PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/16544

Please be advised that this information was generated on 2019-04-07 and may be subject to change.
LIGHT AND ELECTRON MICROSCOPICAL INVESTIGATIONS ON THE SALIVARY GLANDS OF LYMNAEA STAGNALIS L.

H. H. Boer, S. E. Wendelaar Bonga, and N. van Rooyen
Department of Zoology, Free University, Amsterdam, The Netherlands

Received September 12th, 1966

Summary. 1. The salivary glands of Lymnaea stagnalis were investigated by use of a series of histological techniques, including electron microscopy.

2. With the light microscope 9 different cell types could be distinguished in the glandular epithelium. Seven of them (granular cells, pseudochromosome cells, mucocytes I and II, cells with an acidophilic inclusion, grain cells, mixed cells) are regarded as distinct secretory cell types. Of the two remaining types, the basophilic cells probably represent early developmental stages of the secretory cells, whereas the ciliated cells are considered as transporting cells. Transitional stages between secretory cells were not found.

3. The 4 most important cell types appeared to be located in different parts of the gland: the granular cell in the secretory ducts, the pseudochromosome cell in the interlobular ducts, mucocyte II in the intralobular ducts, and mucocyte I in the acini.

4. Because of these results it is concluded that in the gland no "secretion cycles" occur.

5. An enzyme experiment indicated the production of amylase in the granular cell type.

6. The different cell types and their secretion products as seen in the electron microscope, were described. Transitional stages between secretory cell types were not observed. Six of the secretory cell types seem to produce their own secretory material. Two of these material types are found in the mixed cell. Generally, on the basis of the ultrastructure of the endoplasmic reticulum and the Golgi apparatus, 2 groups of cells, probably serous (granular cell, pseudochromosome cell, cell with an acidophilic inclusion) and mucous (mucocyte I and II, grain cell) cells could be distinguished.

Introduction

Pacaut and Vigier (1906) reviewed the literature on the histological investigations of the salivary glands of pulmonates. They stated that the observations had led to different conclusions and interpretations. In later publications, which deal in the main with the glands of Helix pomatia (Krijgsman, 1928; Baecker, 1932; Voitov, 1934), H. aspersa (Blain, 1957), and to a lesser extent of Lymnaea stagnalis (Carriker and Bilstad, 1946; Gabe and Prenant, 1948), no unanimity can be found either.

The investigations have particularly been concentrated on the inventoryization of morphologically different cell types in the glandular epithelium. Furthermore, it has usually been tried to find relationships between the cell types, in order to establish series of genetically related forms. The search for these series was based on the supposition of Nalepa (1883, cf. Pacaut and Vigier, 1906) that several distinct cell types might in fact only be morphological modifications of one functional cell species. Such a cell series, which would reflect the formation of the secretion product, has been called a secretion cycle.

The views of different investigators are rather divergent. Pacaut and Vigier (1906) distinguished in Helix pomatia between 5 cell types. They assumed that there is only one secretion cycle. Krijgsman (1928) also established one secretion cycle. He distinguished, however, between 17 different cell types. The mentioned authors thought that a mucous and a serous series alternate within one secretion cycle.
Salivary glands of Lymnaea stagnalis L.

cycle. Voinov (1934) and Blain (1957), on the other hand, suggested that in Helix pomatia and H. aspersa two secretion cycles occur, a serous and a mucous one.

An extensive study of the salivary glands of Lymnaea stagnalis has been carried out by Gabe and Prentant (1948). They distinguished between 6 different cell types and they established, by using experimental methods, a secretion cycle for some of these cell types. However, their results and conclusions are open to some doubt. For this reason the glands were reinvestigated. To this end many histological staining techniques and an electron microscope were used.

Material and Methods

For the investigations salivary glands of full grown specimens (shell length 30—36 mm) of L. stagnalis, bred in the laboratory were used. The animals were fed on lettuce. In addition a limited number of specimens was taken directly from ditches in the neighbourhood of Amsterdam.

For light microscopy glands were fixed in a number of fixatives, e.g. Bouin and Stieve (Romes, 1948), upgraded in ethanol and amylacetate, and embedded in paraffin wax (m. p. 58° C). Serial sections (thickness 5 μ) were cut, and stained by various histological and histochemical methods, which were carried out as recommended by Pease (1960). For light microscopy also Epon embedded material was used, of which 2 μ thick sections were cut with an ultramicrotome. Staining of Epon sections was usually carried out with toluidine blue (1% in 25% ethanol at a pH > 7).

For electron microscopy techniques as recommended by Pease (1964) were used. The glands were fixed in situ for 10 min in a 4% buffered (pH 7.4) solution of glutaraldehyde. After having dissected the glands out, further fixation was performed in Palade’s buffered OsO₄ mixture and a 4% solution of uranyl nitrate. Ethanol dehydration, immersion in propylene oxide and Epon 812 embedding followed.

Ultrathin sections were cut with glass knives on a Reichert ultramicrotome, picked up on formvar-coated copper grids, and studied in a Zeiss EM 9 electron microscope. In some cases the ultrathin sections were stained with Reynold’s lead citrate (Pease, 1964).

Results

Microscopic Anatomy

The two (6 × 3 mm) salivary glands of Lymnaea stagnalis are situated just behind the esophageal ring against the frontal part of the postesophagus. The secretory ducts run anteriorly to the caudo-dorsal part of the buccal mass (Carriker and Bilstad, 1946). The glands have a bright yellow appearance, due to an alcohol soluble pigment. A weakly developed connective tissue capsule ties both glands dorsally, and in particular ventrally, together. It also connects the glands with the esophagus. The salivary glands must be considered as tubulocinous. The secretory duct branches into 2 or 3 interlobular ducts, each of which penetrates into a gland lobule. Within the lobules these ducts branch into a number of intralobular ducts, which run to the acini.

Light Microscopy

When comparing Epon embedded sections of glands which were fixed in glutaraldehyde and OsO₄, with Bouin fixed and paraffin embedded material, it appeared that in particular the preservation of the secretion granules was much better in the Epon embedded glands. Therefore, a series of fixatives (Bouin, Stieve, Zenker, Baker’s formol and glutaraldehyde) was applied to glands which
were subsequently embedded in either paraffin or Epon. It was found that aldehyde fixation, and especially Epon embedding greatly improved preservation. A disadvantage of the use of Epon sections is the difficulty with which histochemical methods are applied to them (Pease, 1964). However, good results were obtained with toluidine blue staining. For other staining techniques usually paraffin sections were used.

Gabe and Prenant (1948) distinguished — as mentioned in the introduction — between 6 different cell types in the salivary glands of _L. stagnalis_. The terminology used by them will be adopted as far as possible.
Salivary glands of Lymnaea stagnalis L.

In the present study 9 different epithelial cell types will be distinguished, of which 5 correspond to cells described by Gabe and Prenant. The 9 types are: 1. basophilic cell; 2. granular cell; 3. pseudochromosome cell; 4. mucocyte I; 5. mucocyte II; 6. cell with an acidophilic inclusion; 7. grain cell; 8. mixed cell; 9. ciliated cell. In the next section the cell types will be described briefly (Fig. 1).

1. Basophilic Cells. The relatively small (max. 25 μ) ovoid cells do not strictly represent a distinct cell type, since they are considered as early stages of the secretory cells (types 2—8). The nucleus of the basophilic cell has a central position. The cytoplasm is strongly basophilic due to a well developed ergastoplasm. Cells which were basophilic in their entirety, i.e. which did not yet show any sign of the formation of secretion granules, occurred in rather low numbers (< 0.5%).

2. Granular Cells (Fig. 1a). These cells are columnar (20×60 μ), with an excentric nucleus. The cells contain large (4—6 μ) secretion granules and occur regularly and in great numbers.

3. Pseudochromosome Cells (Fig. 1d, e). These cells are ovoid to cubical and measure 20—40 μ. The nucleus has an excentric position. In paraffin sections characteristic bent or rounded strands can be found in the cytoplasm (Fig. 1e). Because these structures are somewhat similar to chromosomes, Prenant (1923) called them pseudochromosomes. In toluidine blue stained Epon sections the pseudochromosomes appear to be more or less round granules (2—3 μ) consisting of a deep blue ring around an unstained, usually polygonal core (Fig. 1d).

4. Mucocyte I (Fig. 1h). The cells are pyramidal, measure ± 15 μ and contain small (1 μ) homogeneous secretion granules. The nucleus is located at the cell basis. The cells occur in fairly great numbers. In paraffin sections the secretion granules appear to be badly preserved.

5. Mucocyte II (Fig. 1j). These cells can attain a diameter of 50 μ. In the small specimens of this cell type (20 μ), the nucleus has a central position, in the larger ones it lies next to the cell membrane. The secretion granules, which measure in the majority of the cells 3—6 μ, contain a filamentous material. In Epon sections this material stains metachromatically (red) with toluidine blue. The metachromasia is not seen in Bouin fixed paraffin sections (Table 1). A number of cells contain very large granules (up to 15 μ). The density of the secretion product in these granules is small. In inadequately fixed and embedded tissue (paraffin) the large granules look like empty vesicles. However, many transitional forms show that cells containing these large empty vesicles must be considered as the last stage of the production of the secretion material of mucocyte II. The cells occur regularly and in great numbers.

6. Cells with an Acidophilic Inclusion (Fig. 1c). The nucleus of these relatively small cells (15 μ) lies at the cell basis. In the cell apex one or two homogeneously staining acidophilic alveoli can be found. The cytoplasm at the cell basis is strongly basophilic. The cells occur in only small numbers irregularly scattered throughout the gland. In paraffin sections they cannot be easily kept apart — unless histochemical tests are applied (cf. Table 1) — from granular cells of which several granules may fuse to one in badly preserved tissue.

7. Grain Cells (Fig. 1g). The cells are columnar to pyramidal and measure 15—25 μ. In glands of animals bred in the laboratory they are infrequently observed (< 1%). In glands from animals taken from ditches their number is consider-
ably larger (± 10%). The nucleus is located eccentrically. The secretion granules have a fairly constant diameter of 1.5—2 μ and stain in Epon sections intensely with toluidine blue and P.A.S.

It may be difficult to keep the grain cells apart from a cell type occurring in the connective tissue capsule of the gland. These cells, often called cells of Leydig (cf. Gabe and Prenant, 1948), are usually small (6—15 μ) and of a rather irregular shape. They may be elongated (15 × 2 μ) or ovoid, and they contain granules of variable size (0.5—3.5 μ) which stain with toluidine blue. Because of the weakness of the basal membrane and the connective tissue capsule, the cells may often be found between the epithelial cells (Fig. 1 i), in particular in the secretory ducts (cf. Pacaut and Vigier, 1906). In the electron microscope the grain cells and the cells of Leydig can easily be distinguished (see below).

8. Mixed Cells (Fig. 1 f). These cells, which are usually ovoid, do not seem to be a regular cell type. However, when they occur in a gland, then usually some hundreds of these cells can be found in a solid group. The cells can attain a length of 70 μ. They are called mixed cells, because they contain two types of secretion granules, viz. pseudochromosomes and granules of the type of mucocytes II. The nucleus can be very large (up to 24 μ).

9. Ciliated Cells (Fig. 1 b). These non-secretory epithelial cells are difficult to discern in paraffin sections. In thin (2 μ) Epon sections they can be observed as very slender cells (1—2 μ wide) located between secretory cells. They occur mainly in the secretory ducts. At the cell basis, but in particular at the cell apex the ciliated cells widen horizontally over the neighbouring cells. From this widened surface area they project cilia (length up to 35 μ) into the secretory ducts. The cylindrical or ovoid nucleus (length 3 to 4 μ) is orientated in the longitudinal axis of the cells. The cytoplasm is vacuolated.

To Bouin fixed and paraffin embedded material various staining techniques and histochemical tests were applied with the aim of distinguishing better between the different cell types. Moreover, it might perhaps be possible to gain, from the results, an insight into the nature of the secretion products.

The results, which are listed in Table 1, point to the reaction of the cytoplasm for cell type 1 (basophilic cells) and for the other cell types to the stain of the secretion products. For cell type 3 the staining reaction of the bent secretion strands (pseudochromosomes) is presented, but not that of their cores, because these are severely damaged in Bouin fixed paraffin sections. The staining reaction of the grain cell is not known in all cases, because this cell type occurs in only small numbers in the gland, so that it is absent from many sections. No results are given for the mixed cells and ciliated cells. The secretion products of the mixed cells react similar to those of the pseudochromosome cell and of mucocyte II, respectively. The ciliated cell is not well preserved in paraffin sections and therefore difficult to distinguish; moreover, these cells are not of a secreting type.

From the foregoing morphological description of the cell types it is evident that some of them (e.g. basophilic cells, pseudochromosome cells) can be distinguished on the basis of morphology alone. For other cell types this is less clear. However, Table 1 shows that these cell types, for example granular cells and cells with an acidophilic inclusion or mucocyte I and II, are distinct on the basis of
Table 1. Staining results of the different cell types. The applied techniques are taken from Romeis (1948) and Pearse (1960).

+ weak reaction; ++ moderate reaction; +++ strong reaction; — negative reaction.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemalum and cosin</td>
<td>Ehrlich</td>
<td>blue</td>
<td>pink</td>
<td>—</td>
<td>blue</td>
<td>pink</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td>Azan</td>
<td>Heidenhain</td>
<td>orange</td>
<td>blue</td>
<td>blue</td>
<td>—</td>
<td>blue +</td>
<td>lila blue +</td>
<td></td>
</tr>
<tr>
<td>Chrome-haematoxylin-phloxin</td>
<td>Gomori</td>
<td>red</td>
<td>blue</td>
<td>blue</td>
<td>light-blue</td>
<td>—</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td>Mucicarmine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Kramer and Windrum</td>
<td>blue</td>
<td>light-blue</td>
<td>purple</td>
<td>light-purple</td>
<td>—</td>
<td>light-blue</td>
<td>—</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>Steedman</td>
<td>—</td>
<td>—</td>
<td>green-blue ++</td>
<td>blue</td>
<td>—</td>
<td>blue +</td>
<td></td>
</tr>
<tr>
<td>P.A.S.</td>
<td>McManus</td>
<td>—</td>
<td>++</td>
<td>+</td>
<td>+++ ++</td>
<td>+</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Performic acid-alcian blue</td>
<td>Adams and Sloper</td>
<td>—</td>
<td>+</td>
<td>+++</td>
<td>--</td>
<td>—</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>Bonhag</td>
<td>+++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td></td>
</tr>
</tbody>
</table>

Their staining reactions. Thus, the acidophilic inclusion of cell type 6 is stained red with the chrome-haematoxylin-phloxin method, the granules of cell type 2, on the other hand, blue-black. Comparable differences are shown for cell types 4 and 5.

As to the chemical nature of the secretion products only tentative conclusions can be drawn. Cell type 4 is undoubtedly a mucous cell (strongly positive to mucicarmine), which seems to produce an acid mucopolysaccharide (e.g., positive with alcian blue after Steedman,  \( \beta \)-metachromasia with toluidine blue). Cell type 5 is probably also a mucous cell (strongly P.A.S. positive, in Epon sections metachromatically staining with toluidine blue), as is perhaps cell type 7. The nature of the secretion products of cell types 2, 3 and 6 is not easily established from the staining results. There are indications that the material of cell types 2 and 6 is proteinaceous in character — e.g., positive reaction with bromphenol blue, and positive staining with alcian blue after oxidation, which suggests the presence of cystine — although the granules may also consist of mixed material, since they are also P.A.S. positive. This seems also true for the pseudochromosome cells. However, because the core of the granules of this cell type is damaged in paraffin sections, no further statements about these structures can be made. The section on the electron microscopy of the cells will give further information about the nature of the secretion granules.
The morphological description and the staining results indicate the presence of 9 different epithelial cell types in the salivary glands of *L. stagnalis*. Seven of them seem to be secretory cells.

The basophilic cells are considered as the first stage in the development of the secreting cells. Gabe and Prenant (1948) usually observed a much higher percentage (2—16.5%) of this cell type than is found in the present study (<0.5%). The discrepancy may be caused by the fact that in Epon sections the formation of secretion granules can be observed in a much earlier stage of development of the cells, so that identification of rather young cells on the basis of the type of secretion granule is possible. Moreover, it might well be that Gabe and Prenant have classified the ciliated cells, which they did not distinguish as a distinct cell type, with the basophilic cells.

It seems evident from the morphology and the staining results that the as different regarded cell types 4 and 5 (mucocytes I and II) should be identified respectively with the "mucocyte peu évoluté" and the "mucocyte évoluté" of Gabe and Prenant, who considered them, however, only as stages of the same cell type.

Very large mucocytes II were shown to contain empty vesicles in paraffin embedded tissue. In Epon sections, however, the vesicles appeared to contain material similar to that found in smaller mucocyte II granules. Therefore the cells are regarded as a developmental stage of this cell type. It seems very likely that these large cells should be identified with the alveolar cells which were described by Gabe and Prenant as a distinct cell type.

Mixed cells have not been described for *L. stagnalis* in earlier work. However, in *Helix aspersa* comparable cells have been observed by Blain (1957), who stated: "Cette notion de cellule mixte, qui du temps de Kriegsman, s'opposait à la théorie classique, laquelle distinguait cellule muqueuse et cellule séreuse, est maintenant confirmée tant chez Vertébrés que chez les Invertébrés" (p. 501).

**Location of the Cell Types within the Gland**

Of 4 animals, which were starved for 48 hrs, the glands were fixed in glutaraldehyde and embedded in Epon. Every 100 μ, a 2 μ thick section was cut perpendicular to the longitudinal axis of the glands. The toluidine blue stained sections were studied light microscopically. In all sections every cell, except for the ciliated cells, was counted.

It appeared that the walls of the secretory ducts may be lined, up till the buccal mass, with secretory epithelium. However, usually the anterior parts of the ducts consisted of non-secretory squamous epithelium cells.

Because — as was mentioned — the basal membrane is only weakly developed and desmosomes between the epithelial cells are lacking (see also the section on electron microscopy), the epithelium of the secretory ducts, but in particular of the lobules, is not very regular in outline: cells of the connective tissue may be found between epithelial cells and secretory cells outside the epithelium proper. The lumen of the acini is, although well to distinguish, usually small (<1—10 μ).

For the evaluation of the cell counts, the gland was divided into three parts (Fig. 2): I. the secretory ducts up to the first bifurcation; II. the interlobular ducts; III. the lobules consisting of the acini and the intralobular ducts. The fre-
quency of the cell types in these parts was calculated as a percentage of the total number of cells counted.

From Table 2 it is evident that the glandular epithelium consists mainly of 4 cell types: granular cells, pseudochromosome cells, mucocytes I and mucocytes

Table 2. Location of the cell types. For details see text

<table>
<thead>
<tr>
<th>Cell types</th>
<th>part I</th>
<th>part II</th>
<th>part III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>secretory ducts</td>
<td>interlobular ducts</td>
<td>gland lobules</td>
</tr>
<tr>
<td>1. Basophilic cell</td>
<td>0.5</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>2. Granular cell</td>
<td>89.0</td>
<td>2.4</td>
<td>—</td>
</tr>
<tr>
<td>3. Pseudochr. cell</td>
<td>1.5</td>
<td>84.3</td>
<td>13.9</td>
</tr>
<tr>
<td>4. Mucocyte I</td>
<td>—</td>
<td>0.9</td>
<td>53.1</td>
</tr>
<tr>
<td>5. Mucocyte II</td>
<td>0.6</td>
<td>4.7</td>
<td>24.3</td>
</tr>
<tr>
<td>6. Cell with acidoph. inclusion</td>
<td>—</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>7. Grain cell</td>
<td>—</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>7a. Cell of Leydig</td>
<td>8.4</td>
<td>3.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>8. Mixed cell</td>
<td>—</td>
<td>1.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Total no. of cells counted</td>
<td>1,745</td>
<td>4,545</td>
<td>16,962</td>
</tr>
</tbody>
</table>

II. The grain cells were observed regularly but in small numbers. Cells with an acidophilic inclusion and mixed cells occur infrequently and irregularly.

As far as the three parts are concerned, it is shown that part I, the secretory ducts, consists mainly of granular cells. Only in the transitional area to part II
some pseudochromosome cells occur. Furthermore cells of Leydig, some mucocytes
(although, the latter cells were all found in the connective tissue outside the
epithelium), and some unidentified ("basophilic") cells were observed. For part II,
the interlobular ducts, the dominating cell type is the pseudochromosome cell.
Other cell types occur in only small numbers. In the rather small transitional area
to part I, only a few granular cells were found. The transition to part III is less
sharp. In this area mucocytes II and also some mucocytes I were observed. Part
III can be divided into intralobular ducts and acini. In the intralobular ducts the
most prominent cell types are the mucocyte II and the pseudochromosome cell.
The epithelium in the acini consists almost exclusively of mucocytes I; besides, a
small number of grain cells was found. In part III also a fairly great number of
mixed cells occurred.

Thus, the four most important cell types are located in different areas of the
gland, although regions of overlap do exist. The granular cells occur in the secre­
tory ducts, the pseudochromosome cells mainly in the interlobular ducts, the muco­
cytes II in the intralobular ducts and the mucocytes I in the acini. This distribution
was confirmed by many observations in all glands (40) investigated, not only for
animals which had been starved for a long period of time (3 weeks), but also for
glands which had been fixed shortly (1/2—2 hrs) after the snails had eaten (lettuce).
However, in the latter glands, the basophilic parts of most cell types were more
prominent, whereas the part of the cell containing secretion granules was smaller.

The staining results indicate that no close relationship between the cell types
can be based on the nature of their secretion products (Table I). The study of the
distribution of the cell types proves that they are not closely topographically
related either: the most important cell types occur in different areas of the gland.
Because of these results it would seem unlikely that the cell types which were
described as distinct, would in fact be morphological modifications of one or two
functional cell species, and constitute one or two secretion cycles. Therefore, the
opinion of Gabe and Prenant (1948) that there is, in the salivary glands of
L. stagnalis, the following secretion cycle: — basophilic cell → pseudochromosome
cell → alveolar cell → granular cell (→ secretion) → basophilic cell etc., is not shared
in the present study.

Amylase Producing Cells

According to Carriker (1946) in the salivary glands of L. stagnalis amylase is
produced. Because it appeared that the most important cell types are located in
different areas of the gland, it seemed possible to correlate a special cell type with
the production of the enzyme, by testing the amylase activity of different parts of
the gland. To this end of 10 animals, starved for 48 hrs, the glands were cut into
three parts, viz. the secretory ducts, the interlobular ducts and the gland lobules.
It was tried to obtain of part 1 and 2 about the same quantity of tissue, while of
part 3 a larger quantity was used. The material was homogenized in glass tubes.
To each of the tubes a phosphate-buffered (pH 7.2) starch solution (1 %) was added.
After 2 and 6 hrs of incubation JKJ was added to part of the solutions, and a
decrease of the intensity of the blue colour, as compared to a control starch-JKJ
solution, was regarded as an indication for amylase activity. It appeared that in
tube 1 (secretory ducts) already after 2 hrs no blue colour developed — the solution
remained entirely colourless — indicating that the starch was fully digested. In tube 2 (interlobular ducts) only after 6 hrs a slight amylase activity was observed, whereas tube 3 (gland lobules) showed no activity at all. It can be concluded from these results that the most important cell type in the secretory ducts, the granular cell, produces amylase.

**Electron Microscopy**

Before and again after cutting ultrathin sections for use in the electron microscope, 2 μ thick sections were cut which were studied with the light microscope for identification and localization of the different cell types.

In general the majority of the secretory cells in glands of starved animals were crowded with maximum sized secretion granules. This stage in the development of the cells is regarded as mature. In glands fixed shortly (½—2 hrs) after feeding of the snails, the general features of some cell types (pseudochromosome cell, mucocyte II) appeared to have changed considerably. Cells could be found in which only a few mature secretion granules were left, while the number of granules of smaller size (immature granules) had increased. In these cells the endoplasmic reticulum and the Golgi apparatus usually showed definite signs of activation. Other cell types (granular cell, mucocyte I) did not show so clearly these structural changes. Whether the remaining secretory cell types (cells with an acidophilic inclusion, grain cells, mixed cells) had been activated or not, could not be established. As mentioned, these cells occur not very regularly, so that only a few specimens of them were studied with the electron microscope.

Transitional stages between secretory cell types were never found on the submicroscopical level.

In the next section the ultrastructure of the cell types will be described.

1. **Basophilic Cells.** In the section on light microscopy it was suggested that the basophilic cells have to be considered as early stages of secretory cells. This supposition was confirmed on the ultrastructural level, because in almost every section of a “basophilic” cell, signs of the formation of some kind of secretion product was observed. Besides, in sections without such granules, the cells could be identified on the basis of the shape of cell organelles like the endoplasmic reticulum and the Golgi apparatus.

2 and 3. **Granular Cells and Pseudochromosome Cells.** The ultrastructure of the most important cell organelles of these two cell types shows similarities, whereas the secretion granules are very distinct.

The cellular membranes of both cell types do not show special differentiations. The cell apices are usually covered by horizontal expansions of ciliated cells (Fig. 4). The mitochondria are oval or oblong and not numerous (Fig. 3, 5).

The granular endoplasmic reticulum consists in resting cells of stacks of parallel membranes (which can be interpreted as flattened tubules), lying between the numerous secretion granules (Fig. 5, 6). The endoplasmic reticulum of activated

---

1 Although secretion is undoubtedly reduced in starved animals, it probably does not stop entirely, since even after a starvation period of 14 days, secretion products were found in the secretory ducts. This indicates a regular production and release of secretion material, even if no food is eaten, and may account for the fact that some of the cell types are difficult to activate by feeding the animals.
pseudochromosome cells reacts by developing many cisternae (Fig. 7, 3), which contain electron-lucent material. HERLANT (1964) considered the number and size of these kind of cisternae as a measure for cell activity. Accordingly, in cells with an increased number of mature secretion granules, the number of cisternae had decreased considerably.

The Golgi apparatus consists of characteristic agranular lamellae, frequently bulb-shaped at their ends, and of Golgi vesicles which measure 500—1500 Å in the pseudochromosome cell and 300—500 Å in the granular cell. The contents of the lamellae and the vesicles are electron-dense. Furthermore in the Golgi area usually larger vesicles can be observed, which might be interpreted as prosecretion granules (Fig. 3).
Fig. 4. Electron micrograph of parts of two granular cells (GR) and of two ciliated cells (CC). The granular cells are densely packed with secretion granules (sg). At the top of the left granular cell the secretion process is indicated (arrow). c cilia, v vacuole-like space of ciliated cell, l lumen of secretory duct. x 6680. Bar represents 2 μ.

Fig. 5. Electron micrograph of part of a granular cell (right) and a ciliated cell (left). The small secretion granules (ssg) are more electron dense than the mature one (msg). v vacuole-like space of ciliated cell, er endoplasmic reticulum, m mitochondrion. x 13 500. Bar represents 2 μ.
The best results in preserving the secretory granules of the granular cell type were obtained when a prolonged glutaraldehyde fixation (20—30 min) preceded OsO₄ fixation. With increasing granular size, the electron density of the homogeneous secretory material decreases (Fig. 5). In mature stages the whole cell is crowded with 4—6 μ measuring granules (Fig. 4).

In active pseudochromosome cells transitional stages between the electron dense prosecretion granules in the Golgi area and the characteristic "pseudo-chromosomes", which consist of a homogeneous electron dense ring and one or more polygonal cores, can be observed. The material of the cores shows a crystalline, hexagonal pattern (Fig. 6). Especially in mature cells, which are particularly encountered in glands of starved animals, granules without the characteristic structure — which probably had been disintegrated — were found.

4. Mucocyte I. This cell type is characterized by the presence of microvilli extending into the acinar lumen (Fig. 9). The mitochondria, which are found throughout the cell in connection with the endoplasmic reticulum, are polymorphic, especially as far as the arrangement of the cristae is concerned. The granular endoplasmic reticulum is usually poorly developed. Dilatations (500 Å wide) of the membranes may occur, but large cisternae as found in the pseudochromosome cell (Fig. 7) were never observed. When compared to granular cells and pseudochromosome cells, Golgi fields are not only more numerous, but also of an entirely different shape in mucocyte I (cf. Fig. 10 and 3). The lamellae lie farther apart so that Golgi vacuoles are formed. Their content is electron transparent. Golgi vesicles of uniform size (400—500 Å) surround the Golgi bodies.

The globular secretory granules, which consist of fine granular material, measure about 1 μ (Fig. 9, 10, 12). They are not very electron opaque, although they may contain small dense areas at their periphery, which are probably artifacts due to fixation.

5. Mucocyte II. Except for some villiform projections at the cell apex, no differentiations of the cell membrane were observed. The mitochondria are similar to those of the pseudochromosome cells. In early stages, the granular endoplasmic reticulum is very regular in outline and consists of a system of tubules (Fig. 8). Wide cisternae were seldom found. The Golgi apparatus is quite similar to that of mucocyte I. Large Golgi vacuoles and many Golgi vesicles (400—600 Å) containing an electron-lucent material, can be found (Fig. 11).

Small secretion granules (≥ 1 μ) consist of homogeneous material. Medium sized granules contain a product consisting of densely packed filaments (Fig. 13). In the largest granules (6—10 μ), the density of the filamentous material is considerably less (alveolar stage).

6. Cell with an Acidophilic Inclusion. Of this cell type only a small number of specimens was studied in the electron microscope. The mitochondria are similar to those of the pseudochromosome cell. The granular endoplasmic reticulum was observed as stacks of parallel cytomembranes. Signs of synthetic activity, like the presence of cisternae were not found. The Golgi apparatus is quite similar to that of the granular cell and the pseudochromosome cell. Large Golgi vacuoles were not found. Golgi vesicles (500 Å) are numerous.

The secretory granules measure 5—10 μ and contain a fine granular material.
Fig. 6. Part of a pseudochromosome cell containing mature secretion granules (sg) consisting of a core with a crystalline pattern surrounded by an electron dense ring. $\times$ 26 500. Bar represents 0.5 $\mu$.

Fig. 7. Electron micrograph of part of a pseudochromosome cell, representing the endoplasmic reticulum of an actively synthesizing cell. Note large cisternae. $\times$ 24 000. Bar represents 0.5 $\mu$.

Fig. 8. Tubulous endoplasmic reticulum of an active mucocyte II. $\times$ 34 500. Bar represents 0.5 $\mu$. 
7. Grain Cell. The general picture of the cell organelles in this cell type shows great similarity with the mucocytes I and II, although there are also differences. The mitochondria, for example, show frequently an irregular shape. The granular endoplasmic reticulum may show rather well developed cisternae in actively synthesizing cells. In mature cells the endoplasmic reticulum is not different from that of mucocyte I. The Golgi apparatus of active cells is strongly developed and lies between the endoplasmic reticulum and the secretion granules. In the Golgi fields prosecretion granules can be observed. Small Golgi vesicles (\( \varnothing \approx 500 \text{ Å} \)) are not numerous.

The secretion granules measure 1.5—2 \( \mu \). The limiting membrane of the granules is often irregular. The secretory material is fine granular and somewhat more electron opaque than that of the mucocyte I secretion granules (Fig. 12).

In the section on light microscopy it was mentioned that it is rather difficult to keep this cell type apart from the cells of Leydig. However, in the electron microscope both cell types are easily distinguished, since the cells of Leydig contain granules consisting of fairly electron-dense material.

8. Mixed Cell (Fig. 14). This cell type contains, as mentioned, two types of secretion granules: "pseudochromosomes" and granules of the type found in mucocytes II. The mitochondria are similar to those of the pseudochromosome cells, which is also true for the well developed granular endoplasmic reticulum. An
Salivary glands of *Lymnaea stagnalis* L.

Fig. 11. Electron micrograph of an active mucocyte II. The Golgi complex (gc) is strongly developed and consists mainly of very large vacuoles, er endoplasmic reticulum, gv small Golgi vesicles, nu nucleus. × 20,500. Bar represents 1 μ.

Endoplasmic reticulum consisting of tubules as found in mucocyte II, was not observed in the mixed cell. Two types of Golgi fields occur, which are entirely similar to that of the pseudochromosome cell and mucocyte II, respectively.

The "pseudochromosomes" are not different from those already described (comp. Fig. 14 with Fig. 6). However, usually many homogeneous granules — i.e. granules which have not developed a ring and a core — varying in size from small prosecretion granules to small "pseudochromosomes", are present. Large granules without the characteristic pseudochromosome structure were not found (cf. the section on the pseudochromosome cell).

All kinds of mucocyte II granules, except for the alveolar stage, may occur in mixed cells. A conspicuous feature of the cell is furthermore, that the filamentous
Fig. 12. Electron micrograph of mucocytes I (1) and grain cells (2) around an acinar lumen (l). sg secretion granules, c cilia, mv microvilli. × 6500. Bar represents 2 μ.

Fig. 13. Two mucocytes II containing secretion granules (sg). The secretion material consists of more (lower cell) or less (upper cell) densely packed filaments. × 7200. Bar represents 2 μ.
material of the mucocyte II granules was frequently found in the cytoplasm between the cisternae of the endoplasmic reticulum, i.e. not granule-bound (Fig. 14).

Usually granules of one type lie together in a certain area of the cell. The transition between these areas is not sharp.

9. Ciliated Cells. At the apex of this cell type cilia and also some microvilli can be observed (Fig. 4). The cilia show the characteristic structure with one pair of filaments in the centre and 9 pairs at the periphery and a basal body (cf. Fawcett, 1961).

![Fig. 14. Electron micrograph of part of a mixed cell, ps secretion granule of the pseudochromosome type, muc secretion granule of the type of mucocyte II, filamentous secretion material, er endoplasmic reticulum. × 14 400. Bar represents 1 μ.](image)

The mitochondria are small (200—1000 μ), but rather numerous, especially in the cell apex. The cristae mitochondriales are orientated in the longitudinal axis (Fig. 5). The endoplasmic reticulum and the Golgi apparatus are poorly developed. The cells are characterized by large spongeous vacuole-like spaces (Fig. 4, 5).

Summarizing the results, it can be stated that neither on the light microscopic level, nor on the ultrastructural level, transitional forms between the 7 secretory cell types occur in the salivary gland of *L. stagnalis*. This is confirmed by the fact that, in general, different cell types occur in different parts of the gland. It was, on the other hand, observed that each cell type — except for the mixed cell — produces its own granule type, of which several developmental stages may occur in the same cell. It can therefore be concluded that 6 different secretion products are released.
The histochemical results give only slight information about the nature of these products. It was supposed that the materials of mucocyte I and II and of the grain cell are mucous products, whereas those of the other cell types are serous. For the granular cell this was confirmed by the enzyme experiment, which showed that this cell type produces amylase.

The ultrastructural information on the shape and the development of the granular endoplasmic reticulum seems to support the suggested classification of the cells in mucous and serous types. In immature stages of the supposed serous cells (granular cells, pseudochromosome cells, cells with an acidophilic inclusion), the endoplasmic reticulum consists of many large cisternae, indicating active production of proteins (cf. Haguenau, 1964). In the supposed mucous cells (mucocytes I and II, grain cells) the endoplasmic reticulum is usually not so well developed. The Golgi apparatus of the serous cells is relatively small, and does not consist of large vacuoles. This type of Golgi apparatus is found in many protein producing cells (cf. Zeigel and Dalton, 1962). The well developed Golgi apparatus as found in the mucous cells, on the other hand, shows similarity with the Golgi bodies of polysaccharide producing cells (cf. Peterson and Leblond, 1964; Lane and co-workers, 1964).

The ultrastructure of the mixed cell is in accordance with the supposition that this cell type produces a mucous (granules of the type of mucocytes II), as well as a serous (pseudochromosomes) secretion material.

References


Salivary glands of *Lymnaea stagnalis* L.


Dr. H. H. Boer
Zoologisch Laboratorium, de Boelelaan 1087
Amsterdam-Buitenveldert Niederlande