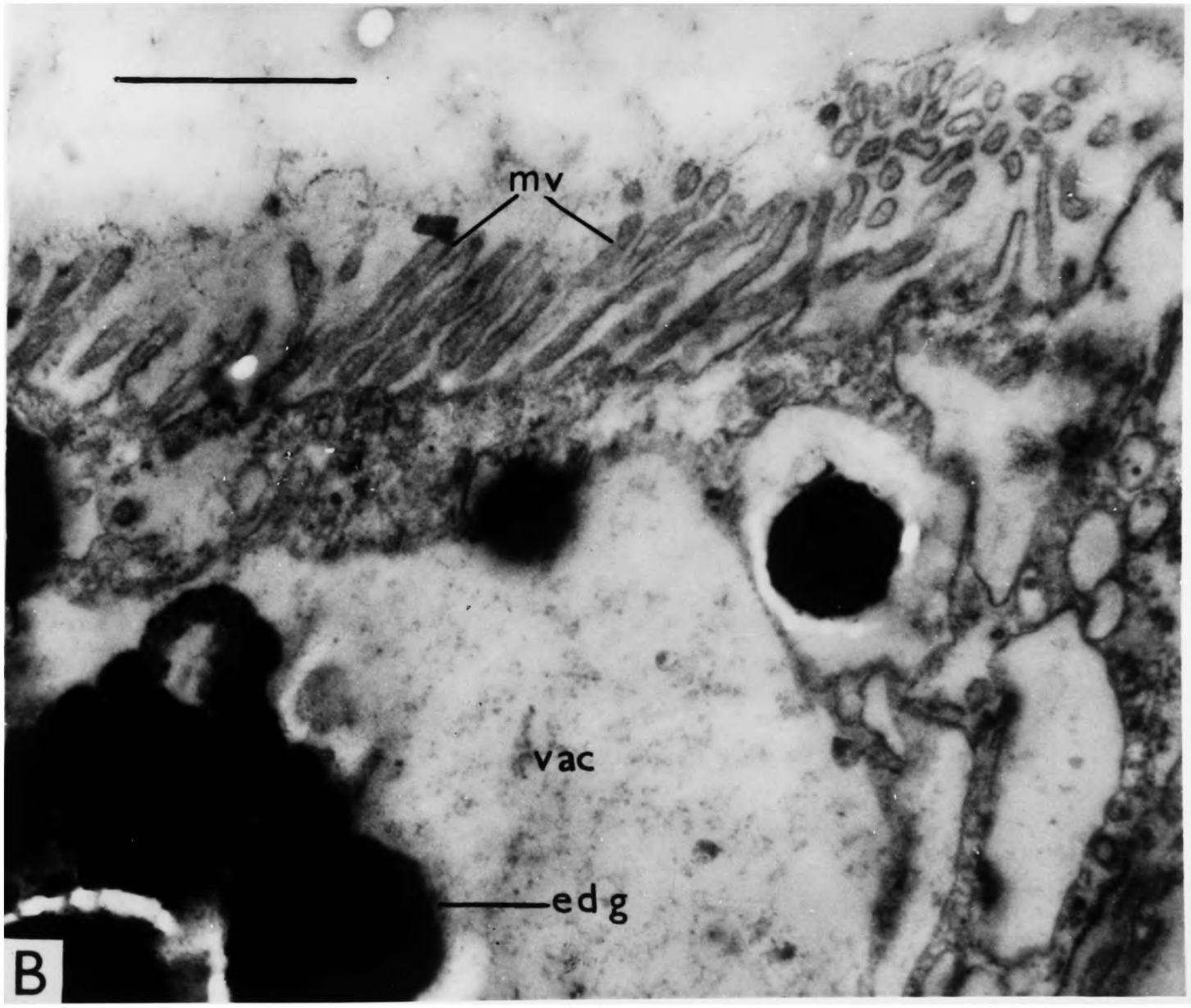
B. Part of an excretory cell (Succinea).

Both micrographs are of tissue fixed for 1h in 1% osmium tetroxide in distilled water, and stained with uranyl acetate.

edg, electron dense granule; m, mitochondrion; mv, microvilli; t, tubules; vac, vacuole..

The scale on each micrograph represents 1 μ.


t
m
t
edg
A


mv
vac
edg
B

particularly towards the base of the cell, there are a few cisternae of ergastoplasm. A few mitochondria, with the typical fine structure, are sometimes present. The apical surface of excretory cells often bears microvilli; however, they may be completely absent, in which case the cytoplasm is practically empty. The vacuole of an excretory cell contains one or more large round homogeneous electron dense bodies, similar to the bodies found in the large vacuoles of early excretory cells.

Succinea putris

Only the differences between the fine structure of digestive gland cells of Succinea and Helix are described here.

In the digestive cells, the 'green' granules show a granular sub-structure, instead of the homogeneity found in Helix. The yellow granules of Succinea differ from the 'green' granules only in their greater electron density; there is no change in sub-structure as found in Helix (Fig.1B).

The spherules in the calcium cells of Succinea (Fig.2A) differ radically from those of Helix. As in Helix, the spherules are bounded by a single membrane, immediately within which is a space of low electron density, 0 to 430 mμ wide. The central part of the spherule is full of granules, about 7 mμ across; sometimes the granules are less densely packed in the centre. A few spherules have no material in the centre, possibly because it has been torn out in sectioning.

In Succinea, as in Helix, there are often membrane-bounded

bodies with granular contents in the cytoplasm around the nucleus of calcium cells. In Succinea, some of these bodies contain an irregular mass of granular material of high electron density.

Testacella maurei

Compared with Helix and Succinea, the microvilli of the digestive gland cells of Testacella are longer and slightly thicker, averaging 95 mμ in diameter and 1.33 μ in length. The longest microvillus measured was 1.86 μ long. The fine structure of the microvilli is the same as that found in Helix and Succinea, but in Testacella the central filaments of the microvilli can often be seen to extend for up to 200 mμ into the underlying cytoplasm. Branched microvilli occasionally occur.

Microtubules often occur in digestive and calcium cells of Testacella. They are mostly about 14 mμ in diameter, but vary between 10 and 20 mμ. Although only short lengths are usually visible, they may extend for up to 2.0 μ.

Mitochondria in Testacella are larger than those in Helix and Succinea, averaging 1.17 by 0.51 μ, and often extending up to 1.7 μ long. The cristae are rather sparse: they are often sinuous, and run in all directions, even in the same mitochondrion. Much of the matrix of the mitochondria is occupied by granular material.

In digestive cells, there are sometimes small invaginations in the apical cytoplasm, between the microvilli, which are presumably pinocytosis channels. The small vesicles in the apical cytoplasm of

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digestive cells usually have a lining of fluffy material on the inner surface of their limiting membranes, as is occasionally found in Helix and Succinea (see p.8) (Fig.1A). At high magnifications, this lining is seen to consist of filaments, 30 to 55 mμ high, with indistinct outlines.

Lipid droplets are numerous in digestive cells. Relatively few occur apical to the nucleus; these are 0.6 to 1.6 μ in diameter. Most of the cytoplasm basal to the nucleus consists of nothing but lipid droplets, 0.8 to 2.2 μ in diameter; usually there is no material between the droplets, and often there are large empty spaces, which are probably an artifact.

The calcium cells of Testacella differ from those of Helix and Succinea in that they contain numerous large membrane-bounded bodies in the apical cytoplasm (Fig.2B). These bodies vary from about 0.63 μ to about 2.5 μ in diameter, and average about 1.34 μ. The contents of these bodies are granular, and are separated from the membrane by a clear zone , 40 to 280 mμ wide.

The calcium spherules of Testacella resemble those of Helix.

Intercellular connections

The plasma membranes of all cells have a three-layered structure. Where the plasma membranes of two cells are adjacent, the three layered structure appears asymmetrical in tissue fixed in formaldehyde-sucrose; the inner dark layer appears to be about twice the thickness of the outer dark layer. At the most apical point where

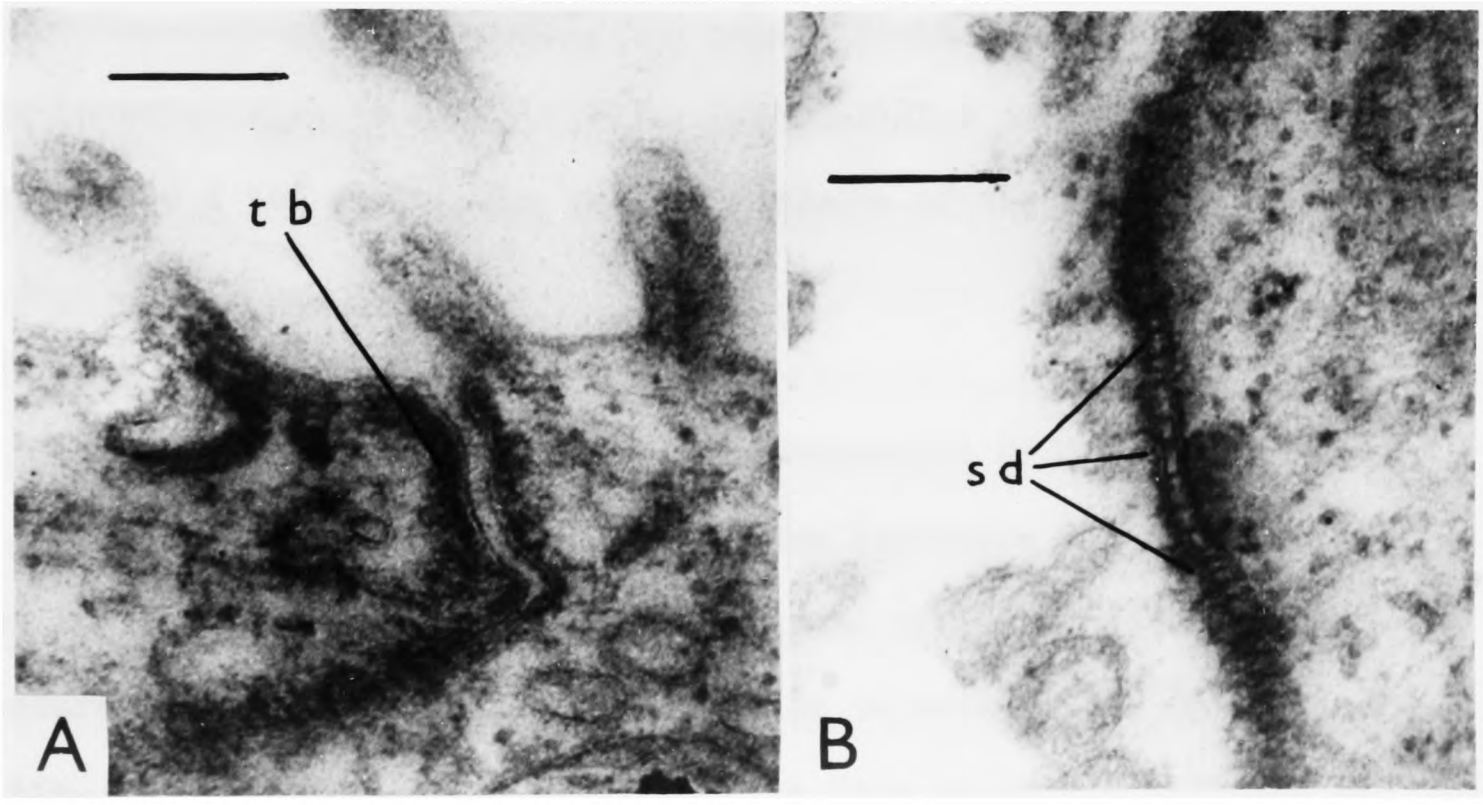
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Fig. 4. Intercellular connections. A. Terminal bar. B. Septate desmosome. In both micrographs the structure of the unit membranes can be clearly seen.

Both micrographs are of tissue fixed for 1h in 1% osmium tetroxide in distilled water, and stained with uranyl acetate.

sd, septate desmosome; tb, terminal bar.

The scale on each micrograph represents 0.2 μ.


t b
A
s d
B

the plasma membranes of two cells are adjacent, there is a mass of electron dense material on the inner surfaces of the membranes (Fig.4A); this resembles the terminal bar described in mammals (Palay & Karlin, 1959). There is no indication of any linkage between the adjacent membranes at this point. Further from the luminal surface of the cells, the adjacent plasma membranes are often obscured by a mass of granular material, which often has transverse striations; in other places cross-connections between the two plasma membranes can be seen (Fig.4B). These cross-connections resemble the septate desmosomes described in other invertebrates by Wood (1959). Intercellular connections appear to extend to 2 to4 μ from the luminal surfaces of the cells.

DISCUSSION

The fact that there is no fundamental difference between the fine structure of cells fixed in osmium tetroxide and of cells fixed in formaldehyde-sucrose provides strong evidence that the fine structure described in this paper is a good representation of what exists in life. The most important difference in fine structure produced by the different fixatives is the presence of a granular ground substance in digestive cells in tissue fixed in formaldehyde-sucrose; and it seems unlikely that the ground cytoplasm would be empty in life, as found after fixation in osmium tetroxide.

On the whole there is a good correlation between the results of this electron microscopical study, and light microscopical studies of Helix, Succinea and Testacella (Sumner, 1965a; Paper 2).

For example, the cytoplasm of thin cells often contains RNA, and the Mann-Kopsch method shows the Golgi apparatus immediately apical to the nucleus. With the electron microscope, the cytoplasm which contains RNA is found to contain ergastoplasm; and immediately apical to the nucleus there is a typical lamellar-vacuolar field of the Golgi apparatus.

A variety of axial structures have been found in microvilli from mammalian epithelia, usually either filaments or tubules (Palay & Karlin, 1959; Brown, 1962; Hansson & Herman, 1962; Millington & Finean, 1962; Kjaerheim, 1963; McNabb & Sandborn, 1964). A wide variety of methods of fixation was used by these workers. Different types of axial structure have also been found in species studies here. The predominant form of axial structure, both here and in mammals, is one or more filaments; but since in Helix different axial structures can be found in the different microvilli of the same cell, it seems probable that the axial structures of microvilli are highly labile.

David & Götze (1963) described the fine structure of the cytoplasm of digestive and of calcium cells as being the same; this is clearly not so, as shown in this study, and it seems probable that David & Götze only described digestive cells. Their description agrees largely with what has been found here for digestive cells. However, they describe the small vesicles found throughout the cytoplasm of digestive cells, particularly apically (see p.8), as agranular endoplasmic reticulum. There seems to be no reason for this, and in fact

these vesicles do not appear to be components of any system generally distributed in cells. The presence of amorphous material on the inner surface of the membrane of these vesicles suggests that they might be pinocytotic vesicles. (Howett, 1965). It is interesting that the absorbing cells of the gastroderm of hydroids have a similar fine structure to the digestive cells of pulmonates, and that in hydroids the small vesicles in the apical cytoplasm are phagocytic vesicles (Beauthier, 1963; Afzelius & Rosen, 1965).

The yellow granules of digestive cells, which consist of lipofuscin (Sumner, 1965a), have a similar fine structure to lipofuscins from various mammalian tissues (Duncan et al., 1960; Wissner & Novikoff, 1960; Malkoff & Strehler, 1963). The fact that the yellow granules are made up of smaller granules no doubt accounts for their granular appearance when seen with the light microscope (Sumner, 1965a). The similarity in fine structure of 'green' and yellow granules in Succinea provides further evidence for the derivation of yellow granules from 'green' granules (Sumner, 1965b). In view of this, it is surprising that there is no resemblance between the fine structure of 'green' and of yellow granules in Helix.

According to David & Götze (1963) the mitochondria of digestive cells have longitudinal cristae. Although in this study mitochondria with longitudinal cristae have been found, in calcium as well as in digestive cells, mitochondria with transverse or oblique cristae are more usual. André (1962) also found considerable variation

in the direction of the cristae in undifferentiated cells of the germinative epithelium of the ovotestis in Testacella, so it seems that great variation in the direction of the cristae may be characteristic of pulmonates.

As would be expected, the cytoplasm of calcium cells, which contains a high concentration of RNA (Rumner, 1965a), is found to be full of ergastoplasm. However, electron microscopy does not give much information about the structure of the spherules in calcium cells. Presumably the highly electron-dense material in the spherules in Helix and Testacella is calcium compounds; no highly electron dense material is present in Succinea, where calcium is usually absent from the spherules. (Paper 2).

It is not clear what the membrane-bounded bodies with granular contents found around the nucleus in calcium cells are. They show some resemblance to the fine structure of lysosomes, as described by Baams & van Rijssel (1961), but no acid phosphatase has been found in these sites (Paper 8). The presence of this enzyme is characteristic of lysosomes (de Duve, 1963).

Early excretory cells are clearly derived from calcium cells. The ergastoplasm has broken down and instead there are the short bent tubules characteristic of early excretory cell cytoplasm; numerous intermediate stages can be found between cells where the cytoplasm is full of ergastoplasm, and where it is completely replaced by tubules. The typical spherules of calcium cells have apparently

changed into round, homogeneously electron-dense bodies in vacuoles. Early excretory cells are evidently what appear in light microscopical preparations as calcium cells with yellow lipofuscin granules in place of the normal spherules. Fully developed excretory cells have similar lipofuscin granules which are round and homogeneously electron-dense. Thus the lipofuscin granules in these cells have a different fine structure from the lipofuscin granules of digestive cells, and of mammalian cells (Duncan et al., 1960; Sumner & Novikoff, 1960; Walkoff & Strehler, 1963). It is not obvious what sort of differences between the lipofuscins of digestive and of excretory cells this difference in fine structure could reflect, but it might be due to a different physical state of the material from which the lipofuscin is formed. The fine structure of early excretory cells and of excretory cells is completely consistent with the view that they are degenerate and derived from calcium cells (Sumner, 1965a).

It is interesting that the intercellular connections found in the digestive glands of Helix, Succinea and Testacella are similar to those found in animals belonging to a large variety of unrelated phyla. Septate desmosomes have been found previously in mammals, coelenterates, platyhelminths, annelids and echinoderms (Wood, 1959) and in insects (Tsubo & Brandt, 1962; Wiener et al, 1964; Locke, 1965), but apparently have not been found in molluscs before.

The large membrane-bounded bodies which are present in the apical cytoplasm of calcium cells in Testacella are without doubt

protein granules. These are much more numerous in Testacella than in Helix or Succinea (Paper 2), where they have not been found by electron microscopy.

The numerous bodies in the digestive cells of Testacella which are blackened by the Mann-Kopsch method (Paper 2), are evidently not lamellar-vacuolar fields, which are very scarce in electron micrographs of these cells. It seems most likely that it is the mitochondria which are blackened.

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PAPER 4

EXPERIMENTS ON PHAGOCYTOSIS AND LIPID ABSORPTION IN THE ALIMENTARY SYSTEM OF *HELIX*

(Accepted for publication in the Journal of the Royal Microscopical Society).

SYNOPSIS

Carmine particles and lipids are taken up by the digestive cells of the digestive gland of Helix. These substances are taken up by phagocytosis, and the apical and "green" granule vacuoles (Sumner, 1965) of the digestive cells are phagocytosis vacuoles. Carmine eventually becomes incorporated into the small yellow granules, showing that they are derived from the "green" granules. Lipids are also taken up by the epithelia of the digestive gland ducts, intestine, and rectum. Phagocytosis has not been found in these sites, and the process appears to resemble that found in vertebrates.

INTRODUCTION

There has been much uncertainty about the site and mechanism of food absorption in pulmonates. Cuénot (1892, 1899) introduced soluble colourants and iron saccharate into the gut of pulmonates, and found that they were taken up by the digestive gland, but not by the intestine. Jordan (1918) described phagocytosis in the digestive gland of Helix, and this was confirmed by the careful studies of Rosen (1941, 1952). Nevertheless, Krijgsman (1928), Horstadius (1933), and Horstadius & Horstadius (1940) failed to find any evidence of phagocytosis in the digestive gland of Helix.

Jordan (1918) stated that no active absorption or

phagocytosis occurred in the intestine of Helix, across the walls of which substances can easily diffuse (Jordan & Begemann, 1921). Guardabassi & Ferreri (1953) showed that absorption of lipids occurred throughout the intestinal epithelium, and also in the epithelium of the digestive gland ducts, but they did not find any uptake of lipid in the digestive gland itself. Uptake of lipid by digestive gland cells has been described in a nudibranch (Millott, 1937), but in chitons (Fretter, 1937) and lamellibranchs (George, 1952) lipid absorption has only been found in other sites.

All these studies were concerned primarily with establishing the existence of absorption and phagocytosis. Therefore, as well as investigating whether or not lipid absorption occurs in the digestive gland of Helix, the fates of absorbed and phagocytosed materials after they had entered the cells were studied, to establish the functions of different cell types and their inclusions.

MATERIAL AND METHODS

The snail Helix aspersa was used throughout the present study. The snails were lightly anaesthetized with chloroform, and the food material injected into the oesophagus with a fine pipette, by way of the mouth. This method made certain that the snail received the food material, and the time at which the food entered the snail

was also known. It could be seen through the skin that the food material immediately passed back to the crop.

Carmine, olive oil, and milk were the food materials used. Carmine consists mainly of carminic acid bound to protein, and is insoluble in distilled water (Baker, 1960); thus it may be regarded as an insoluble coloured protein. Its use for studying phagocytosis has been criticized, because it is soluble in alkalis (Horstadius, 1933), but this criticism does not seem to be valid, as carmine can be found as particles throughout the alimentary system. In this study carmine was injected into several snails as a suspension in distilled water, and portions of digestive gland and intestine were fixed in formaldehyde-saline or in 96 per cent ethanol, at 1, 5, 9, and 14 days after the injection. The tissue was embedded in paraffin; sections were cut at 8μ, and examined unstained.

In the experiment with olive oil, two injections were made, 1¼ and 2½ h before fixation; portions of digestive gland, intestine and rectum were fixed in formaldehyde-calcium, post-chromed, embedded in gelatine, sectioned at 10μ, and coloured with Sudan black (Baker, 1956). Snails injected with milk were fixed 25 min or 1h after injection. They had previously been starved for 66 to 69 days, to remove the lipid from the digestive gland cells (Sumner, 1965). Portions of digestive gland, crop

and intestine were fixed in formaldehyde-calcium and post-chromed, or in Lewitsky-saline, and embedded in gelatine, sectioned at 10µ, and coloured with Sudan black (Baker, 1956). Formaldehyde-calcium with post-chroming was found to give better results than Lewitsky-saline.

Tissues from snails injected with lipid were compared with tissues from control snails which had not been injected with lipid.

RESULTS

The terms used here for digestive gland cells and their inclusions are those used in an earlier paper (Sumner, 1965).

Carmine particles are taken up only by the digestive cells of the digestive gland, and not elsewhere in the digestive gland, nor by the intestinal epithelium. One day after injection, very small carmine particles are present in the apical cytoplasm of digestive cells. 5 days after injection carmine particles can be seen in vacuoles in the apical part of the cells; apart from the contents, these vacuoles are identical with those containing the "green" granules. At this stage, and also 9 days after injection, carmine also occurs in the basal part of the cell, forming granules with the same vacuolar or granular sub-structure that the small yellow granules of the same cell have. 14 days after injection, there are three kinds of coloured granule in the basal part of digestive cells,

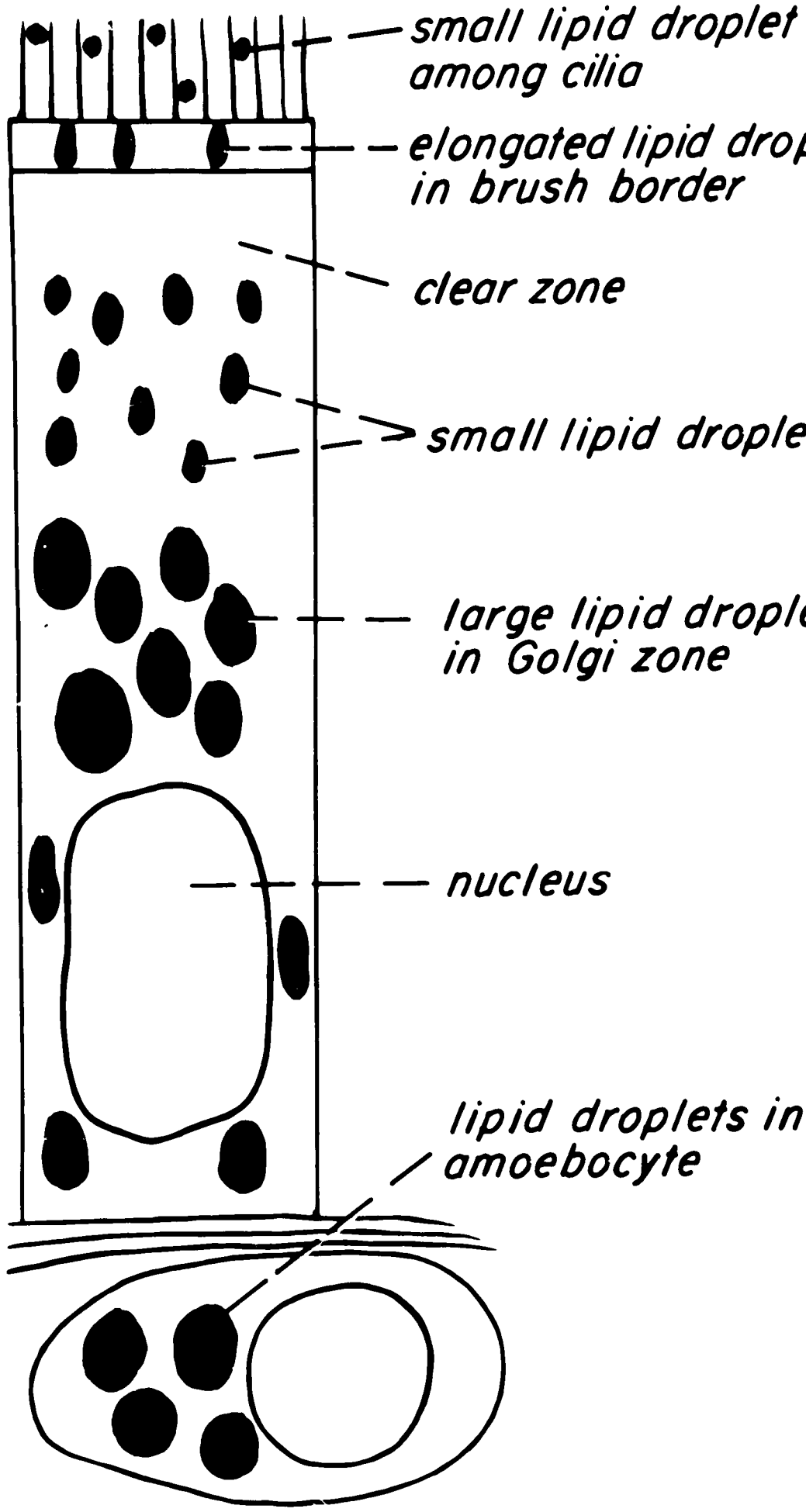


Fig. 1. Diagram showing the distribution of lipid in an intestinal epithelial cell which is absorbing lipid. Not to scale.

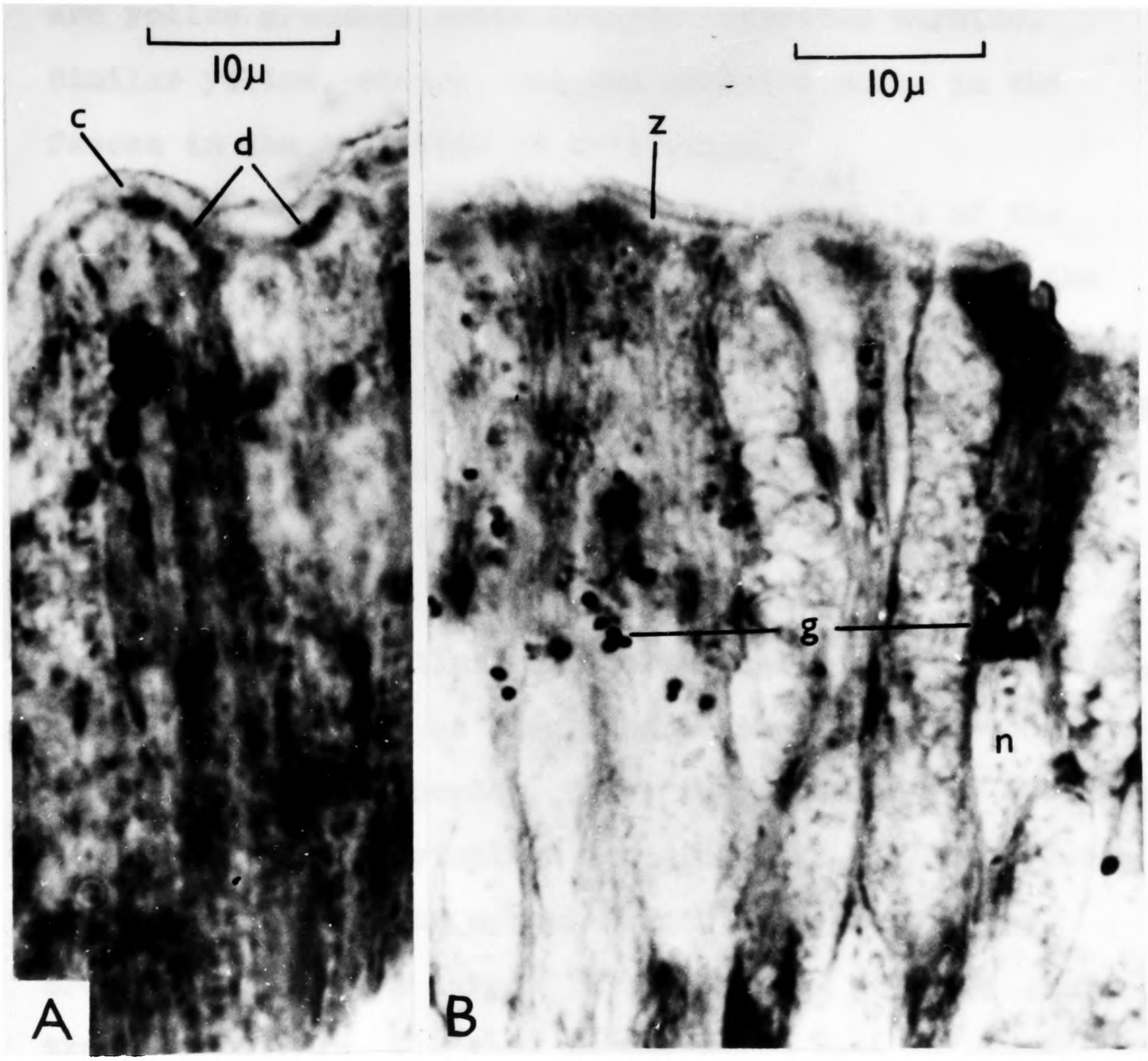


Fig. 2. The distribution of absorbed lipid in intestinal epithelial cells.

c, cilia; d, lipid droplets in brush border; g, large lipid droplets in Golgi zone; n, nucleus; z, clear zone beneath brush border.

all with a similar granular sub-structure. Some granules are yellow, some orange, and some red. The orange granules are yellow granules which have incorporated carmine. Similar yellow, orange, and red granules occur in the faeces in the intestine at this stage.

Lipids are taken up by epithelial cells of the digestive gland ducts, intestine and rectum, and by the digestive cells of the digestive gland. No lipid is taken up by epithelial cells of the crop. A little lipid seems to be taken up by the calcium and excretory cells in the digestive gland, but they are certainly not so important in lipid absorption as the digestive cells.

In the intestinal epithelial cells (figs. 1 and 2), lipid first appears as elongated droplets, up to 0.8μ long, in the brush border. Below the brush border there is a narrow layer of cytoplasm containing no lipid droplets; below this layer, the cytoplasm contains small lipid droplets. There are larger lipid droplets just apical to the nucleus (fig. 2); this is the Golgi zone in these cells (Guardabassi & Ferreri, 1953). Frequently, the connective tissue immediately below the intestinal epithelium contains numerous amoebocytes full of lipid droplets. Lipid absorption in the digestive gland ducts and rectum produces a similar appearance, but has not been so fully studied.

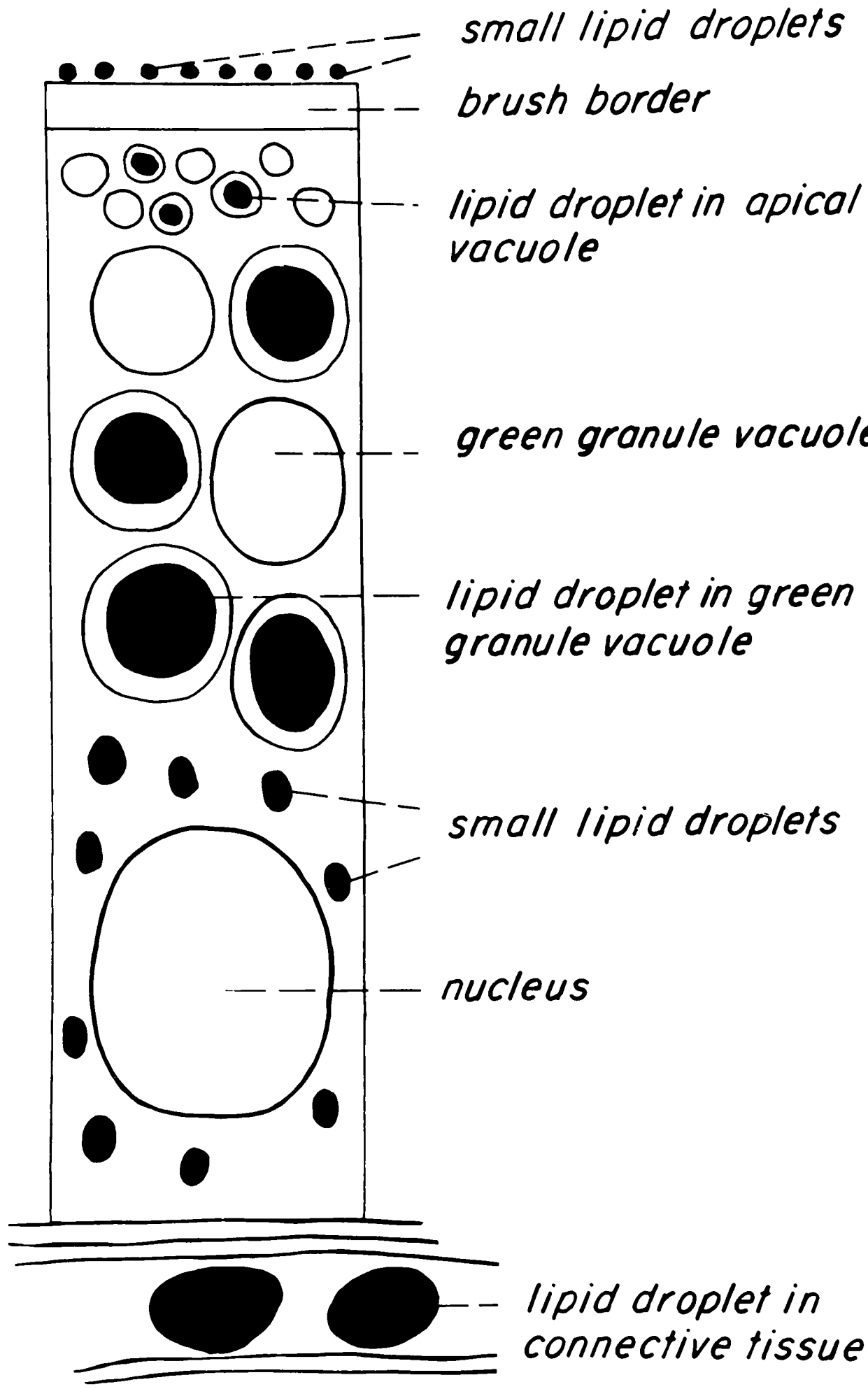


Fig.3. Diagram showing the distribution of absorbed lipid in a digestive cell of the digestive gland. Not to scale.

The intestinal epithelial cells of control snails, which have not been injected with lipid, contain a much smaller number of lipid droplets. These lipid droplets are mostly basal, with a few elsewhere in the cytoplasm. There are no large lipid droplets in the Golgi zone of control snails.

In the digestive cells of the digestive gland, the following stages can be distinguished (fig. 3). Firstly, small lipid droplets, 1 to 2μ in diameter, appear in the apical vacuoles. Next, the "green" granule vacuoles become filled with lipid, which forms large droplets up to 15μ in diameter (fig. 4). Later, small lipid droplets, about 1μ in diameter, appear in the cytoplasm in the basal half of the cells. Finally, lipid droplets accumulate in large numbers in the connective tissue surrounding the digestive gland tubules. Lipid absorption only occurs in those digestive cells which possess a brush border.

Digestive cells of control snails which have been starved for 65 days contain only a few small lipid droplets throughout the cytoplasm; there is never any lipid in vacuoles in digestive cells except when they have been injected with lipid. After 65 days' starvation, very few lipid droplets remain in the connective tissue surrounding the digestive gland tubules.

It is characteristic of snails which have been

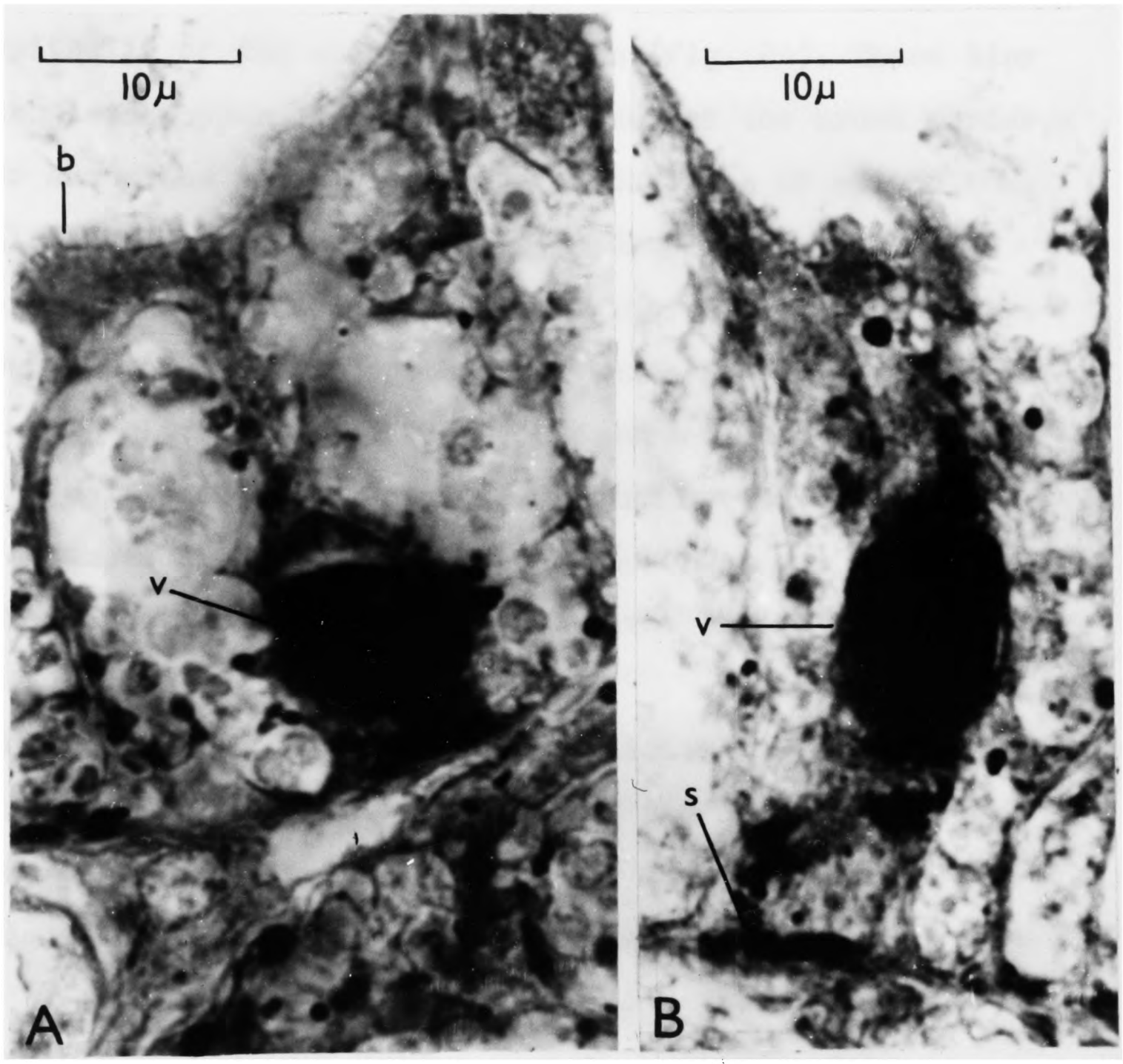


Fig. 4. Absorbed lipid in digestive cells of the digestive gland.

b, brush border; s, lipid droplets in the sub-epithelial connective tissue; v, large lipid droplet in vacuole.

injected with lipid that tiny lipid droplets, about 0.4μ in diameter, are present on the free surfaces of the epithelia of the alimentary system (fig. 2A). These tiny droplets appear to be on the surface of the brush border, or among the cilia, depending on the type of epithelium. Although the appearance of these droplets may be a preliminary to lipid absorption, they also appear on the surface of the crop epithelium, where no absorption occurs.

Olive oil appears to be absorbed more readily by the epithelia of the digestive gland ducts, intestine and rectum, while milk appears to be absorbed more readily by the digestive cells of the digestive gland.

DISCUSSION

The results show that carmine particles and lipids are taken up by the digestive cells of the digestive gland, and that lipid is also absorbed by the epithelia of the digestive gland ducts, intestine and rectum.

The digestive cells of the digestive gland are clearly phagocytic, taking up both carmine and lipid as particles. These experiments show that both the apical and "green" granule vacuoles are phagocytosis vacuoles. The change in form of carmine as it passes down the cell suggests that the protein part of it is being digested, the carminic acid becoming attached to the "green" granules. Rosen (1952) found that particles ingested by digestive

gland cells were digested as they passed down the cell, but his methods could not show the change from "green" to yellow granules.

The morphological appearance of lipid absorption in the digestive gland ducts, intestine and rectum of Helix resembles closely the appearance described in mammals (Baker, 1951; Palay & Karlin, 1959). It thus seems likely that the mechanism of lipid absorption in these epithelia is similar to that found in the intestinal epithelium of mammals. It is now generally accepted that in mammals lipid is taken up as small particles by pinocytosis, and possibly also partly as soluble products of hydrolysis (Palay & Karlin, 1959; Wotton, 1963; Napolitano & Kleinerman, 1964; Strauss, 1963, 1964). It is not, of course, possible to demonstrate pinocytosis of lipid in the epithelia of the ducts, intestine and rectum of Helix without electron microscopical studies. Lipase is present in the crop fluid of Helix (Graetz, 1929; Holden & Tracey, 1950; Myers & Northcote, 1958), just as it is present in the intestine of mammals, but there is no evidence that it plays an important part in the digestion of triglycerides. According to Myers & Northcote (1958) the lipase of Helix may act on lipoproteins; certainly it does not prevent lipid reaching either the digestive gland or the intestine and rectum as insoluble droplets. Thus there seems to be no

reason why pinocytosis of particles of lipid should not occur in the digestive gland ducts, intestine and rectum of Helix.

It is interesting that lipid from milk appears to be taken up more readily by the digestive gland cells, and olive oil more readily in the other sites. The protein component of milk may promote its phagocytosis in the digestive gland. Rosen (1952) has shown that the presence of protein induces phagocytosis in the digestive gland of Helix; and proteins are known to induce pinocytosis (Rustad, 1961), which is essentially similar to phagocytosis (Holter, 1961).

I thank Dr. J.R.Baker, F.R.S., for his help and supervision during the course of this work; and Professor J.W.S.Pringle, F.R.S., for accommodating me in his Department.

This work was done during the tenure of a D.S.I.R. research studentship; I thank them for financial assistance.

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PAPER 5

THE EFFECT OF DRUGS ON SECRETION IN THE ALIMENTARY SYSTEM OF HELIX

INTRODUCTION

In the course of cytological studies on the alimentary system of the snail Helix aspersa, granules of a particular kind were found in the calcium cells of the digestive gland (Sumner, 1965a) and in certain cells (intestinal secretory cells) of the intestinal epithelium (Sumner, in preparation). It was thought that these granules might be discharged from the cells. It was desired to establish with certainty whether this was so.

There have been very few experiments on the action of drugs on secretion in invertebrates. In insects, Lebedeff (1899) and Ōka (1930) found that pilocarpine produces strong discharge of the salivary gland secretion. Monti (1899) and Pacaut & Vigier (1906) studied the effect of pilocarpine on the salivary gland of Helix, and found that it produced changes in the cells similar to those produced by feeding, but more pronounced. Yonge (1926) injected pilocarpine into the lamellibranch Pecten, and found that it produced a great increase in the number of colourless granules in the epithetial cells of the style sac. Thus it appears that pilocarpine stimulates secretion in molluscs. However, there is no reason to suppose that it is the only substance to do so, or the most effective. It has been found that a wide variety of substances excite or inhibit nerve cells in molluscs (Kerkut & Walker, 1961, 1962), and it is

possible that many nerves in molluscs are not cholinergic (Bacq, 1947; Ze. - Nagy & Salanki, 1963), and would thus be unaffected by pilocarpine.

The present study was therefore undertaken with two aims in view; to establish whether the granules of the calcium cells and intestinal secretory cells were discharged from the cells; and to find what drugs affected the secretions in the alimentary system.

MATERIAL AND METHODS

The following cell types from the alimentary system of the snail Helix aspersa were studied; the mucous cells of the epithelia of the crop, stomach, and intestine; the calcium cells of the digestive gland; and the intestinal secretory cells.

The drugs used were pilocarpine hydrochloride, atropine sulphate, adrenaline hydrochloride, and 5 - hydroxy - tryptamine (5HT) in the form of serotonin creatinine sulphate. All the drugs except pilocarpine were used in a solution containing 0.5 mg/ml and were injected in such a quantity as to result in a concentration in the snail of 0.025 mg/g of snail. The solution of pilocarpine contained 2.5 mg/ml, and the final concentration of pilocarpine in the snail was 0.25 mg/g. Pilocarpine was used at a higher concentration than its antagonist atropine as this has been done in previous experiments (Menzies, 1952) and is recommended for medical and veterinary use (Anon, 1948; Stecher, Finkel, Siegmund & Szafranski, 1960). All the solutions were made up in distilled water, and injected through the foot of the snail. As controls, snails were injected with a

quantity of distilled water equivalent to the quantity of drug solution that would have been injected into a snail of that weight. The snails were starved for a few days before the experiments, to clear the alimentary system of food and other materials, which might themselves produce discharge of secretions.

Snails injected with pilocarpine or with atropine were killed 20 min., and 1, 1½, 2½ and 5 h after injection. Some snails were injected with atropine, and then with pilocarpine 10 min. later; the snails were then killed at the same intervals after the pilocarpine injection as when the drugs were used alone.

To study the effects of large doses of pilocarpine and atropine, two other experiments were done. In one experiment, a snail was given three injections of pilocarpine, at the usual dosage, at hourly intervals, and killed 1 h after the third injection (i.e. 3 h after the first injection). In the other experiment, pilocarpine and atropine were used at ten times the usual concentration; the drugs were either used alone and the snail killed 1 h after injection; or else an injection of atropine was followed 10 min. later by an injection of pilocarpine, and the snail killed 1 h after the second injection. Snails injected with adrenaline were fixed 20, 30 and 40 min., and 1 and 2 h after injection, and those injected with 5HT 30 min., 1 h and 2 h after injection. The most suitable times for killing the snails were found by experience.

When the snails were killed, the appropriate organs were dissected out, fixed in formaldehyde-saline overnight, embedded in paraffin, and sectioned at 8μ. Mucus was shown by the periodic acid/Schiff test (McManus, 1948), and the granules of calcium and intestinal secretory cells by the DMAB/nitrite test for tryptophane (Adams, 1957).

RESULTS

Since the intestinal secretory cells of pulmonates do not seem to have been described before, they will be described briefly here. A full description will be given elsewhere (Sumner, in preparation).

The intestinal secretory cells occur in the second half of the intestine, in the loop which is on the outer surface of the digestive gland. This is the part of the intestine which immediately precedes the rectum. The epithelium of this part of the intestine consists of numerous intestinal secretory cells, usually separated from each other by non-secretory cells; there are also a few mucous cells. The secretory cells are very conspicuous as they are wider than the non-secretory cells. They have basiphil cytoplasm, and the apical half of the cell is full of granules which react positively to the DMAB/nitrite test for tryptophane. The nucleus is round, instead of elongated like the nuclei of the other epithelial cells.

Pilocarpine and atropine

Injection of pilocarpine caused discharge from the cells of all

the cell inclusions studied. In all the experiments discharge of the mucus was observed throughout the gut; at times the discharge was particularly strong in the crop, but this was not consistently so. Up to 1½ h after injection of pilocarpine, the protein granules of the calcium cells increased in number and size, with occasionally slight discharge of granules; this was so even after injection of ten times the usual dose. At 2½ and 5 h after injection, and when three injections were given, strong discharge of protein granules occurred; the granules were found in the stomach and in the ducts of the digestive gland, and sometimes the number of granules in the calcium cells was reduced. Injection of pilocarpine also caused discharge of the intestinal secretory granules; the discharge was generally slight, but stronger 20 min. and 5 h after injection.

Injection of atropine did not normally cause discharge of the cell inclusions studied. There was usually a very slight discharge of mucus and of intestinal secretory granules, but not more than in the controls. Strong discharge of mucus occurred 20 min. and 5 h after injection. In the calcium cells of the digestive gland, protein granules usually increased in size and number as a result of injection of atropine, but they were only found to be discharged 20 min. and 5 h after injection, and then not strongly.

When an injection of atropine was followed by an injection of pilocarpine, the effect on the discharge of mucus and of intestinal secretory granules was virtually the same as that found after injection of atropine alone; there was normally no appreciable discharge.

However, the protein granules were generally discharged to some extent; they were found in the stomach, and sometimes in the ducts, at all times after the injections, and also after injection of ten times the usual dose. Usually there were few protein granules in the calcium cells.

Adrenaline

Injection of adrenaline caused discharge of all the cell inclusions studied. At 20, 30 and 40 min. after injection, mucus was discharged throughout the gut, but at 1 h and 2 h after injection, the discharge of mucus was largely restricted to the crop. The discharge of mucus could also be observed in the living animal, as there was a strong discharge of mucus from the skin, beginning a short time after injection. At all periods after injection of adrenaline, protein granules were found in the stomach, although few were present there 20 min. after injection. Protein granules were found in the ducts of the digestive gland up to 1 h after injection, but were not always present there after 30 min. after injection. 20 min. after injection, protein granules were present in the lumen of the digestive gland tubules themselves. Protein granules were usually rather scarce in the calcium cells, but were sometimes numerous from 40 min. after injection onwards.

Intestinal secretory granules did not seem to be discharged up to 40 min. after injection of adrenaline. Slight to strong discharge occurred 1 h after injection, and the discharge was still strong 2 h after injection.

5 - hydroxy - tryptamine

Injection of 5HT caused discharge of mucus and of protein granules. There often seemed to be slight discharge of intestinal secretory granules, but this was not appreciably stronger than in the controls.

Discharge of mucus was slight 30 min. after injection. 1 h after injection discharge was strong in the crop, and 2 h after injection discharge of mucus was strong in all parts of the gut studied. Protein granules were always numerous in the stomach of snails injected with 5HT. 30 min. and 1 h after injection protein granules were also present in the digestive gland ducts, but scarce in the calcium cells. 2 h after injection no protein granules were found in the ducts, but they were numerous in the calcium cells.

Controls

The control snails never showed as much discharge of secretions as was found when it was induced by injections of drugs. A slight discharge of mucus was often found in the gut, particularly in the crop. Discharge of protein granules and of intestinal secretory granules was very rare in control snails, and always very slight.

DISCUSSION

The results show firstly, that the protein granules of the calcium cells in the digestive gland, and the granules of the intestinal secretory cells can be discharged from the cells in which

they are formed; and secondly, that pilocarpine, adrenaline and 5HT all cause discharge of various secretory products from cells in the alimentary system of Helix.

It has frequently been stated that one of the functions of the digestive gland of gastropods is secretion of enzymes into the crop and stomach (Cuénot, 1892; Krijgsman, 1925, 1928; Fretter, 1939; Evans & Jones, 1962 a), and there are several reports of the same enzyme occurring in the digestive gland and in the crop fluid (Holden & Tracy, 1950; Dodgson & Powell, 1959; Evans & Jones, 1962 a & b). It has been thought that enzymes were secreted by the digestive and excretory cells (Barfurth, 1883; Krijgsman, 1925, 1928; van Weel, 1950), but it has been shown that these have phagocytic and excretory functions respectively, (Fretter, 1939; Rosen, 1952; Sumner, 1965 a & b). It has since been suggested that the calcium cells of gastropods might be the cells responsible for secretion of enzymes (Kerkoja, 1961, 1964; Sumner, 1965 a). The present work shows that the granules of the calcium cells of Helix may be discharged from the cell and pass into the stomach. It remains to be established whether these granules contain enzymes.

Although all the drugs used, except atropine, cause widespread discharge of secretory products, the different drugs vary in their actions. Pilocarpine readily causes discharge of mucus throughout the gut, and discharge of protein granules, but has a much weaker

action on the intestinal secretory granules. Adrenaline causes discharge of mucus throughout the gut, but the discharge is noticeably stronger in the crop; and it does not cause discharge of intestinal secretory granules until 1 h after injection, although discharge then becomes strong. 5HT resembles adrenaline in that it tends to cause stronger discharge of mucus in the crop than elsewhere; however, it apparently has no effect on intestinal secretory granules. Except for the action of adrenaline on intestinal secretory cells, no particular drug is associated with the discharge of any particular secretory product. Thus, with this exception it is impossible to say what the chemical transmitters involved in the innervation of these organs might be. The intestinal secretory cells are apparently innervated by adrenergic nerve fibres. It should be noted that Kerkut & Walker (1962) found that more than one chemical transmitter might excite a particular nerve cell.

Atropine clearly has some inhibitory effect on the stimulatory action of pilocarpine, but the inhibition does not seem to be complete. In particular, some discharge of protein granules occurs when an injection of atropine is followed by one of pilocarpine. In general, no discharge of secretions occurs when atropine alone is injected. The inhibitory action of atropine on discharge of secretion which is stimulated by pilocarpine suggests innervation by cholinergic nerves (Bacq, 1947; Gaddum, 1953). It must be emphasized, however that the other drugs used cause discharge of the same secretions as pilocarpine does.

SUMMARY

1. Pilocarpine, when injected into the snail Helix, causes discharge of mucus throughout the alimentary system, discharge of protein granules from the calcium cells of the digestive gland, and slight discharge of the granules of the intestinal secretory cells. Its effects are inhibited by atropine. Adrenaline produces similar effects to pilocarpine, but its action on the intestinal secretory granules is stronger. 5HT causes discharge of mucus and of protein granules, but not of intestinal secretory granules.

2. The protein granules of calcium cells and the intestinal secretory granules are secretory products which are discharged into the lumen of the alimentary system.

I thank Dr. J. R. Baker, F.R.S., for his help and supervision during the course of this work; and Professor J. W. S. Pringle, F.R.S., for accommodating me in his Department.

This work was done during the tenure of a D.S.I.R. research studentship; I thank them for financial assistance.

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PAPER 6

THE CYTOLOGY AND HISTOCHEMISTRY OF THE DIGESTIVE GLAND CELLS OF SOME FRESHWATER LAMELLIBRANCHS

SYNOPSIS

The cells of the digestive gland tubule epithelium of the freshwater lamellibranchs Anodonta, Sphaerium and Unio were studied. There are two main types of cell: digestive and basiphil cells. Digestive cells are highly vacuolated, and closely resemble digestive cells in other classes of mollusca. Basiphil cells have a high concentration of RNA in the cytoplasm, and a conspicuous Golgi apparatus. They are the cells considered by previous workers to be undifferentiated cells. The basiphil cells have apically small granules which contain protein; the cells are believed to be secretory, and the apical granules the secretory product. Sphaerium, and possibly the other species studied, have groups of cells bearing very long flagella, among the groups of basiphil cells at the blind end of the tubules.

INTRODUCTION

The digestive gland tubule epithelium of Eulamellibranchs is widely believed to consist of only one type of cell. There are crypts of darkly staining cells which exhibit mitosis; these are thought to be undifferentiated cells which replace the mature vacuolated cells as they are lost from the epithelium (Cuthell, 1912; Yonge, 1925, 1926 a and b; Graham, 1931; Mansour, 1946 b; Morton, 1956). A few workers have suggested that the darkly staining cells are a distinct type, which bear cilia or flagella (Owen, 1955; Saleuddin, 1964, 1965). Flagellated cells, distinct from the darkly staining cells, have also been reported (Saleuddin, 1965).

The vacuolated cells of the lamellibranch digestive gland are absorptive and phagocytic (Yonge, 1926 a & b; Owen, 1955, 1959; Morton, 1956). Although it has often been stated that the digestive gland is also secretory (Cuthbil, 1912; Mansour, 1946 a & b; Mansour-Bek, 1946; Ballantine & Morton, 1956), no specialised secretory cells have been described, and the absorptive cells have been suggested as the source of digestive enzymes (Mansour-Bek, 1946). If the epithelium of the lamellibranch digestive gland consists of only one type of cell, then it differs fundamentally from the digestive glands of most other molluscs (Cuénot, 1907; Graham, 1932, 1938; Fretter, 1937, 1939; Gabe & Prenant, 1949; Pugh, 1963; Hartoja, 1964; Sumner, 1965 a), although Morton (1959) has stated that only digestive and undifferentiated cells are present in Scaphopoda.

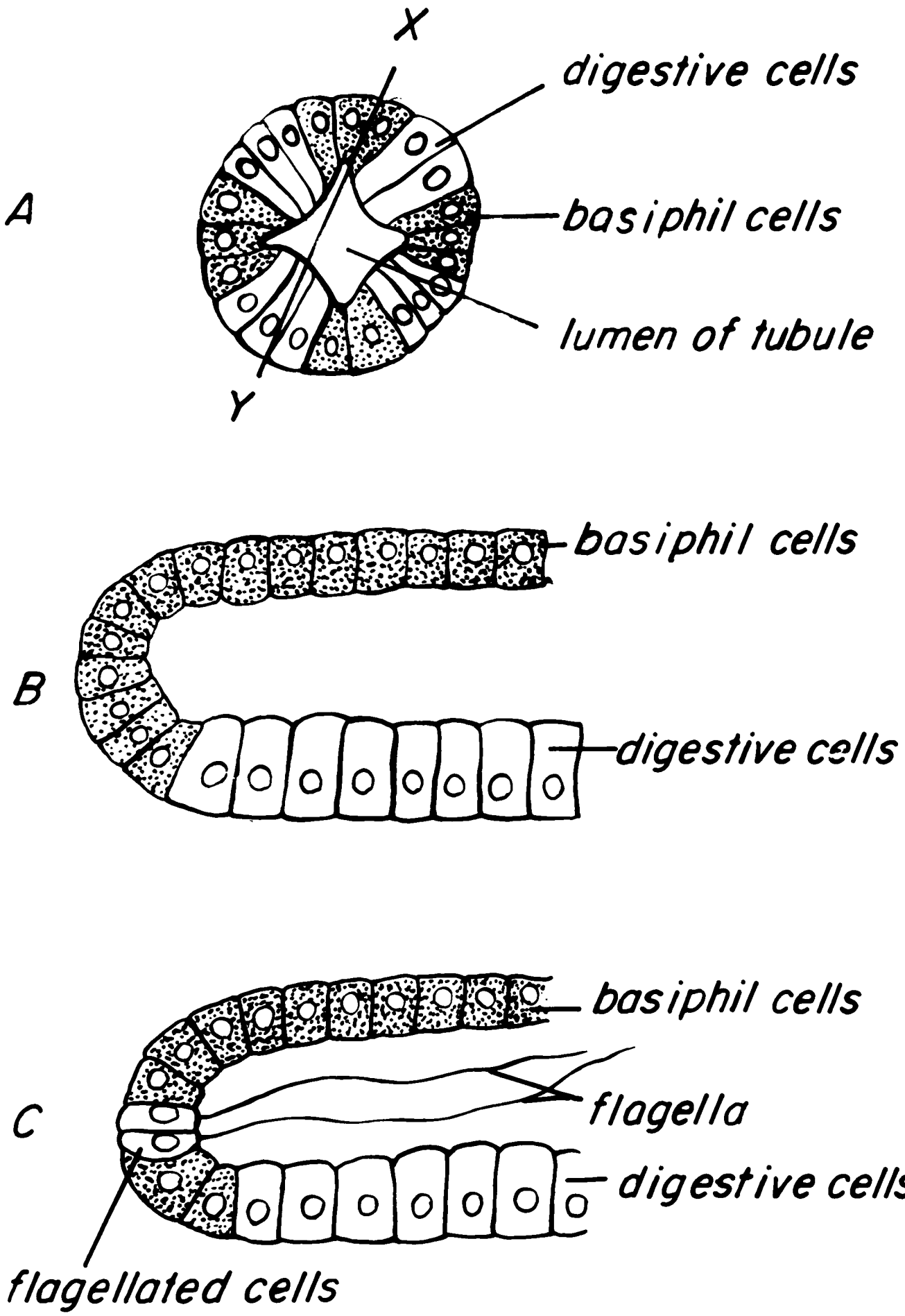
It was decided to carry out a detailed cytological and histochemical study of the digestive gland of some freshwater lamellibranchs, as such information is not available, and it was felt that it would help in deciding what cell types were present, and what their functions were.

MATERIAL AND METHODS

The species studied in detail was Anodonta anatina; Sphaerium corneum and Unio tumidus were studied for comparison. All the animals used were feeding. The work was confined to the tubule epithelium of the digestive gland of these species.

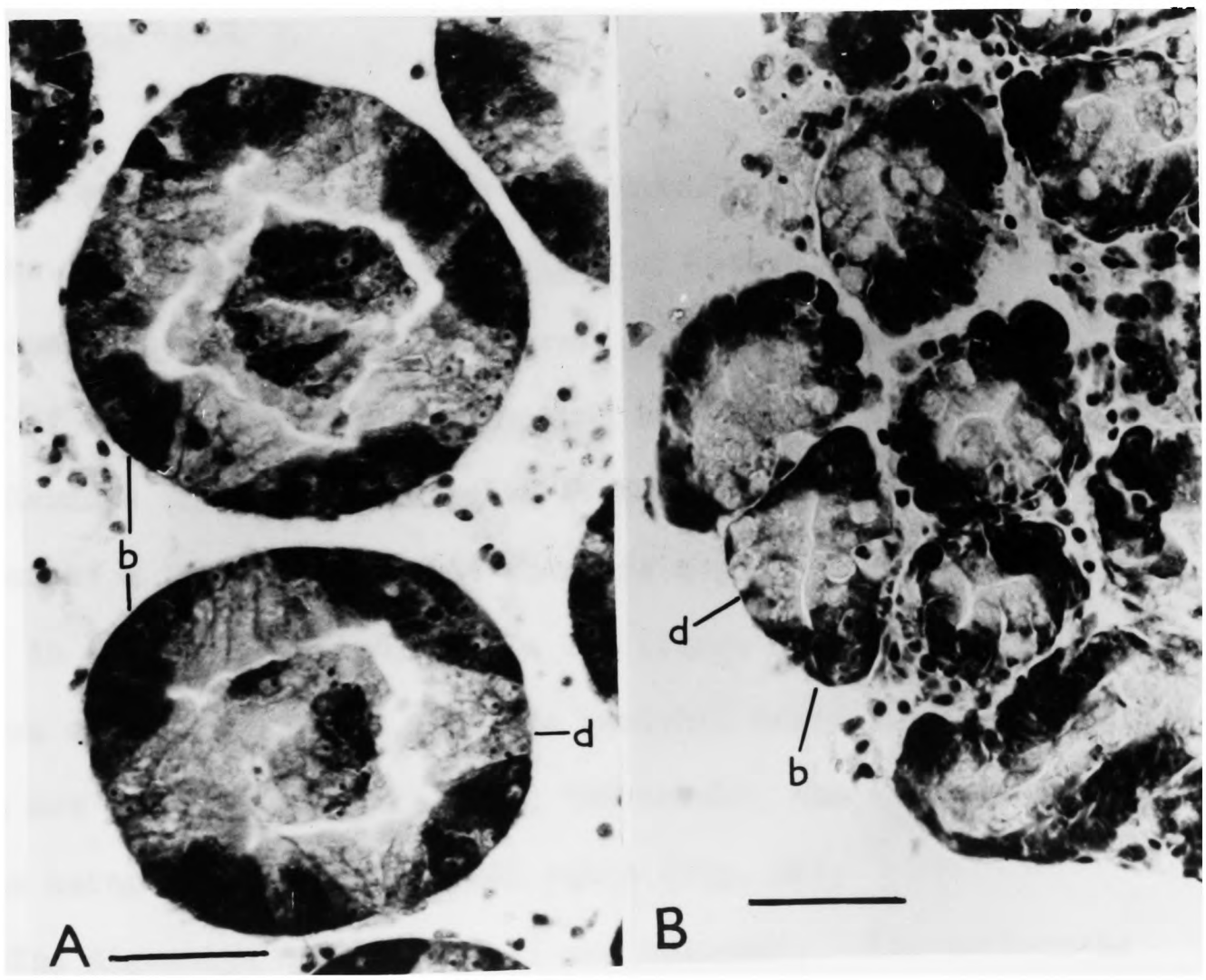
See over

Fig. 1. Diagrams showing the structure of the digestive gland tubules. A, cross section. B, longitudinal section, through the planeXY in fig. 1A, of a tubule of Anodonta or Unio. C, longitudinal section, through the plane XY in fig. 1A, of a tubule of Sphaerium.


X
digestive cells
A
basiphil cells
lumen of tubule
Y
basiphil cells
B
digestive cells
basiphil cells
C
flagella
digestive cells
flagellated cells

See over

Fig. 2. Micrographs of cross sections of digestive gland tubules. A, Anodonta. B, Sphaerium. b, basiphil cells; d, digestive cells.


b
d
A
d
b
B

The cytological and histochemical methods used, except those for lipofuscins, are summarized in table 1, and the tests for lipofuscins in table 4.

RESULTS

The results of the histochemical tests, other than those for lipofuscins, are summarized in table 2. The dimensions of the cells are given in table 3.

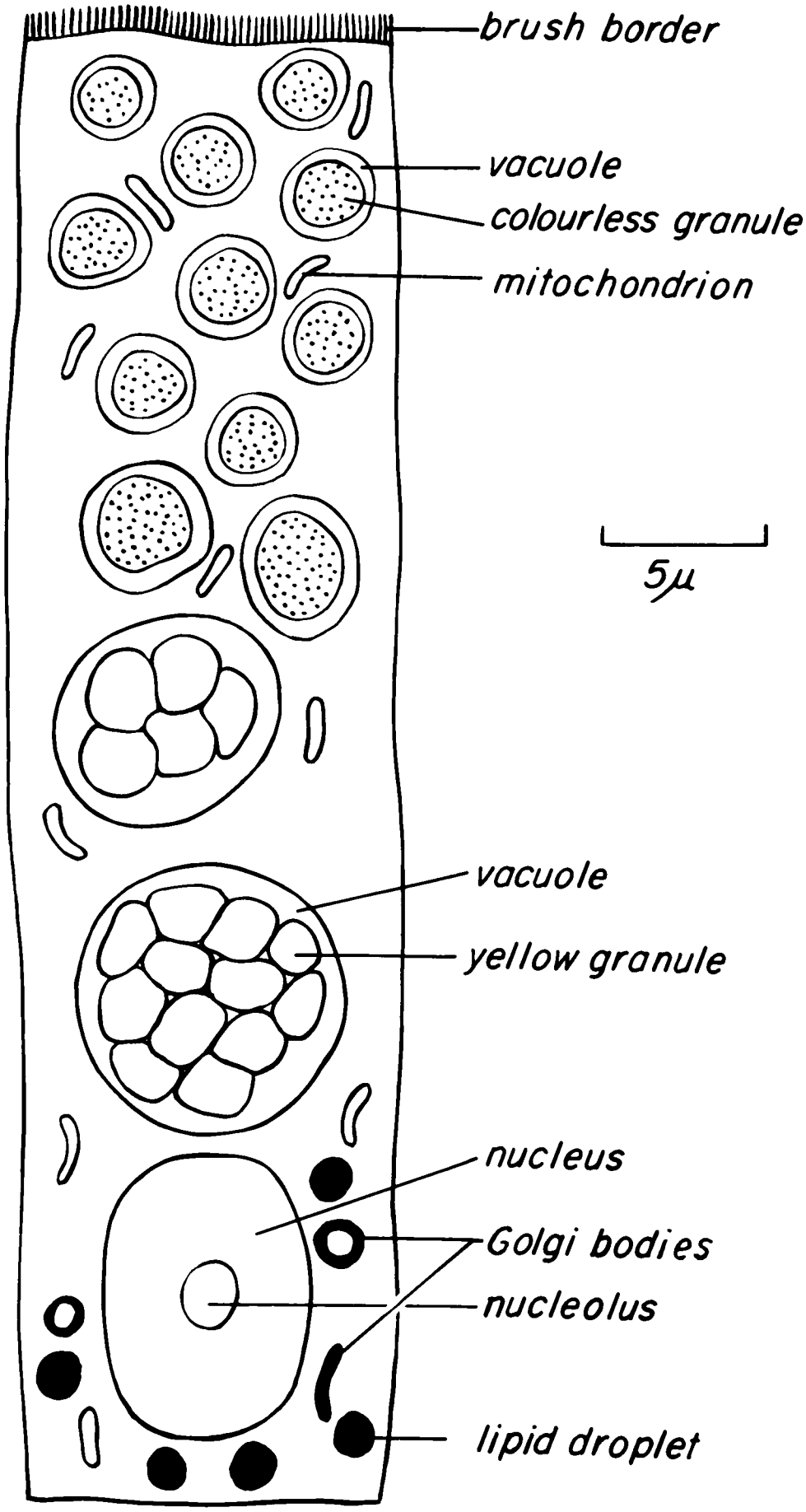
Anodonta

Two main types of cells are present in the epithelium. These are the digestive cells (Keulenzellen of Gutheil (1912); absorbing or vacuolated cells of most authors), and the basiphil cells (young cells of many authors; Cryptenzellen of Gutheil (1912); dark cells of Saleuddin (1964); crypt cells of Saleuddin (1965)). A cross-section of a tubule shows that there is a number of crypts, usually four, in which the basiphil cells lie (figs. 1A & 2). A longitudinal section of a tubule shows that the basiphil cells and the digestive cells are arranged in bands along the tubule, the blind end of the tubule being lined with basiphil cells (fig. 1B).

The digestive cells (fig. 3) are columnar. The nucleus is usually basal, although it may be up to half way up the cell; it contains a single nucleolus. The cytoplasm apical to the nucleus is full of vacuoles. These are small near the apex of the cell but a single one often occupies the whole width of the cell just apical to the nucleus. All the vacuoles contain granules, which are only

See over

Fig. 3. Diagram of a digestive cell of Anodonta.


brush border
vacuole
colourless granule
mitochondrion
5μ
vacuole
yellow granule
nucleus
Golgi bodies
nucleolus
lipid droplet

slightly smaller than the vacuoles themselves. Most of the vacuoles are about 3μ in diameter; those just apical to the nucleus are up to 8μ in diameter. Most of the granules in the vacuoles are colourless, while the granules in the vacuoles just apical to the nucleus are usually yellow; sometimes all the granules are colourless, and sometimes all are yellow. The results of the histochemical tests carried out on the yellow granules are summarized in table 4.

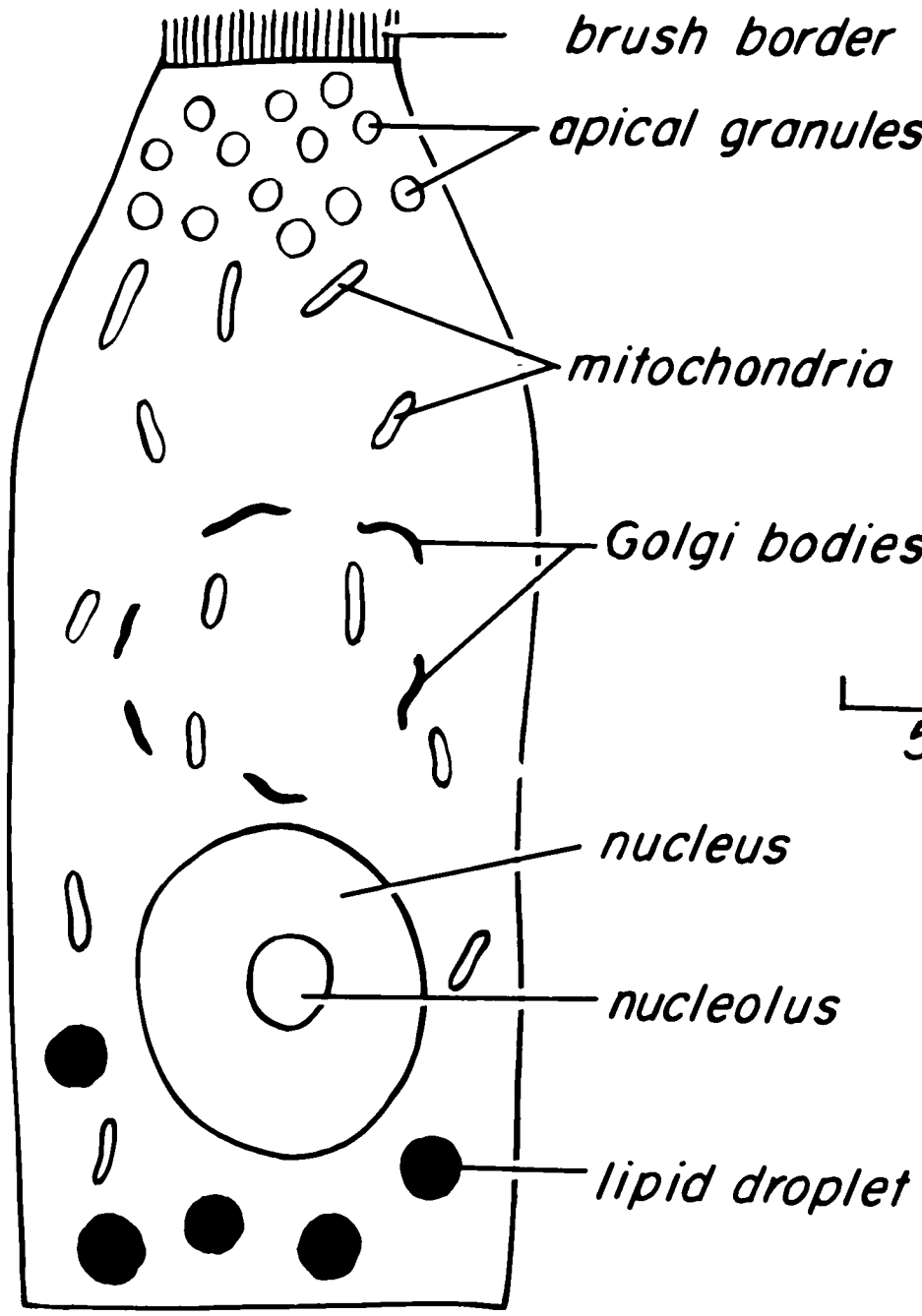
The apical surfaces of the digestive cells are usually rounded and project into the lumen of the tubule; more rarely they are straight and level with each other and bear a brush border, 0.6 to 0.8μ high.

Mitochondria are present in moderate numbers throughout the digestive cells, without being concentrated in or absent from any particular region. They are long and thin, being up to 2.4 in length, and 0.4μ or less wide. The Golgi apparatus consists of narrow threads up to 2μ long, or rings to 1.5μ in diameter, in the region just apical to, and at the sides of, the nucleus.

Basiphil cells (fig. 4) are distinguished by the basiphilia of their cytoplasm, which is due to the presence of RNA (see table 2). Basiphil cells are conical or columnar, and although shorter than the digestive cells reach the lumen. The nucleus is basal and rounded; usually only one nucleolus is visible in it. The cytoplasm of basiphil cells is not vacuolated, but may appear finely granular. Some or all of these cells bear a brush border, 1 to 2μ high.

See over

Fig. 4. Diagram of a basiphil cell of *Anodonta*.


brush border
apical granules
mitochondria
Golgi bodies
5μ
nucleus
nucleolus
lipid droplet

Basiphil cells may be divided into two classes. Those on the outside of the groups in the crypts are most strongly basiphil, and those in the middle of the groups are only moderately so. The basiphil cells with the most basiphil cytoplasm have a number of apical granules, 0.4 to 0.8μ in diameter. These granules may be stained with iron haematoxylin (particularly after fixation in fluids containing osmium tetroxide), with acid fuchsin in Metzner's method, and with aldehyde fuchsin after permanganate oxidation. 10 to 20 granules can usually be seen in a section of a single cell. Mitosis occurs infrequently in those basiphil cells that have less basiphil cytoplasm. These cells do not have apical granules.

Basiphil cells have moderate to large numbers of mitochondria throughout the cytoplasm, often rather more numerous just apical to the nucleus, in the Golgi zone (see below). The mitochondria are 1.2 to 2.4μ long, and 0.4μ or less thick. The Golgi apparatus of basiphil cells typically appears as a group of contorted black lines, about 1.5μ long, in or enclosing a mass of cytoplasm which is greyer than the rest in Mann-Kopsch preparations. This group of lines is apical to, or sometimes beside the nucleus, and may occupy an area up to 8μ across. Focusing up and down shows that the lines are in fact plates.

Lipid droplets, about 1.5μ in diameter, occur in small numbers basally, in both digestive and basiphil cells.

Sphaerium

As well as digestive and basiphil cells, Sphaerium has a third type of cell. Among the basiphil cells at the blind end of a tubule

there is a small group of flagellated cells (fig. 10). These cells are probably fusiform. They are narrowest apically, and widen out to about 4μ half-way down the epithelium, where the nucleus lies. Although the cells have not been traced down to the basement membrane, it seems likely that they reach it. The nuclei are elongated along the long axis of the cell, and are about 6.4 by 3.6μ . Only one to three flagella are visible in each tubule, and each flagellated cell probably has only one flagellum, which may be up to 32μ long. The cytoplasm stains strongly with acid fuchsin in Metzner's method, but no mitochondria have been seen.

The digestive cells of Sphaerium differ from those of Anodonta in two respects. The vacuoles in the cells are larger, being up to 12μ in diameter. Yellow granules have not been found in the vacuoles, all of which contain colourless granules.

The basiphil cells of Sphaerium resemble those of Anodonta, but apical granules have not been identified in them with certainty.

Unio

As in Sphaerium, flagella are present in the lumen of tubules, but they have not been traced to any cell in Unio.

The digestive cells of Unio resemble those of Anodonta very closely in all respects.

A third class of basiphil cells is present in Unio, as well as the two classes found in the other species. Some of the basiphil cells have a large vacuole, up to 18μ in diameter, but more often only 9 to 13μ in diameter. The vacuole contains a yellow granule, similar in

histochemical properties to those in digestive cells (see table 4). The nuclei of these cells appear to lie in the vacuoles, on the surface of the yellow granules; they have a normal content of DNA.

DISCUSSION

The results show clearly that there are two main types of cells in the tubule epithelium of the digestive gland in Anodonta, Sphaerium and Unio: digestive cells and basiphil cells. It has been clearly shown that the digestive cells are absorptive and phagocytic (Yonge, 1926 a & b; Owen, 1955, 1959; Morton, 1956; Rosen, 1965). They show a great resemblance in structure to the digestive cells in the digestive glands of other molluscs, where these cells are also absorptive and phagocytic (Jordan, 1918; Hirsch, 1924; Peczenick, 1925; Fretter, 1937, 1939; Graham, 1938; Rosen, 1941, 1958; Sumner, 1965 b).

The following hypothesis is put forward to account for the observed features of the basiphil cells. The basiphil cells with a relatively low content of RNA and no apical granules are immature. This is suggested by the occurrence of mitosis in these cells, as well as the low RNA content and lack of apical granules. The cells with apical granules and the highest content of RNA are mature secretory cells. The high RNA content and prominent Golgi apparatus are characteristic of protein secretory cells. The apical granules are the secretory product, possibly digestive enzymes. They contain protein with disulphide groups, which is typical of enzymes, (Neurath, 1965).

The basiphil cells found in Unio which have vacuoles containing yellow granules are degenerate; they seem to be undergoing similar changes to those found in the calcium cells of Stylommatophora. The calcium cells develop into excretory cells which have a large vacuole containing one or more yellow granules (Thiele, 1953; Sumner, 1965a; Paper 3). The basiphil cells show a striking resemblance to the calcium cells of the Stylommatophoran digestive gland, the only major difference being the absence of calcium spherules. Both cell types are apparently secretory (Sumner, 1965a; Paper 9).

There is no evidence that the basiphil cells replace digestive cells lost from the epithelium, as has been suggested (Guthiel, 1912; Yonge, 1923, 1926 a & b; Graham, 1931; Mansour, 1946 b; Morton, 1956). No intermediate stages between basiphil and digestive cells have been described in the literature, nor have they been found in this study. If the basiphil cells undergo degenerative changes, they will themselves need replacing.

As well as the two main cell types, Sphaerium, and probably Unio, possess flagellated cells. Although many descriptions of lamellibranch digestive glands do not mention ciliated or flagellated cells (Guthiel, 1912; Yonge, 1923, 1926 a & b, 1928; Graham, 1931; Morton, 1956), they are mentioned sufficiently often to make it seem likely that they are present in most forms (Potts, 1923; Yonge, 1939; Owen, 1955, 1956; Saleuddin, 1964, 1965). It has been said that the flagella are processes of what are called here basiphil cells (Owen, 1955, 1956;

Saleuddin, 1964, 1965), but it seems more likely that they are processes of specialized flagellated cells among groups of basiphil cells, as found here in Sphaerium. Saleuddin (1965) described flagellated cells which were distinct from basiphil cells. It has not been found here that basiphil cells themselves bear flagella.

The pigment of the yellow granules of digestive cells, and of basiphil cells in Unio, has not been identified with certainty. By comparison with Stylommatophora, one would expect the pigment to be lipofuscin (Sumner, 1965a); the pale colour of the granules, and the weakly positive or negative reactions to tests which are normally positive for lipofuscins (see table 3), would then suggest an early stage of oxidation. Zacks (1955) described a pigment of the lipofuscin type in several tissues of the lamellibranch Venus mercenaria, but did not state whether the digestive gland was one of those tissues.

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TABLE 1. CYTOLOGICAL AND HISTOCHEMICAL METHODS

Test	Abbreviation	Fixative	Thickness of Sections (μ)	Reference	Method to show
Iron haematoxylin	-	Ze, Ch, F-Na, M	6, 8	-	General cytology
Ehrlich's haematoxylin & phloxine	-	Ze, F-Na	6, 8	-	General cytology
Oxidation + aldehyde fuschin	-	Ze3, F-Na	6, 8	Dawson, 1953	Apical granules
Crystal violet	-	San.	14	Baker & Jordan, 1953	Mitotic chromosomes
Metzner	-	Ch, M.	4, 6	Meves, 1911	Mitochondria
Mann-Kopsch	-	M	4	Baker, 1933	Golgi apparatus
Feulgen	Feul.	Ze	6	Feulgen & Rossenbeck, 1924	DNA
Pyronine/ methyl green	PMG	Ze3	6	Jordan & Baker, 1955	RNA
Ribonuclease + PMG	RNase	Ze3	6	Bradbury, 1956	RNA
Periodic acid/Schiff	PAS	Ze, F-Na	6	MacManus, 1948	Carbohydrates and some other substances
Diastase + PAS	Diast.	Ze	6	Lillie & Greco, 1947	Glycogen

TABLE 1. (Continued)

Test	Abbrev-iation	Fixative	Thickness of Sections (μ)	Reference	Method to show
Millon	Mill.	F-Na	8	Baker, 1956a	Tyrosine
DMAB/nitrate	DMAB	F-Na	8	Pearse, 1960	Tryptophan
Peracetic acid/alcian blue	PAAB	F-Na	8	Pearse, 1960	Cystine
Peracetic acid/Schiff	PAAS	Ze,F-Na	6, 8	Pearse, 1960	(Unsaturated (lipids
Sudan black	SB	F- Ca/PC	10	Baker, 1956b	Lipid
Acid haematein	AH	F- Ca/PC	10	Baker, 1946 .	Phospholipoid
Cobalt substitution	Co	FMP	10	-	Calcium
Calcium red	Ca R	FMP	10	McGee-Russell, 1955	Calcium
Perls' test	Perl	F-Na	8	Pearse, 1960	Iron

The following abbreviations are used for fixatives:

Ch, Champy; F-Ca/PC, formaldehyde-calcium, with post-chroming;

FMP, formaldehyde-methanol-pyridine; F-Na, formaldehyde-saline;

M, Mann; San, Sanfelice; Ze, Zenker; Ze3, Zenker for 3 h.

TABLE 2. RESULTS OF HISTOCHEMICAL TESTS (EXCEPT THOSE FOR YELLOW GRANULES).

Test	All Cells			Digestive Cells			Basiphil Cells		
	Nuclei	Nucleoli	Basal Lipid Droplets	Ground Cytoplasm	Apical Cytoplasm	Granules in Vacuoles	Ground Cytoplasm	Apical Cytoplasm	Apical Granules
Feul	+++	0	-	0	0	0	0	0	0
PMG	0	++	-	+	+	+	++/+++	0	0
RNase	0	0	-	0	0	0	0	0	0
PAS	-	-	-	+	++	++	+	+	+++
Diast	-	-	-	+	++	++	+	+	+++
Mill	+	-	-	+/++	+/++	+/++	+	+	-
DMAB	-	-	-	+	++	+/++	+	+	-
PAAS	-	-	-	+	+	0/+	+	+	+++
PAAS	-	-	-	+	+	+	+	+	-
SB	0	0	+++	0	0	0	0	0	0
AH	0	+++	0	0/++	0/++	0	+++	-	-
Co	0	0	-	0	0	0	0	0	0
Ca P	0	0	-	0	0	0	0	0	0
Perl	0	0	-	0	0	0	0	0	0

The following symbols are used:

0, no reaction; -, no observation; + weakly positive reaction;

++, moderately positive reaction; +++, strongly positive reaction;

/, indicates variation, e.g. 0/++ indicates a reaction varying

between none and moderately positive.

TABLE 3. DIMENSIONS OF CELLS, THEIR NUCLEI, AND THEIR NUCLEOLI

	Anodonta	Sphaerium	Unio
Digestive Cells			
Height	44.5	27.2	40.8
Width	10.5	-	10.4
Nucleus	8.5 x 6.1	-	6.4 x 6.4
Nucleolus	2.0 x 1.7	-	2.0 x 1.6
Basiphil Cells			
Height	27.5	24.0	20.0
Width	10.6	-	10.4
Nucleus	7.2 x 6.2	-	8.0 x 7.2
Nucleolus	2.0 x 1.8	-	2.4 x 2.4

All dimensions are in μ.

TABLE 4. HISTOCHEMICAL TESTS APPLIED TO YELLOW GRANULES

Test	Fixative	Thickness of Section (μ)	Reference	Result
Basiphilia (Toluid-line blue, pH 3.5)	Ze	6	-	0 or +
Periodic acid/Schiff	Ze	6	MacManus, 1948	+
Diastase, followed by periodic acid/Schiff	Ze	6	Lillie & Greco, 1947	+
Peracetic acid/ Schiff	Ze	6	Pearse, 1960	0
Sudan black	F-Ca/PC	10	Baker, 1956b	0
Acid haematein	F-Ca/PC	10	Baker, 1946	0
Long Ziehl-Neelsen (24h differentiat-ion)	F-Na	8	Pearse, 1960	0
Peracetic acid/ alcian blue	F-Na	8	Pearse, 1960	++
Millon	F-Na	8	Baker, 1956a	+
Perls test	F-Na	8	Pearse, 1960	0

The same abbreviations are used as in tables 1 and 2.

PAPER 7

THE FINE STRUCTURE OF THE DIGESTIVE GLAND CELLS OF ANODONTA

SYNOPSIS

The digestive gland cells of the lamellibranch Anodonta anatina have been studied by electron microscopy. The fine structure of the digestive cells in Anodonta resembles that of cells with similar functions in hydroids and pulmonates. The mitochondria have few cristae and a matrix of low electron density. The basiphil cells contain much ergastoplasm and have numerous lamellar-vacuolar fields. There are membrane-bounded bodies which represent the apical granules seen with the light microscope; similar bodies are seen in association with the Golgi apparatus. Towards the apical surface, the cells are joined together by septate desmosomes; basally there are considerable intercellular spaces into which amoebocytes may penetrate.

INTRODUCTION

In a recent light-microscopical study of the digestive gland cells of freshwater lamellibranchs (Sumner, 1966b), a number of features were described for the first time. It seemed desirable to study the same tissue with the electron microscope, not only to confirm the presence of these features, but also to compare the fine structure of the digestive gland cells of Anodonta with that of the digestive gland cells of pulmonates (Sumner, 1966a). The results of light microscopical studies suggest that there might be considerable similarities. At that level, the digestive

cells of Anodonta and Helix appear very similar in structure; and it seems likely that the basiphil cells of Anodonta (Sumner, 1966b) resemble the calcium cells of Helix in being secretory cells (Sumner, 1966c).

MATERIAL AND METHODS

The tubule epithelium of the digestive gland of the freshwater lamellibranch Anodonta anatina was studied. The gland was dissected out and small portions fixed in cold 1 per cent osmium tetroxide in distilled water for 1h. The tissue was then passed directly to 70 per cent ethanol, dehydrated, and embedded in araldite. Silver sections were cut with a Huxley ultramicrotome, mounted on grids, and stained with uranyl acetate by the method of Gibbons & Grimstone (1960). The sections were examined in an Akashi TMS 50 electron microscope.

RESULTS

The same two types of cells can be recognized with the electron microscope as with the light microscope. These are the digestive and the basiphil cells (Sumner, 1966b).

Digestive cells

The free surface of the digestive cells often bears microvilli, although they are occasionally absent. The microvilli are short, averaging about 640mμ long and 100mμ wide. Central filaments are frequently visible in the microvilli.

The apical cytoplasm contains numerous small vesicles, 145 to 510μμ in diameter (fig. 1A). The inner surface of the membrane of these vesicles bears a fringe. The space between the vesicles is usually rather empty, with only a few scattered granules in it. Frequently there are short lengths of microtubules, particularly just below the brush border.

Further down the cell there are large vacuoles, 2.0 to 3.34μ in diameter (fig. 1A). These vacuoles contain granular material of rather low electron density; normally there is no distinct granule lying in the vacuole. There are also small vesicles, identical with the small vesicles in the apical cytoplasm. Near the cell membrane there are often a few cisternae of ergastoplasm. The nuclei of digestive cells are a short distance above the base of the cell, and present the appearance usually found in electron micrographs. The outer nuclear membrane sometimes bears ribosomes. In the cytoplasm around the nucleus, there may be a few lipid droplets, about 1.0μ in diameter. The Golgi apparatus also occurs in this region, and consists of one or two typical lamellar-vacuolar fields (Hirsch, 1961).

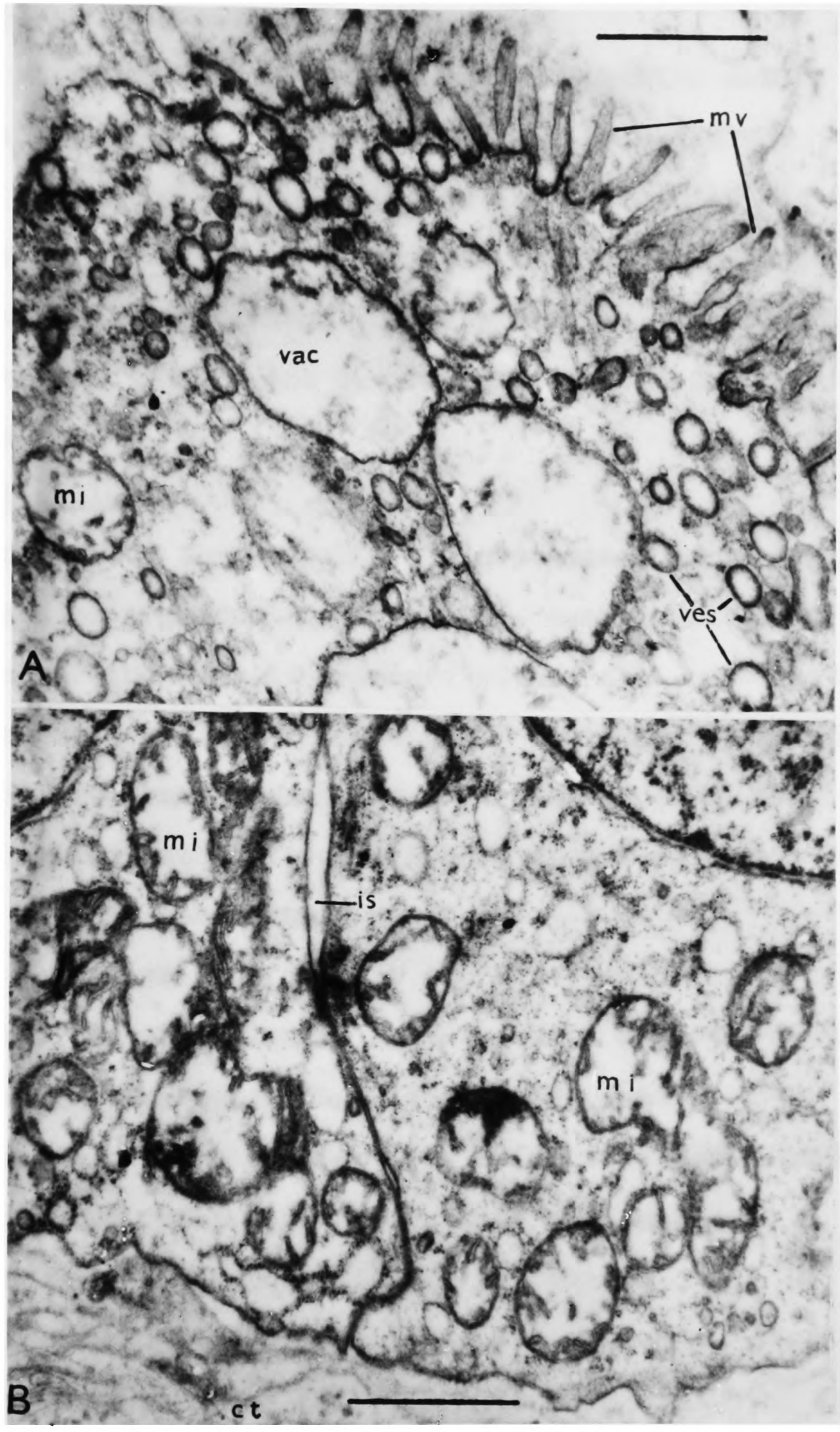
The cytoplasm basal to the nucleus consists largely of granular material (fig. 1B); many of the granules are 10 to 14mμ across, and may be free ribosomes. Towards the sides of the cell there are a few cisternae of

..e over.

Fig. 1. Digestive cells. A. Apical cytoplasm, bearing microvilli; and more basal cytoplasm, with large vacuoles. B. Basal cytoplasm. (Fixed in 1 per cent osmium tetroxide in distilled water, and stained with uranyl acetate).

ct, connective tissue; is, intercellular space; mi, mitochondrion; mv, microvilli; vac, vacuole; ves, vesicles.

The scale on each micrograph represents 1 μ.


mv
vac
mi
ves
A
mi
is
mi
B
ct

ergastoplasm with attached ribosomes.

Mitochondria occur throughout digestive cells, particularly basally. They average 1.11 by 0.38µ and usually appear largely empty (fig. 1B). They have few short cristae, which are orientated in all directions even in the same mitochondrion. The matrix has a very low electron density, and the mitochondria have a swollen appearance.

Basiphil cells

The cytoplasm of the basiphil cells contains large quantities of ergastoplasm (fig. 2B), except apically. The cisternae of the ergastoplasm are either oval, and about 200mµ across, or else elongated and about 40mµ thick. The elongated cisternae frequently have swollen ends. The cisternae seem rather loosely packed when compared with tissues such as mammalian pancreas.

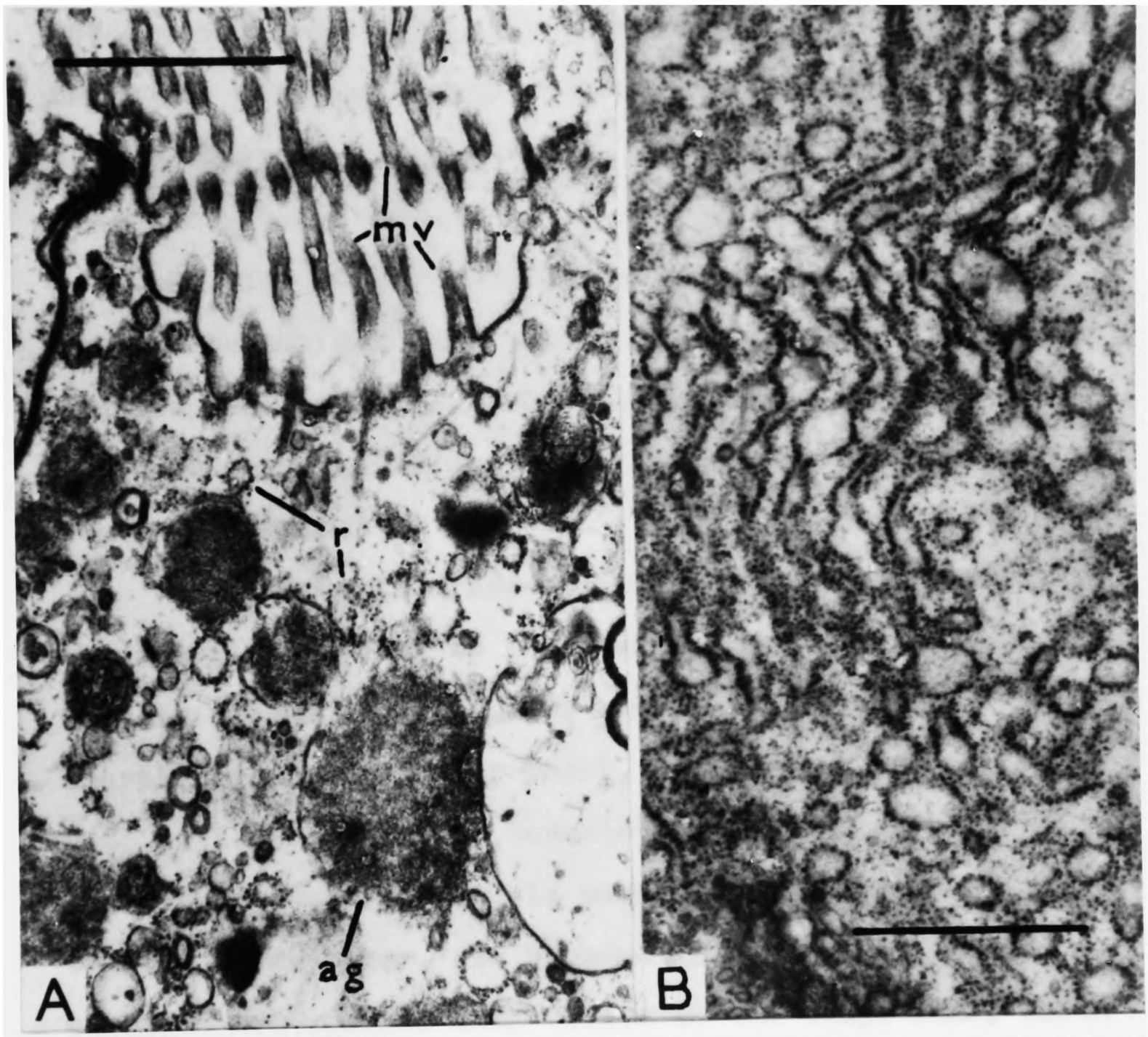
The apical surfaces of the basiphil cells bear microvilli, slightly longer than those of digestive cells. They contain very conspicuous filaments running along their length, and extending up to 365mµ into the underlying cytoplasm. The filaments appear to be arranged both centrally and peripherally in the microvilli.

The apical cytoplasm (fig. 2A) contains several membrane-bounded bodies with granular contents, averaging 650mµ in diameter. The granular contents are separated

Fig. 2. Basiphil cells. A. Apical cytoplasm, showing apical granules. B. Ergastoplasm. (Fixed in 1 per cent osmium tetroxide in distilled water, and stained with uranyl acetate).

ag, apical granule; mv, microvilli; r, ribosomes.

The scale on each micrograph represents 1 μ.


mv
r
ag
A
B

from the membrane by a narrow space, about 70mμ wide. Occasionally bodies of this type can be found further down the cell, particularly near the Golgi apparatus. As well as the membrane-bounded bodies, the apical cytoplasm contains several vacuoles, ranging in diameter from 165mμ to 1.66μ. The larger vacuoles contain lumps of electron-dense material and small granules. There is rather little ergastoplasm apically, but it nevertheless extends right up to the apical surface of the cells, in between the other inclusions.

Numerous lamellar-vacuolar fields can be found in the cytoplasm apical to the nucleus. The Golgi cisternae usually appear straight, but many are curved, often sharply; they are rather short, and usually extend for 0.63 to 1.03μ. The most conspicuous feature of the lamellar-vacuolar fields is numerous small granules, 40 to 60mμ in diameter, and bounded by a membrane; larger vacuoles are scarce. Many bodies in the Golgi zone, which otherwise resemble the membrane-bounded bodies, apparently lack a membrane, but may be partly surrounded by small granules resembling those found in the lamellar-vacuolar fields, or else may have numerous short lengths of microtubules originating from them. Occasional short lengths of microtubules occur elsewhere in the basiphil cells.

Mitochondria occur throughout the basiphil cells, and generally resemble those of digestive cells. They average 1.11 by 0.46μ. Some of them have few cristae and a swollen appearance, like the mitochondria of digestive cells; while others do not appear swollen, and have more numerous cristae. The cristae run in all directions, even in the same mitochondrion. Many have longitudinal cristae, and in some there are oblique cristae. The cristae almost never extend right across the mitochondrion.

A few lipid droplets are present in the basal part of the cell.

The nucleus of basiphil cells has a double membrane. The outer membrane bears ribosomes. Pores have not been found in the nuclear membrane.

Intercellular appearances

At the most apical point where two cells are adjacent, there is an electron-dense mass on the inner surface of the membranes of both cells; this extends for 200 to 250mμ, and corresponds to the terminal bar described in mammals (Palay & Karlin, 1959). Immediately below this region, the plasma membranes of adjacent cells are joined by numerous cross-connections, 18 to 22mμ apart. The structure resembles that of septate desmosomes (Wood, 1959). Where the adjacent membranes are sectioned obliquely, so that the membranes themselves are not visible, cross-striations are still

visible. The cross-connections appear to extend for at least 2μ.

Towards the bases of the cells, the adjacent plasma membranes often cease to be closely apposed, and an intercellular space appears. These spaces are often narrow, most of them being about 200mμ wide. Sometimes, however, they may be much wider, in the region of 1μ. Usually the intercellular space is completely empty, but in one case there appears to be an amoebocyte in the space between two basiphil cells.

DISCUSSION

The results of electron microscopy confirm that there are two distinct types of cells in the tubule epithelium of the digestive gland of Anodonta.

The digestive cells of lamellibranchs have been shown to take part in absorption and phagocytosis (Yonge, 1923, 1926a & b; Owen, 1955), and it is thus not surprising that they have a similar fine structure to cells having the same function in hydroids (Gauthier, 1963; Afzelius & Rosen, 1965) and in pulmonate molluscs (Sumner, 1966a). It is surprising that only a little diffuse granular material occurs in the large vacuoles of digestive cells. With light microscopy the same vacuoles are seen to contain colourless or yellow granules which almost fill the vacuole (Sumner, 1966b). It may be that the fixative used

in this study (1 per cent osmium tetroxide in distilled water) is not a satisfactory fixative for some non-membranous components of cells. In this study, and in an earlier study on pulmonates (Sumner, 1966a), it was also found that the ground substance of the cytoplasm of digestive cells was largely lost after using this fixative, although in Helix formaldehyde-sucrose (Baker, 1965) preserved the ground substance.

The mitochondria of digestive cells, and to some extent those of basiphil cells, present a swollen appearance. However, there is no evidence of swelling in any of the other cell components. While it may be that the mitochondria are particularly susceptible to factors causing swelling, it is possible that in fact the mitochondria of Anodonta do have an empty-looking matrix, with rather few short cristae. The great variation found in the direction of the cristae may be characteristic of molluscs, as it also occurs in various cells of pulmonates (André, 1962; Sumner, 1966a).

The basiphil cells have the fine structure typical of protein-secreting cells, such as mammalian pancreatic exocrine cells. The ergastoplasm and Golgi apparatus are both very well developed. The membrane-bounded bodies found in the apical cytoplasm of basiphil cells are clearly the apical granules found with the light microscope

(Sumner, 1966b), and thus it is interesting that they resemble the protein granules of pulmonates (Sumner, 1966a). It is clear that the basiphil cells are not undifferentiated cells, as was formerly thought (Cuthell, 1912; Yonge, 1923, 1926a & b, 1928, 1939; Mansour, 1946; Morton, 1956).

The membrane-bounded bodies in the apical cytoplasm are clearly associated with the Golgi apparatus. It is interesting that some similar bodies do not appear to be surrounded by a membrane, but by objects resembling the small Golgi vesicles or by numerous microtubules. There is no clear evidence as to what the function of microtubules might be, although Slautterback (1963) found them associated with the nematocysts of Hydra, which are a secretory product just as the apical granules of Anodonta are.

The presence of septate desmosomes in the digestive gland, and also in the digestive gland ducts (Sumner, unpublished), indicates that they must be widespread in molluscs, since they also occur in pulmonates (Sumner, 1966a). The separation of cells basally doubtless allows amoebocytes to penetrate into the epithelium. Graham (1931) illustrates amoebocytes in the epithelium of the digestive gland, and according to Yonge (1926a) their function is to carry away material absorbed by the digestive gland cells.

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PAPER 8

THE DISTRIBUTION OF PHOSPHATASES IN THE DIGESTIVE GLAND CELLS OF CERTAIN MOLLUSCS

SYNOPSIS

The distributions of alkaline and acid phosphatases, and of thiamine pyrophosphatase were studied in the digestive glands of certain pulmonate gastropods and lamellibranchs.

Alkaline phosphatase occurs in the brush border and basement membrane of digestive gland cells. Acid phosphatase occurs in association with the lipofuscin granules of digestive cells, and occasionally in other sites; it has not been found with certainty to be associated with intracellular digestion. Thiamine pyrophosphatase has been found in the Golgi apparatus and plasma membranes of cells, but occurs in the largest amounts in or near the phagocytic vacuoles of digestive cells in the carnivorous slug Testacella.

INTRODUCTION

The presence and distribution of enzymes in the digestive gland cells of molluscs is of great interest in relation to absorptive and secretory processes. It has been shown that the functions of the digestive gland include discharge of secretory products into the lumen of the gland (Sumner, 1966b) and uptake of food materials from the lumen (Hirsch, 1924; Yonge, 1926a & b; Rosen, 1941, 1952, 1965; Sumner, 1965b). It is clearly desirable to know whether the material discharged into the lumen of the gland contains enzymes, and what structures are

concerned with intracellular digestion.

The enzymes selected for study were alkaline phosphatase, acid phosphatase, and thiamine pyrophosphatase (TPPase), all of which are associated with absorptive or secretory processes. Alkaline phosphatase is typically found in the brush border of absorptive cells (Clark, 1961; Chase, 1963). Acid phosphatase is one of the enzymes occurring in lysosomes (de Duve, 1963), and has been used as a marker for these bodies (Goldfischer et al., 1964). Because of the close association of lysosomes with intracellular digestion (Gordon et al., 1963, 1965; Straus, 1964a & b) it was clearly necessary to study the distribution of acid phosphatase. The distribution of TPPase is of interest because of its association with the Golgi apparatus (Novikoff & Goldfischer, 1961).

MATERIAL AND METHODS

The species used were Helix aspersa, Succinea putris and Testacella maugei (pulmonate gastropods), and Anodonta anatina and Unio tumidus (lamellibranchs). The specimens were feeding, or at least had access to food; starved specimens of Helix were also used. Only the digestive glands of these species were studied.

Pieces of digestive gland were dissected out, and fixed overnight either in cold acetone-ethanol (1:1) or in cold formaldehyde-calcium (or cold formaldehyde-sucrose

(Baker, 1965) for Unio). Pieces of tissue fixed in acetone-ethanol were embedded in paraffin, and sectioned at 10μ. Pieces fixed in formaldehyde were rinsed in distilled water, and stored until sectioning in cold sucrose-gum acacia (Holt, 1959); unembedded frozen sections were cut at 15μ.

The test for alkaline phosphatase was carried out on all forms except Succinea. Sections of tissue fixed in acetone-ethanol (and also in formaldehyde-sucrose in the case of Unio) were incubated in Gomori's alkaline phosphatase medium (Gomori, 1952), for periods between ½ and 4½h; the best period was generally found to be 1½h. The reaction product was visualized by testing for calcium by the cobalt substitution method. Control slides were incubated in Gomori's medium without the substrate (sodium β-glycerophosphate).

The test for acid phosphatase was carried out on all forms except Anodonta. Sections of tissue fixed in formaldehyde were incubated in Gomori's acid phosphatase medium (Gomori, 1952). The periods of incubation usually used were 4 and 16h, although periods of 1h were used for Unio and 1½h for Succinea. The reaction product was visualized by dipping in dilute ammonium sulphide solution. Control slides were either incubated in the medium without substrate, or in the complete medium containing 0.1M sodium fluoride as an inhibitor. 0.01M sodium fluoride was found

not to produce complete inhibition of the reaction.

The test for TPPase was carried out on Helix and Testacella, with sections of tissue fixed in formaldehyde-calcium. The method of Novikoff & Goldfischer (1961) was used, and the reaction product visualized by dipping in dilute ammonium sulphide solution. For Helix the most satisfactory period of incubation was 2½h; for Testacella periods of 25 and 45min gave better results than longer periods. Control slides were either incubated in the medium without the substrate, or in the complete medium containing 0.01M uranyl nitrate as an inhibitor.

RESULTS

Pulmonates

Alkaline phosphatase occurs in the apical cytoplasm and brush borders of calcium and excretory cells and some of the digestive cells, and in the basement membranes surrounding the tubules (figs. 1 & 2A).

Acid phosphatase occurs in small granules in the apical cytoplasm of digestive cells (fig. 1) and in association with the yellow granules in the basal vacuoles of digestive cells (figs. 1 & 2D). It is not clear whether the enzyme occurs in the yellow granules themselves, or whether it is chiefly localized at the edges of the vacuoles containing them. Granules in the nuclei are sometimes positive for acid phosphatase.

Thiamine pyrophosphatase (TPPase) occurs on the

See over.

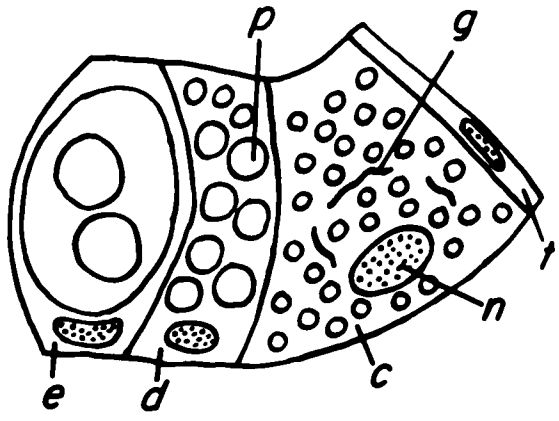
Fig. 1. Diagrams showing the distribution of alkaline phosphatase, acid phosphatase and thiamine pyrophosphatase in the digestive gland cells of pulmonates and lamellibranchs.

Continuous lines and objects that are black all through represent sites of enzyme activity; dotted lines and other objects that are dotted represent structures which do not possess enzyme activity.

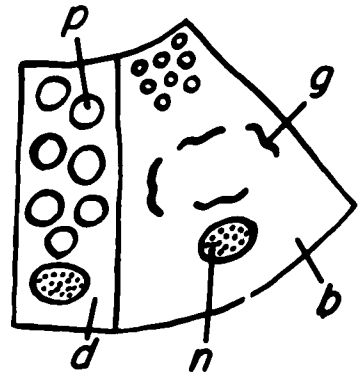
b, basiphil cell; c, calcium cell; d, digestive cell; e, excretory cell; g, Golgi body; n, nucleus; p, phagocytic vacuole; t, thin cell.

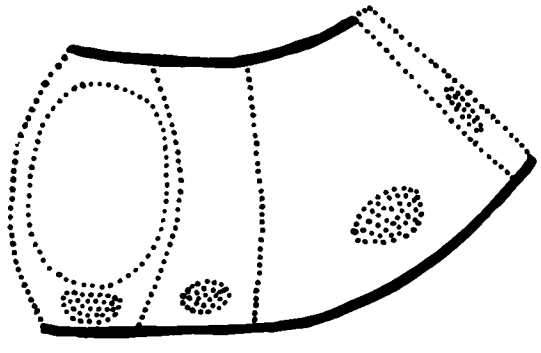
PULMONATES

LAMELLIBRANCHS

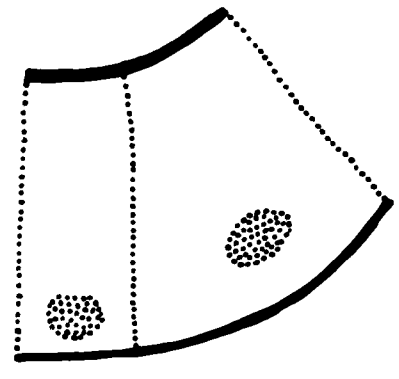


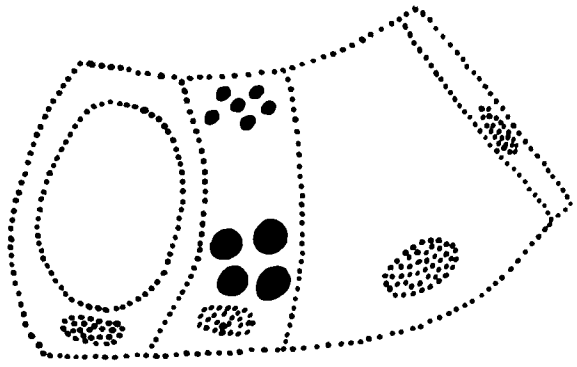
general structure



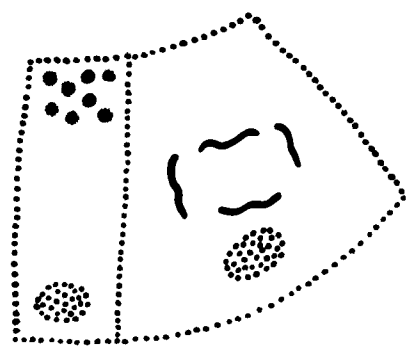


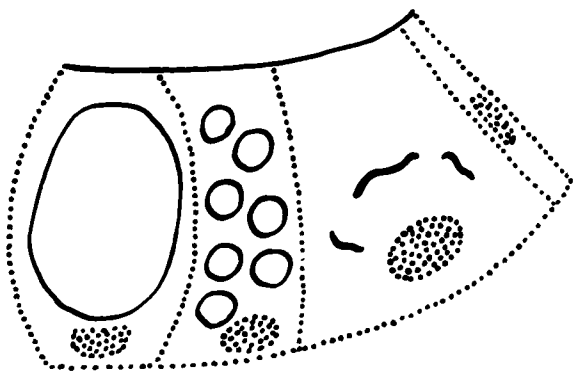
alkaline phosphatase





acid phosphatase





thiamine pyrophosphatase

free surfaces of cells, on the surfaces of the large vacuoles of excretory cells, and parts of the surfaces of the vacuoles of digestive cells (fig. 1). This enzyme also occurs in the Golgi bodies of calcium cells (fig. 1).

In Helix there is also a diffuse positive reaction for alkaline phosphatase throughout the calcium cells (fig. 2B). There is a weak positive reaction for alkaline phosphatase in the yellow granules of digestive cells, but this is also present in the controls. Acid phosphatase occurs in most of the calcium spherules (fig. 2C). This enzyme is often absent from the yellow granules of digestive cells, particularly when the snail is feeding or had been starved for less than 49 days, and is always absent from the apical cytoplasm.

The calcium and excretory cells of Succinea have granules, up to 1.2μ across, in the apical cytoplasm, which are positive for acid phosphatase. These granules occasionally appear as rings. They do not correspond to any inclusion known to be present in these cells.

In Testacella acid phosphatase also occurs as large lumps (3 to 5μ across) just basal to the nuclei of calcium cells, and on the surfaces of the vacuoles and yellow granules of excretory cells. Testacella has no acid phosphatase in the basal vacuoles of its digestive cells. TPPase occurs as numerous round bodies in the apical half

See over.

Fig. 2. A. Alkaline phosphatase in the digestive cells of *Helix*.

B. Alkaline phosphatase in a calcium cell of *Helix*.

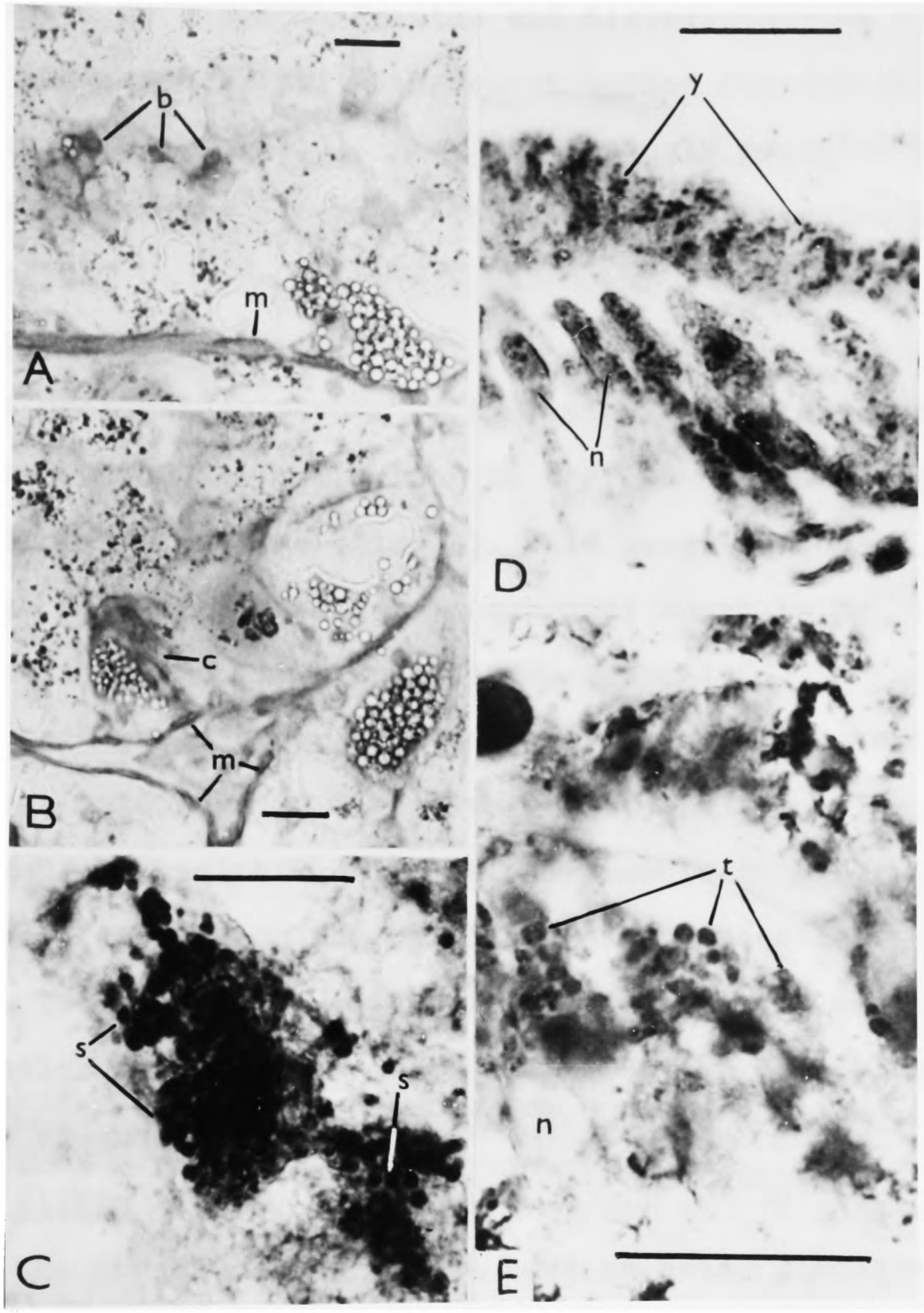
C. Acid phosphatase in the calcium spherules of the calcium cells of *Helix*.

D. Acid phosphatase in the yellow granules of digestive cells of *Succinea*.

E. Thiamine pyrophosphatase in the digestive cells of *Testacella*.

b, brush border; *c*, calcium cell; *m*, basement membrane; *n*, nucleus; *s*, calcium spherules; *t*, site of TPPase; *y*, yellow granules.

The scale on each micrograph represents 20 μ.


b
m
A
c
m
B
s
s
C
y
n
D
t
n
E

of the digestive cells (fig. 2E). These bodies usually have a more strongly positive outline. The outline of these bodies corresponds in size and distribution to the objects blackened by the Mann-Kopsch method for the Golgi apparatus (Sumner, 1966g); the less strongly positive part of these bodies may represent the phagocytic vacuoles of these cells.

Lamellibranchs

Alkaline phosphatase occurs in the apical cytoplasm and brush borders of all cells, and in the basement membranes surrounding the tubules (fig. 1). This reaction occurs only in tissue fixed in formaldehyde-sucrose; there is no reaction in tissue fixed in acetone-ethanol.

Acid phosphatase occurs as very small granules in the apical cytoplasm of digestive cells, and in plates and threads in basiphil cells which resemble in size, shape and distribution the Golgi bodies of these cells (Sumner, 1966g)(fig. 1). After incubation for 4h, some of the vacuoles in digestive cells which contain colourless granules are positive.

Control slides

None of the structures described here as being positive for alkaline and acid phosphatases and thiamine pyrophosphatase are positive in control sections.

DISCUSSION

Although the function of alkaline phosphatase is not certainly known, it is typically associated with the free border of absorptive cells (Clark, 1961; Chase, 1963), and is probably concerned with transport of materials across cell membranes (Danielli, 1952). The localization of this enzyme at the free border and in the basement membrane of digestive gland cells of molluscs is what one would expect, since the digestive cells, at least, are absorptive and phagocytic (Hirsch, 1924; Yonge, 1926a & b; Rosen, 1941, 1952, 1965; Sumner, 1965b). The fact that not all digestive cells of pulmonates have alkaline phosphatase apically presumably reflects different physiological states; some cells are probably absorbing, while others are about to excrete by discharging the contents of their vacuoles into the lumen (Sumner, 1965a). The diffuse positive reaction for alkaline phosphatase in the calcium cells of Helix seems most likly to be connected with calcium metabolism, possibly in a manner comparable to that in mammalian bone formation (Irving, 1957).

Acid phosphatase typically occurs in lysosomes (de Duve, 1963). These bodies are known to be involved in intracellular digestion (Gordon et al., 1963, 1965; Straus, 1964a & b), and in the production of lipofuscin (Essner & Novikoff, 1960). In the digestive cells of Helix and Succinea, the acid phosphatase is associated with the

yellow granules, which contain lipofuscin (Sumner, 1965a, 1966d), and presumably is concerned in their formation. This is supported by the absence of acid phosphatase in corresponding sites in Testacella, a form in which the digestive cells do not contain yellow granules (although acid phosphatase is associated with the yellow granules of excretory cells in Testacella). The acid phosphatase in the calcium spherules of Helix may similarly be concerned with formation of lipofuscin, as yellow granules can be formed in the same sites as the spherules (Sumner, 1966a).

The small granules in the apical cytoplasm of digestive cells in Succinea, Testacella and Unio, which contain acid phosphatase, are more likely to be concerned with intracellular digestion. Presumably these granules are contained in the apical vacuoles (Sumner, 1965a) in which phagocytosed food material first appears (Sumner, 1965b). It is rather surprising that acid phosphatase does not occur in vacuoles further down the cell, except in connection with the formation of lipofuscin. The distribution of acid phosphatase found here is only partly consistent with the distribution of some lysosomal enzymes in pulmonates as described by various authors (Billett & McGee-Russell, 1965; Nakazima, 1956; Rosenbaum & Ditzion, 1963; Muller, 1965). It is difficult to account for the differences, but a fairly consistent finding is the

association of lysosomal enzymes with the yellow granules of digestive cells.

The occurrence of acid phosphatase in the Golgi apparatus of basiphil cells of Unio is interesting, as this enzyme also occurs in the Golgi apparatus of intestinal secretory cells in Helix (Sumner, 1966a); it does not often occur in the Golgi apparatus, at any rate in mammalian cells (Goldfischer et al., 1964).

TPPase has been found in high concentrations in the Golgi apparatus of vertebrate cells, and has been used as a marker for this organelle (Novikoff & Goldfischer, 1961; Goldfischer et al., 1964). TPPase also occurs in the plasma membranes of many cells (Goldfischer et al., 1964). In general, TPPase occurs in these sites in Helix and Testacella, but in Helix the concentration of the enzyme in the Golgi apparatus is not conspicuously high. In the digestive cells of Testacella, what appears to be the Golgi apparatus has a high concentration of TPPase. However, it has been shown (Sumner, 1966a) that the objects in these cells which are blackened by the Mann-Kopsch method are not lamellar-vacuolar fields (Hirsch, 1961). There may be a relationship between the presence of TPPase and the reduction of osmium tetroxide in these cells, without these properties being localized in any particular structure. It has previously been found that TPPase is not exclusively localized in the Golgi apparatus in some invertebrates

(Lane, 1963; Lee, 1963; Meek & Lane, 1963).

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This work was done during the tenure of a D.S.I.R. research studentship; I thank them for financial assistance.

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PAPER 9

INTESTINAL SECRETORY CELLS IN PULMONATE MOLLUSCS

SYNOPSIS

As well as ciliated and mucous cells, the intestinal epithelium of pulmonates contains secretory cells. The intestinal secretory cells have much RNA in the cytoplasm, a conspicuous Golgi apparatus, and numerous secretory granules. The secretory granules consist largely of protein, with possibly a little phospholipid.

Alkaline phosphatase occurs in the free border of the intestinal epithelial cells, and in the pigmented granules of the ciliated cells. Acid phosphatase occurs in the pigmented granules of the ciliated cells, and in the immature secretory granules of the intestinal secretory cells.

INTRODUCTION

In the course of studies on the digestive system of molluscs, it was found that in the pulmonate snail Helix part of the intestinal epithelium contained cells which were characterized by the presence of numerous granules which contain protein. It has been shown that the granules in these cells are discharged under the influence of certain drugs (Sumner, 1966), and thus the cells have been called intestinal secretory cells. Since these cells do not seem to have been described before, at any rate in pulmonates, it was felt that a full description should be given.

Most authors describe only two cell types in the intestinal epithelium of pulmonates: ciliated cells and mucous cells (Argaud & Bounoure, 1910; Spiro, 1911; Morton, 1955). The ciliated cells are usually stated to contain coloured droplets or granules, although von Haffner (1924) considers that the cells which contain the coloured granules are distinct from the ciliated cells. Guardabassi & Ferreri (1953) describe what they consider to be secretory cells in the intestine of Helix, but the granules of the cells they describe contain alkaline phosphatase, which indicates that they are distinct from the type of cell to be described here.

The present study had two aims: to locate the part of the intestine in which the intestinal secretory cells lie (since they certainly do not occur throughout the intestine); and to describe in detail the cytology and histochemistry of these cells.

MATERIAL AND METHODS

To determine in which part of the intestine the secretory cells lie, the whole digestive system was dissected out and fixed in formaldehyde-saline. A diagram of the course of the intestine through the digestive gland was drawn. The intestine itself was then dissected out from the digestive gland, divided into segments, and a careful note made of the original position of each segment. Each segment was then embedded in paraffin, sectioned

longitudinally at 8μ, and tested histochemically for tryptophan by the DMAB/nitrite method (Pearse, 1960). This was done with the snails Helix aspersa and Theba cantiana.

The intestines of the following species were studied to see whether secretory cells were present: Limnaea stagnalis (Basommatophora); Agriolimax reticulatus, Arion hortensis, Helix aspersa, Milax budapestensis, Succinea putris and Theba cantiana (Stylommatophora).

For routine cytological study, portions of the appropriate part of the intestine were fixed in Zenker or Champy, embedded in paraffin, and sectioned at 6μ. Sections of tissue fixed in Zenker or Champy were stained with Heidenhain's iron haematoxylin; in addition, sections of tissue fixed in Zenker were stained with Ehrlich's haematoxylin and counterstained with phloxine. Mitochondria were stained by Metzner's method (Meves, 1911) in 4μ sections of tissue fixed in Champy. The Golgi apparatus was shown by the Hann-Kopsch method (Baker, 1933); osmication was carried out at room temperature, and the best period of osmication was found to be 17 days. The histochemical tests used are listed in table 1.

Histochemical tests for the enzymes acid and alkaline phosphatase were carried out on the whole intestinal epithelium, by the methods of Gomori (1952).

For the acid phosphatase test, portions of intestine were fixed overnight in cold formaldehyde-sucrose (Baker, 1965), rinsed in cold distilled water and stored in cold sucrose-gum acacia (Holt, 1959). 15μ frozen sections were cut, and attached to slides using the method of Baker (1945). Sections were incubated in Gomori's acid phosphatase medium for 1h or 4h, and the reaction product visualized by dipping in weak ammonium sulphide solution. The shorter incubation period was better as the detail was more easily visible. Control sections were incubated in Gomori's medium containing 0.1M sodium fluoride as an inhibitor.

For the alkaline phosphatase test, portions of intestine were fixed overnight in cold acetone-ethanol (1:1), embedded in paraffin, and sectioned at 10μ. The sections were incubated in Gomori's alkaline phosphatase medium for ½h or 1½h, and the reaction product visualized in the usual way. Control sections were incubated in Gomori's medium lacking the substrate, sodium β-glycerophosphate.

RESULTS

In Helix and Theba intestinal secretory cells occur in the final segment of the intestine, immediately preceding the rectum (C to D in fig. 1). This is the part of the intestine which is on the outer surface of the digestive gland. Towards the end of this part of the intestine, the secretory cells become fewer. At any rate in Theba, a few

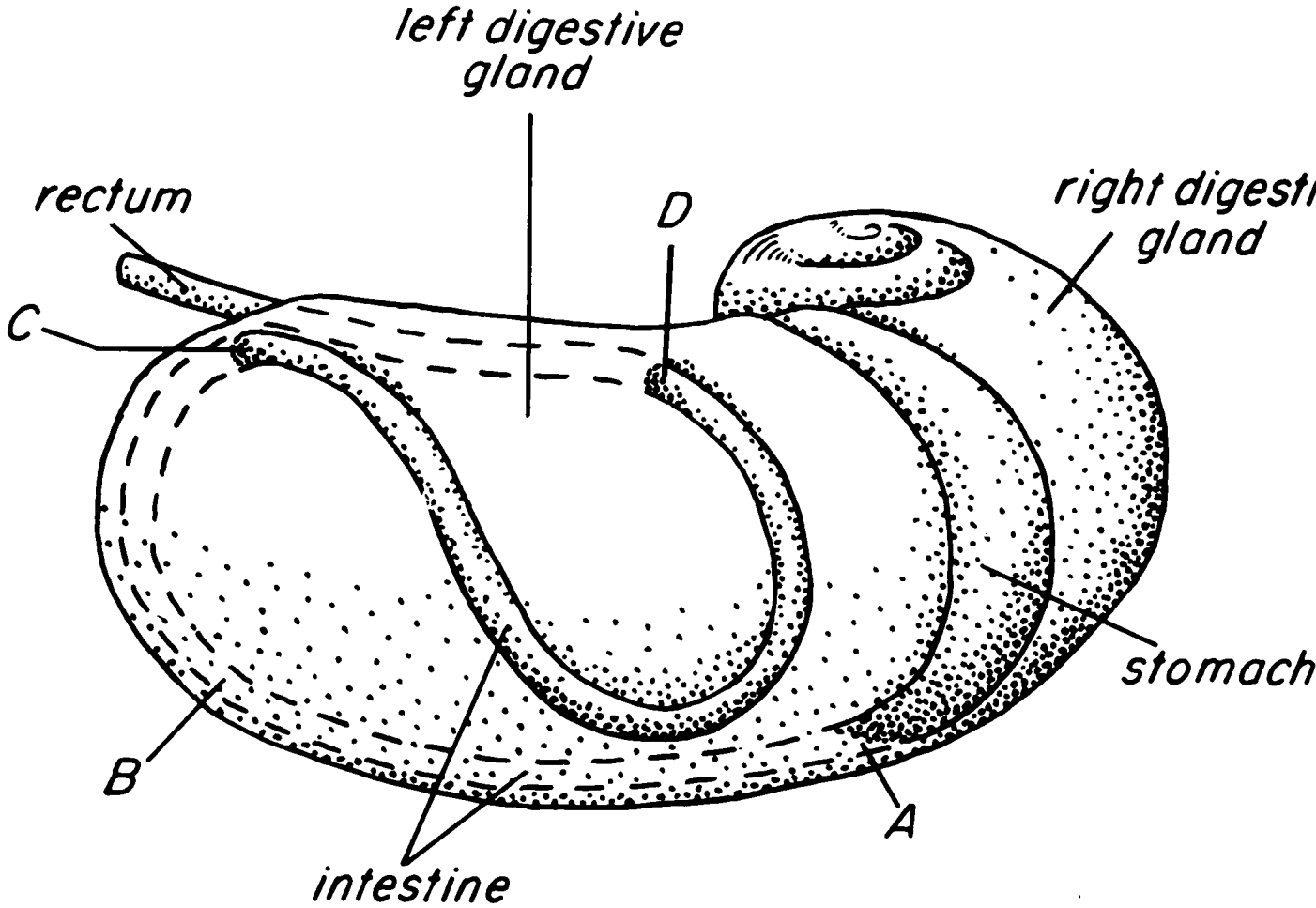


Fig. 1. Diagram showing the course of the gut through the digestive gland in a pulmonate snail (e.g. Helix, Theba). The intestine runs from A to D.

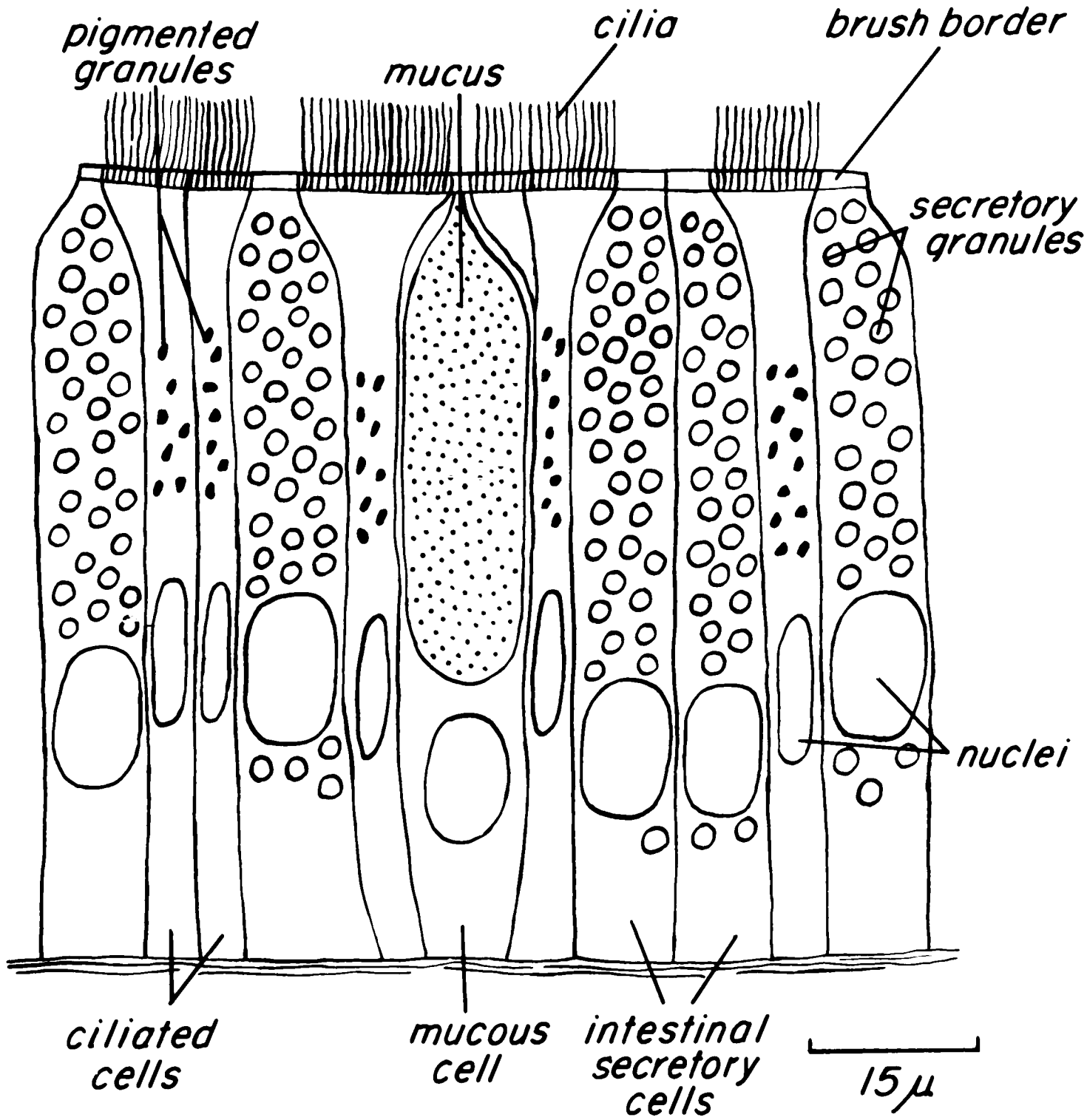


Fig. 2. Diagram of the histology of that part of the intestinal epithelium which contains intestinal secretory cells.

secretory cells occur in the preceding section of the intestine (B to C in fig. 1).

Intestinal secretory cells occur in all the species examined.

The epithelium of the part of the intestine where the intestinal secretory cells occur is made up of three types of cells: there are roughly equal numbers of secretory and ciliated cells, and there are also mucous cells, which are much scarcer (fig. 2). The ciliated cells are narrower than the secretory cells, giving the impression of greater numbers of secretory cells than of ciliated cells; in fact the two types of cells roughly alternate (figs. 2 & 3A).

The ciliated cells usually bear cilia apically, as well as a brush border, but the cilia are sometimes absent in fixed preparations. It is not known whether this is so in life. In the cytoplasm between the nucleus and the free border of the cell there is usually a group of small yellowish or brownish granules.

Intestinal secretory cells

The dimensions of the various parts of the cells are listed in table 2, and the results of the histochemical tests in table 3.

Intestinal secretory cells are columnar, tapering suddenly to a very narrow neck apically (fig.4). The small area of free surface of the cell appears to bear a

See over.

Fig. 3. Micrographs of intestinal secretory cells.

A. A pyronine/methyl green preparation, showing the distribution of RNA.

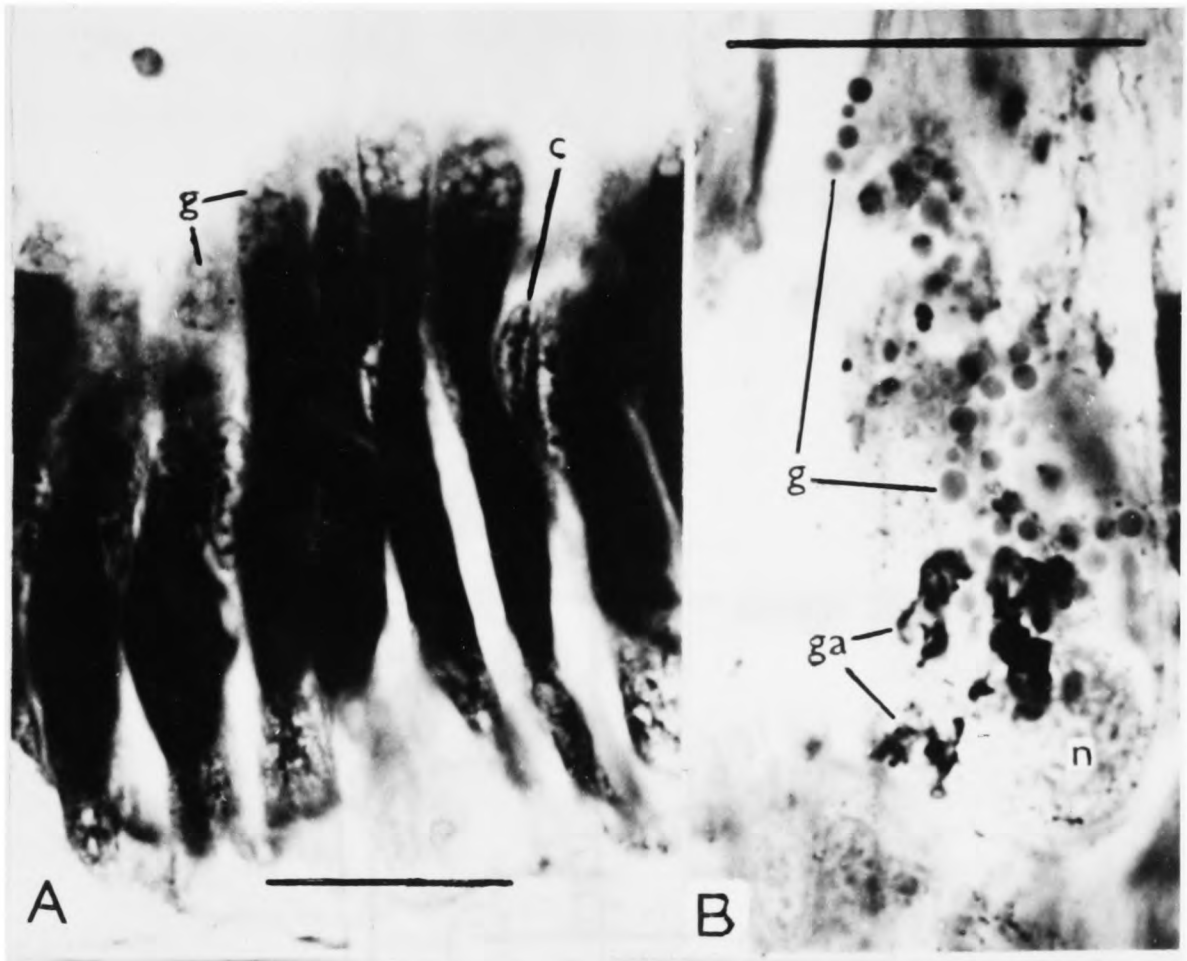
B. A Mann-Kopsch preparation, showing the Golgi apparatus.

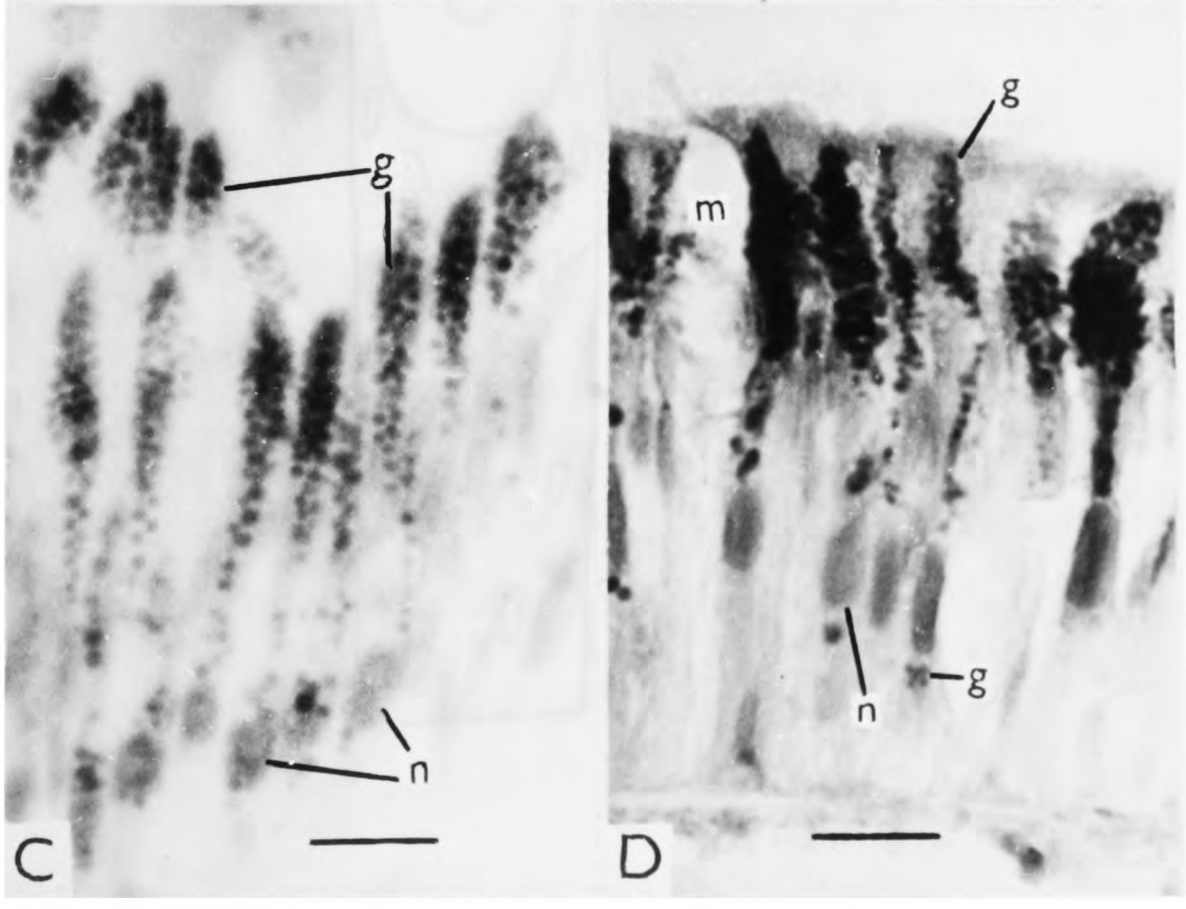
C and D. DMAB/nitrite preparations, showing the tryptophan content of the secretory granules.

All the micrographs are of Helix aspersa, except D, which is of Theba cantiana.

c, nucleus of ciliated cell; g, granules of intestinal secretory cells; ga, Golgi apparatus; m, mucous cell; n, nuclei of intestinal secretory cells.

The scale on each micrograph represents 20 μ.


g
c
A
g
ga
n
B


g
n
C
g
m
n
g
D

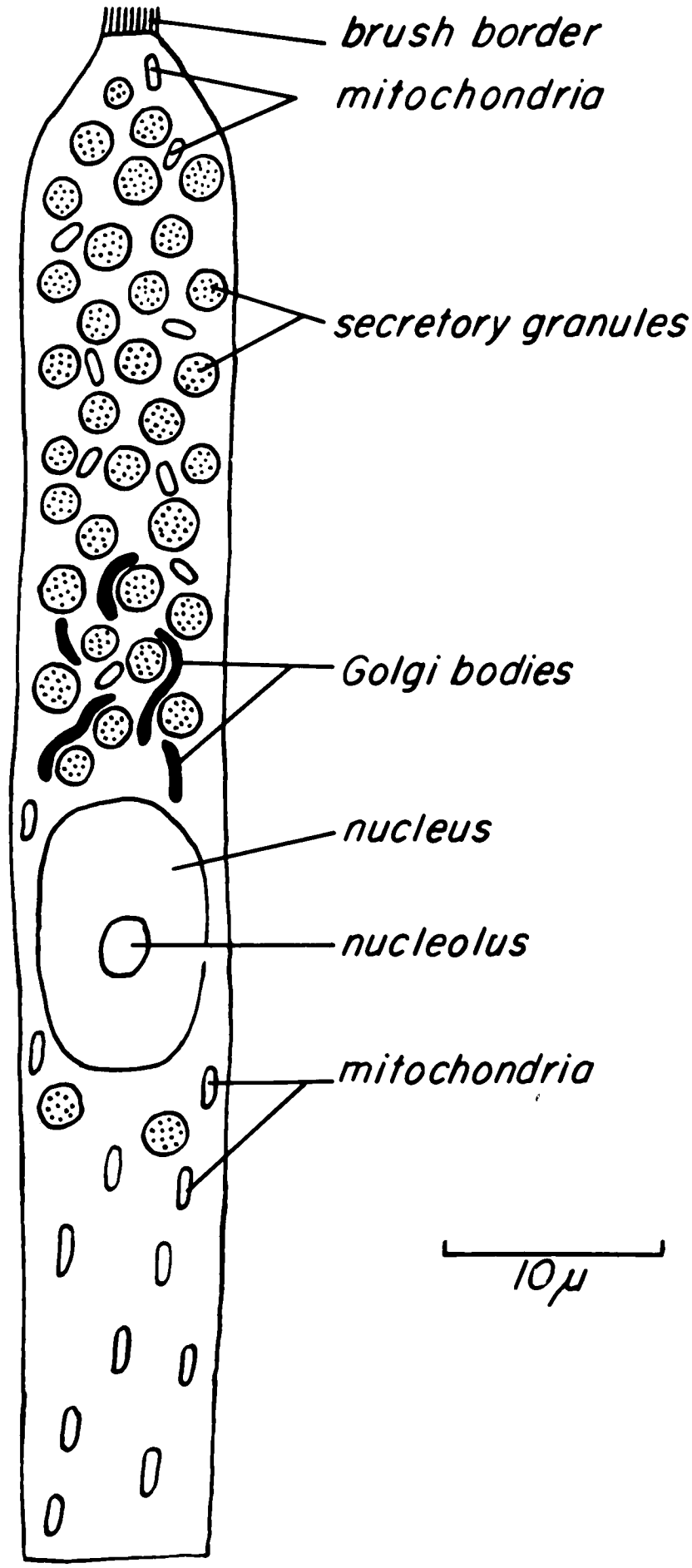


Fig. 4. Diagram of an intestinal secretory cell.

brush border. In shorter cells the nucleus is near the base, although there is always a moderate amount of cytoplasm basal to it; in taller cells the nucleus may be half-way up the cell. One prominent nucleolus is usually visible. The cytoplasm is strongly basiphil, owing to the presence of RNA (table 3; fig. 3A). The granules of the intestinal secretory cells occupy much of the cytoplasm apical to the nucleus, and occasionally there may be one or two granules basal to the nucleus. The granules are characterized by a high content of tryptophan (table 3; fig. 3C & D). 30 to 40 granules are usually visible in a section of a single cell.

The Golgi zone is immediately apical to, and at the sides of the nucleus. The degree of development or of osmication of the Golgi apparatus is very variable. In its simplest form the Golgi apparatus consists of one or two crescentic threads or plates, which may partly enclose a grey area. Sometimes the Golgi apparatus is made up of many of these crescentic bodies. Often the Golgi apparatus appears as a complex network (fig. 3B), apparently formed by the joining up of several crescentic bodies. Secretory granules frequently occur in the Golgi zone, often in close association with the Golgi bodies.

Mitochondria occur almost throughout the cytoplasm of the intestinal secretory cells. Basally to the nucleus

they are numerous and fairly densely packed. They are long and thin (table 2) and orientated along the long axis of the cell. Among and apically to the secretory granules, the mitochondria are shorter and randomly orientated, but are still fairly numerous. Mitochondria are apparently absent where secretory granules are densest.

No lipid droplets have been found in intestinal secretory cells. After coloration for ½h with Sudan black, the mitochondria appear to be coloured. In the supra-nuclear region, there often appear to be crescents, coloured by Sudan black, and partly surrounding secretory granules; these are probably Golgi bodies.

Phosphatases in the intestinal epithelium

Acid phosphatase is absent from the mucous cells of the intestinal epithelium. In the ciliated cells it is localized in numerous small granules, about 1.2 by 0.4µ, elongated along the long axis of the cell (fig. 5A). These granules are concentrated in the supra-nuclear region. In the intestinal secretory cells acid phosphatase occurs in a strongly positive mass just apical to the nucleus (fig. 5A). This mass consists of irregular granules, mostly 1.6 to 2.0µ across, and thick crescentic threads. In the control sections no structures show a positive reaction.

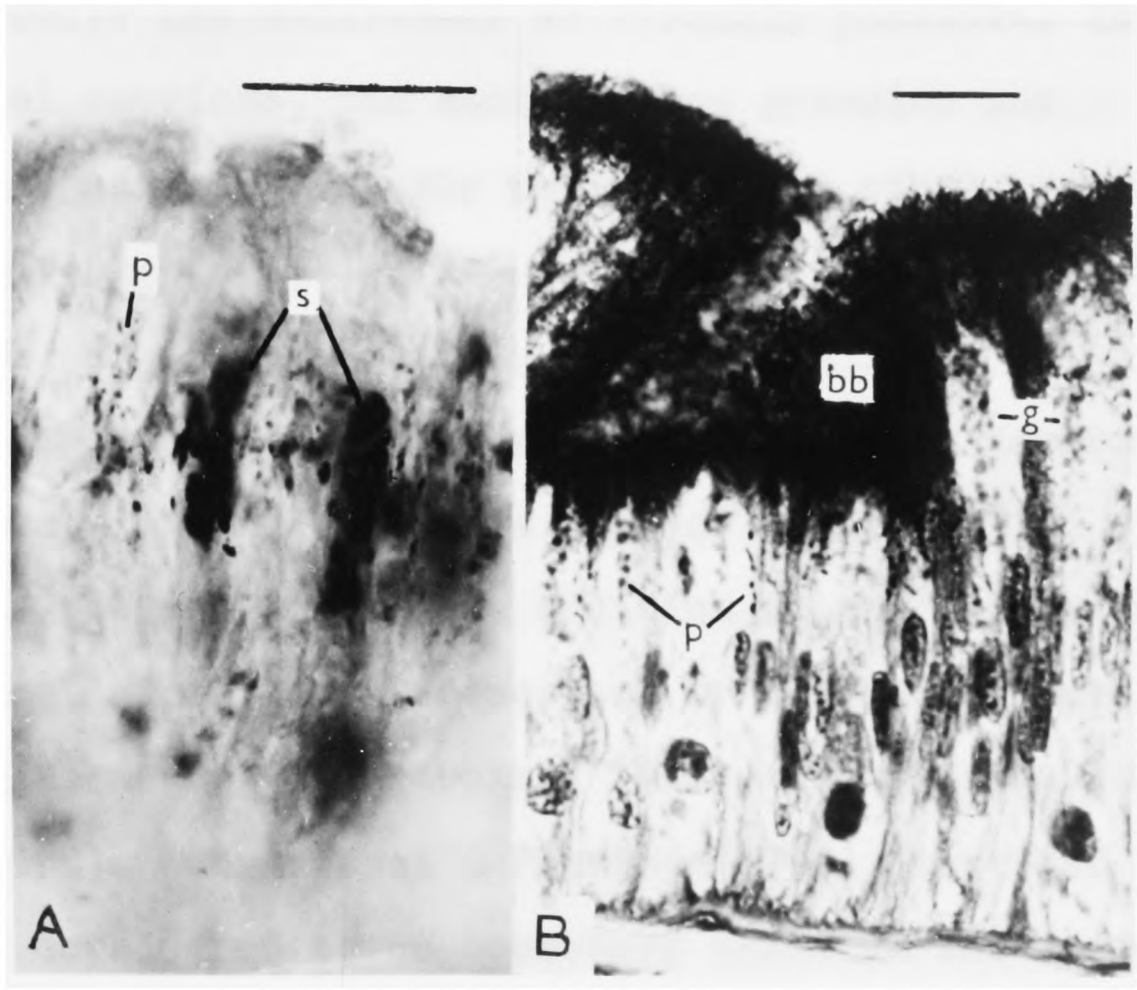
Alkaline phosphatase is present in large quantities in the brush border of the entire intestinal epithelium

See over.

Fig. 5. Micrographs showing the localization of phosphatases in the intestinal epithelium of Rana. A. Acid phosphatase. B. Alkaline phosphatase.

bb, reaction product in brush border; g, granules of intestinal secretory cells (stained with phloxine); p, pigmented granules of ciliated cells; r, reaction product in intestinal secretory cells.

The scale on each micrograph represents 20 μ.


p
s
A
bb
g
p
B

(fig. 5B). After 1½h incubation it also appears in the apical cytoplasm of all cells, and, very weakly, in the most apical granules of intestinal secretory cells; these reactions may be due to diffusion. A weakly positive reaction occurs in the nuclei of all cells, and in the basement membrane. The small yellow granules of the ciliated cells are moderately to strongly positive. In the control sections, the small yellow granules may sometimes appear very weakly positive; all other structures are negative.

DISCUSSION

The final segment of the intestine of pulmonate gastropods clearly contains a distinctive type of cell, the intestinal secretory cell, in addition to the mucous and ciliated cells previously described by various authors (Argaud & Bounoure, 1910; Spiro, 1911; von Haffner, 1924; Morton, 1955). Guardabassi & Ferreri (1953) described what they considered to be secretory cells in the intestine of Helix; these cells contained numerous granules which were strongly positive for alkaline phosphatase. The intestinal secretory cells do not have much alkaline phosphatase in their granules. It seems more likely that Guardabassi & Ferreri were referring to the ciliated cells, the yellow granules of which are numerous and do contain alkaline phosphatase.

Although intestinal secretory cells have not been described before in pulmonates, there are similar cells in other gastropods (Howells, 1944; Gabe, 1952), and probably in the Polyplacophora (Fretter, 1937). The granules of the cells described by Gabe (1952) in heteropods resemble those found here in staining well by mitochondrial methods.

Although the cells are clearly secretory, and the granules are discharged into the lumen of the intestine (Sumner, 1966), it is not clear what their function is. The granules consist largely of protein, with possibly a little phospholipid. The protein contains very little cystine, as shown by the reactions to the performic acid/ alcian blue, Schmorl, and oxidation/aldehyde fuchsin tests. This suggests that enzymes are not present, as these usually contain a considerable amount of cystine (Neurath, 1964); the location of the cells towards the end of the intestine also makes this unlikely. Not much further digestion would be reqired by the time the food reached this part. In other forms it has been suggested that the secretion of the cells helps to bind the faeces together (Howells, 1944). However, in Helix it has not been found that there is a high concentration of tryptophan on the surface of the faeces (Sumner, unpublished) as would be expected if this were so.

The distribution of alkaline phosphatase described here confirms the results of Guardabassi & Ferreri (1953).

Acid phosphatase has been used as a marker for lysosomes, and has been shown to occur in immature secretory granules and in lipofuscin granules (Goldfischer et al., 1964). In the intestinal secretory cells, the granules in the Golgi zone, which presumably are immature, contain acid phosphatase. It is possible that the Golgi apparatus itself contains acid phosphatase, although this needs confirmation by electron microscopy. The Golgi apparatus does not normally contain acid phosphatase (Goldfischer et al., 1964).

The yellow granules of the ciliated cells clearly contain both acid and alkaline phosphatase. If the pigment of the granules is lipofuscin, which is widely distributed in the digestive system of molluscs (Jacks, 1955; Sumner, 1965), one might deduce that they are lysosomal in origin, and that the lysosomes contain alkaline as well as acid phosphatase. Alkaline phosphatase has occasionally been found in lysosomes (Ogawa et al., 1961).

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TABLE 1. HISTOCHEMICAL TESTS USED

Test	Fixative	Thickness of sections (μ)	Method to show	Reference
Feulgen	Ze	6	DNA	Feulgen & Rossenbeck, 1924
Pyronine/methyl green (P/MG)	Ze3	6	RNA	Jordan & Baker, 1955
Ribonuclease + P/MG	Ze3	6	RNA	Bradbury, 1956
Periodic acid/ Schiff (PA/S)	Ze	6	1:2 glycol groups	MacManus, 1948
Diastase + PA/S	Ze	6	Glycogen	Lillie & Greco, 1947
Sudan Black	F-Ca/PC	10	Lipids	Baker, 1956b
Acid haematein	F-Ca/PC	10	Phospholipids	Baker, 1946
Pyridine extraction	WB	10		
DMAB/nitrite	F-Na	8	Tryptophan	Pearse, 1960
Millon	F-Na	8	Tyrosine	Baker, 1956a
Sakaguchi	Ze	16	Arginine	Baker, 1947
Peracetic acid /alcian blue	F-Na	8	Cystine	Pearse, 1960
Oxidation + aldehyde fuchsin	F-Na	8	Cystine and some other substances	Dawson, 1953 Sumner, B.E.H., 1965
Schmorl	F-Na	8	Cystine; some pigments	Pearse, 1960

The following abbreviations are used: F-Ca/PC, formaldehyde-calcium with postchroming; F-Na, formaldehyde-saline; WB, weak Bouin; Ze, Zenker; Ze3, Zenker for 3h.

TABLE 1. AVERAGE DIMENSIONS OF INTESTINAL SECRETORY CELLS

Height of cell	69.1 μ
Width of cell	[illegible] μ
Nucleus	11.9 x 7.[illegible] μ
Nucleolus	2.6 x 2.0 μ
Secretory granules	1.9 x 1.7 μ
Mitochondria	1.8 x 0.4 μ
Golgi zone	7.9 x 5.[illegible] μ
Height of brush border	1.2 μ

TABLE 3. RESULTS OF HISTOCHEMICAL TESTS

Test	Nuclei	Nucleoli	Ground cytoplasm	Apical cytoplasm	Secretory granules
Feulgen	+++	0	0	0	0
Pyronine/methyl green (P/MG)	0	+++	+++	++	0
Ribonuclease + P/MG	0	0	0	0	0
Periodic acid/Schiff	0	0	+	+	0
Diastase + PA/S	0	0	+	+	0
Sudan black	0	0	0	0	0
Acid haematein	0	++	0	0	++ or +++
Pyridine extraction	0	0	0	0	0 to ++
DMAB/nitrite	0	0	+	+	+++
Millon	+	-	+	++	+++
Sakaguchi	+	-	++	++	+++
Peracetic acid/ alcian blue	0	0	+	+	0
Oxidation + aldehyde fuchsin	0	-	+	+	+
Schmorl	0	0	0	+	+

The following symbols are used: 0, no reaction; -, no observation; +, weakly positive; ++, moderately positive; +++, strongly positive.

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