Some Investigations concerning the Glands associated with the Skin of the Mollusc, *Arion hortensis* (Fér.).

Volume I : Text

Volume II : Figures and Plates

Presented by Jean M. Lawrence B.A.(Keele),

for the degree of

Doctor of Philosophy, Keele, 1972.

Vol.I

ACKNOWLEDGEMENTS

I would like to thank Professor A. R. Gemmell for providing the facilities for my research; Dr. Mary Campion for supervising my work for the past three years and reading the proofs of the thesis; Mr. Peter Webster, the electron microscope technician, for his technical advice; and, lastly, the Science Research Council for providing the finance.

ABSTRACT

The structure and histochemistry of the gland cells of the dorsal and ventral surfaces of Arion hortensis were described, together with that of the pedal and caudal glands. This investigation was conducted using a light microscope and standard staining techniques.

The ultrastructure of the pedal gland was investigated and the nature of an unusual tubule - filled endoplasmic reticulum considered and described. The effect of the enzyme pepsin, and of deamination on the ultrastructure of the tubules, was examined.

Lastly, a brief regeneration experiment was conducted, using mantle tissue, to study the origin of the gland cells. The process was first investigated using a light microscope, and then tissue from a limited time sequence was studied using the electron microscope. These last results were compared with the ultrastructure of the normal mantle.

INDEX

G1. GENERAL INTRODUCTION

The aim of this research was to study, in some detail, the nature and possible developmental sequence of the glands associated with the skin of a mollusc.

The mollusc chosen as the subject of the investigation was the slug Arion hortensis.* This is a 'naked' mollusc in that it lacks a shell, and this feature was felt to be desirable bearing in mind that it was the epidermis and its associated glands which were to be examined. The removal of a shell could seriously damage that tissue in certain regions of the body, and would be a tedious operation to carry out before fixation when speed is essential for good preservation. A second advantage of this slug, since many individuals would be required in the course of the research, was its abundance and wide distribution throughout the British Isles (Boycott 1934), particularly in cultivated land.

However, a problem arising from the selection of Arion hortensis was its pigmentation (black over the mantle, back and sides, and orange along the length of the sole and sole margins), which could have obscured vital structures.

The research project fell into three sections. Firstly, a general histological and histochemical survey of the slug was necessary in order to determine the structure and content of the glands involved. Clearly, this would have been an enormous task in electron microscopic terms and therefore the survey was conducted using the light microscope. Also, the number of histochemical methods available to the electron microscopist is limited and so for a more complete study it was necessary to use tissue prepared for light microscopy either by paraffin embedding or by freezing.

Secondly, one area was selected for a detailed electron microscope examination, the pedal gland. This was chosen because of its

* Férussac (1819).

considerable importance in the production of mucus for locomotion, and because it represented a well-defined and limited area for study. Furthermore, an examination of the literature showed that its ultrastructure had never been described.

Lastly, an experiment was conducted to study the regeneration of mantle tissue with the hope that this might throw some light on the origin of the gland cells, even though this would represent development in damaged rather than normal tissue.

Id. IDENTIFICATION

The molluscs were identified using Quick's (1949) key and Taylor's (1894-1900 and 1900-1907) Monograph. Quick described the slime as orange but the results of the current investigation agreed with Taylor's statement that it was the dermal mucus which was orange, the caudal and locomotory mucus being colourless. In fact, the slime produced by the dorsal surface only became obviously orange after 'severe' attack, such as when submersing the live animal in fixative solution, or dissecting it without anaesthetic to obtain the pedal gland tissue. The colouring was due to the discharge of the pigment cells (LM4 iv pp54-60) of the mantle and back.

Fig. 1 illustrates the external features of Arion hortensis.

CM. COLLECTION AND MAINTENANCE OF SLUGS

The slugs were collected in the vicinity of the Departmental greenhouses and gardens and were transferred to an indoor vivarium, a procedure which was not essential since the slugs were available all the year round, but convenient because in very cold weather (when humidity was low), the slugs burrowed beneath the soil and an indoor vivarium removed the necessity for digging. The depth of burrowing was not ascertained accurately, but it was greater than 6cm .

The vivarium was a polythene box, 11.4cm deep, 26.7cm long and 19cm wide, and without a lid. The bottom of the box was filled with 1.25cm of gravel (for drainage), and covered with 5cm of crudely sieved soil. Shelter for the slugs was provided by some large stones, a piece of wood and an inverted Petri dish embedded in the moistened soil. No more than a dozen slugs were placed in this vivarium and they were supplied with a small variety of food which was placed on the buried Petri dish (for ease of cleaning). A 1:1 mixture of oats and chalk bound together with water was a constant feature of the diet, but the other items of food, pieces of cabbage, lettuce, potato or carrot, were fed to the slugs successively and it was not until the carrot was supplied that they ate. Stephenson (1962-1963) had a similar experience and suggested that carrot was preferred because it did not dry out as quickly as the other vegetables, a process which would render the food unpalatable. Cardot (1924) also found that potato and carrot were eaten by Arion hortensis in captivity. Fresh food was supplied three times a week and the soil re-watered whenever necessary. At the same time old food was removed and the surface of the soil lightly raked over to ensure that no hard crust formed. The soil was replaced completely once a month so that excessive contamination by faeces and slime could be avoided. The vivarium was covered by a fine metal grid which allowed air to circulate over the soil but which prevented any slugs

escaping, and was placed in the indoor departmental aquarium, where the animals were protected from frost, snow, direct sun and drying winds whilst still being subject to a day/night cycle and some temperature variations (between 8°C and 15°C). Exposure to a day/night cycle was felt to be desirable, along with high humidity, for the provision of a satisfactory environment for the slug, since Lewis (1969) found in Arion ater that activity was initiated by falling light intensity rather than by any temperature change as thought by Dainton (1954) .... and as far as possible ordinary outdoor conditions were imitated. The aquarium itself was unheated and therefore usually quite cool, although external weather conditions could affect the air temperature due to the presence of windows along the length of the north-facing wall. In these conditions the slugs survived very well, but were generally inactive and stayed beneath the shelter provided, the inactivity probably being due to insufficient humidity in the low temperatures.

For the regeneration experiment the slugs were collected by trapping, since large numbers were required at one time. Flowerpots were inverted over rolled oats or bran and small potatoes on a recently dug potato patch in the Botanic gardens. Numerous slugs of several varieties could be collected in this way.

As wound healing was to be studied, the best possible environmental conditions were required so that the process could take place efficiently, for any environmental deficiency could have a detrimental influence. Pairs of slugs were placed in lidless crystallising dishes, 8cm in diameter by 5cm deep, half-filled with a 'soil' composed of five parts loam to three parts peat to one part sharp sand, plus a little chalk. The soil, after watering, retained its moisture and did not form lumps. The whole dish was covered by terylene netting secured by a rubber band and twelve of these dishes, two deep, were placed in a glass aquarium, 20cm high by 30cm long by

20cm wide, in the bottom of which was 1cm of water. The dishes were raised above the water by standing on inverted Petri dishes and rotated so that no one dish was permanently at the bottom. The top of the aquarium was covered by a double layer of foil to raise a humid atmosphere, but light could still enter laterally, and placed in the departmental aquarium in the conditions described previously. The soil moisture was maintained by watering when necessary and the slugs were fed fresh carrot three or four times a week. These were assumed to be good conditions for the slugs to live in since breeding occurred and the level of activity seemed quite high. Some notes on the appearance of the eggs, clutch size, hatching times and conditions can be found in Appendix I. This culture method was a variation of that used by Stephenson in 1962-1963, but he stacked similar dishes, three deep in wooden racks, in screw top jars which were kept in an outside insectary or an unheated greenhouse.

LM. LIGHT MICROSCOPY

LM.1. INTRODUCTION

In order to examine the epidermis and its associated structures, it was necessary to fix, embed and section the slug. Sections were then treated with various stains and the results tabulated.

The survey was planned from an histological and histochemical point of view and here the choice of fixative can be critical. It must be able to diffuse rapidly, before autolysis begins in the depths of the tissue and must have the ability to coagulate and harden tissue causing diffusible substances to become insoluble. At the same time, the fixative must strengthen the tissue so that it can survive the dehydration and embedding procedures necessary before sectioning and staining can take place. The fixative must also leave the tissues in a state whereby they can be stained easily, and the results be viewed clearly.

The majority of fixatives react with the proteins of the tissue concerned, either in an additive manner so that atoms of the fixative combine with the protein (usually a side chain being involved), thus stabilising it; or, they are non-additive but act in such a way as to change the solubility of the proteins without affecting other properties.

According to Rothmann (1970) the best available fixatives are those which lead to the denaturation of cellular proteins without recognisable precipitation and thus provide an 'equivalent image' of cell structure at any given level of microscopic resolution. However, there is no ideal fixative, only the one best suiting the substance to be studied should be selected (for instance, Baker's Formol-Calcium for lipids) and even then some changes will have occurred. When such specific fixatives are required for a stain or substance, it will be stated in the text.

LM.2. METHODS

In the initial survey a number of fixatives were chosen from the literature, in particular from the works of Gurr (1962), Humason (1962), Drury and Wallingford (1967), and Pearse (1954 and 1968). A general selection of fixatives was necessary because the structure and chemical content of the gland cells in Arion hortensis were unknown; they included Susa, Helly, Alcoholic Bouin (Duboscq-Brasil) and Zenker which were recommended for animal tissues in general. A fifth fixative, Sodium Acetate-Formol (Conklin 1963), was selected because it was stated by Conklin to be very good for the histochemical demonstration of acid mucopolysaccharides. It was prepared by neutralising 10% formalin with sodium bicarbonate and then adding 2 g of sodium acetate; Conklin used calcium carbonate for neutralisation, but since calcium was one of the substances to be tested for in the mollusc, it was felt that another compound should be used.

In the case of all the solutions except the Sodium Acetate-Formol, which was used at $4^{\circ}C$, fixation was carried out at room temperature (approximately $20^{\circ}C$).

Since most of the fixing, washing, dehydrating and embedding programmes were for mammalian tissues, a timetable for Arion hortensis was worked out on these mammalian techniques, but with the realisation that considerable alterations in timings might be necessary in the light of this pilot run.

Depending on the fixative employed the slugs were washed, dehydrated, cleared in toluene (a clearing agent acting as an intermediate between the slug in alcohol and the slug in the embedding medium), and embedded in paraffin wax (plus Ceresin, congealing at about $55^{\circ}C$). Any soil particles adhering to the slug were removed after fixation since their presence in the wax block could blunt the Knife used for sectioning. It

was necessary to clean the fixed animal since brushing of the live animal caused large amounts of slime to be expelled.

The following table shows the routine instituted for each fixative.

Table I : Timetable for fixing, washing, dehydrating and embedding of tissue

Fixative	Length of fixation	Washing in Dist. water	Alcohols				Toluene	Wax I 60°C	Wax II 60°C
			50%	70%	90%	Abs.			
Susa	16 standing	-	-	2 A x2	1½ A x2	1½ A x1	2¼ A x1	1½	1½
Alcoholic Bouin	16 standing	-	-	"	"	"	"	"	"
Helly	"	6	2 A x1	16 A	"	"	"	"	"
Zenker	"	"	"	"	"	"	"	"	"
Sodium Acetate-Formol	" 4°C	"	"	"	"	"	"	"	"
Carnoy	16½ standing	-	-	-	-	2 A x2	2 A x3	1	2
Lillie's 10%	16½ standing	2 A	-	1½ A x1	1½ A x1	1½ A x3	2 A x3	1½	1½
Formol-Calcium	17 standing	1 RT	-	½ A x2	½ A x2	1½ A x3	1½ A x3	1	1½

Figures show time in hours. RT = Wash in Running Tap Water

A = Agitated

x2 = Number of changes of solution

Standing = Without agitation

The slugs were embedded finally in small rectangular polythene pots, and the resulting block of wax could be trimmed to a size suitable for attaching onto the microtome mounting blocks.

Serial sections of 5, 6, 8, 9 and 10 µm were cut depending on the histochemical test to be applied. A ribbon of such sections was floated onto warm distilled water (which had previously been boiled to remove air) contained in a crystallising dish. The sections extended and flattened, and then a cleaned slide, smeared lightly with glycerin/albumen was slid under them. The slide was lifted out of the water at an angle and drained, with the sections adhering to it. When dry, the slides were transferred to an open slide box and placed in a 37°C oven; they were then ready for staining.

Using Mallory's Triple stain the quality of fixation and penetration of the wax was assessed.

The tissues in Susa sectioned well and penetration of the wax through the slug was complete. At the microscopic level, the cilia around the sole were very regularly arranged and well preserved, as were the cells in the depths of the body; nor did there appear to be any excessive shrinkage or swelling in the tissue. Furthermore, when placed in Susa the slug seemed not to contract excessively, whereas in Alcoholic Bouin the animal contracted into a 'U'-shape, which rendered sectioning rather difficult. Moreover, in Bouin the central part of the animal was not properly impregnated with wax, possibly due to insufficient clearing in toluene or to an insufficient embedding time, and to correct this there would have to be a modification of the schedule listed in Table I.

Fixation in Helly's fixative was not good either, the cells appearing to be rather empty as if material had been lost from them and the epidermal layer was torn. This latter could be due to brittleness of the tissue induced perhaps by too long a fixation time.

The tissue fixed in Zenker sectioned very well and fixation seemed good, but in the central part of the body where the animal was thickest due to contraction, the sections were brittle. This could indicate that the clearing and embedding times were not long enough, and a modification in the schedule would again be necessary. Lastly, the Sodium Acetate-Formol fixed tissue, whilst sectioning well, did not give a satisfactory cellular appearance, the tissue appearing very contracted and damaged.

Thus although several of the above fixatives might have been useful with altered schedules, the Susa fixative was adopted for the general survey since adequate results were obtained with the existing times. Before Susa (also Zenker and Helly) fixed sections could be stained it was necessary to remove the mercuric precipitate formed and this was done by immersing the slide in alcoholic solutions of iodine and sodium thiosulphate (Humason 1962).

The last of the fixatives characterised in Table I were special solutions employed for specific stains, and the stains and the other procedures used in the survey, (with the references for the schedules) are as follows:-

<u>For connective tissue</u>:-

Mallory's Triple stain (Humason 1962):-

I Methyl Blue

II Acid Fuchsin

III Orange G

For Elastic Tissue:-

Weigert's Elastin Stain (Puchtler and Sweat 1960)

Carnoy fixation.

For Polysaccharides and Mucopolysaccharides:-

A. Staining methods for above substances, excluding Fluorescent techniques.

(i) 0.5% Alcian Blue, in 3% acetic acid (pH 2.5) with Ehrlich's Haematoxylin. (Humason 1962)

(ii) a. 1% Alcian Blue 8 GX ... pH 0.5

b. 1% Alcian Blue 8 GX ... pH 1.0

Sections from a. and b. blotted dry.

c. 1% Alcian Blue 8 GX ... pH 2.5

(Lev and Spicer 1964)

(iii) Alcian Blue 8 GX (AB 8 GX), Critical Electrolyte Concentration (CEC) technique. (Scott and Dorling 1965, and Quintarelli and Dellovo 1965).

(iv) a. AB8GX (0.2M Magnesium Chloride)-PAS

b. AB8GX (0.5M Magnesium Chloride)-PAS

c. AG8GX (1%)-PAS

(Andersen, Møllgård and von Bülow 1970)

(v) a. Methylation ... 20°C for 24 hours

b. Saponification... 1 hour

both followed by 0.5% AB pH 2.5. (Humason 1962)

(vi) Drastic Methylation... 4 hours at 60°C and 12 hours at 60°C, both followed by Aldehyde fuchsin. (Pearse 1968)

(vii) a. Methylation using Thionyl Chloride. (Stoward 1967).

b. Saponification a low temperatures for 2 hours. (Stoward 1967).

a. and b. followed by Alcian Blue 8GX pH 1.0 and 2.5.

(viii) a. Mowry's Colloidal Iron Method, plus Control. (Humason 1962)

b. Mowry's Colloidal Iron -PAS. (Mowry 1963)

(ix) Best's Carmine. (Humason 1962)

(x) PAS + Diastase Control. (Humason 1962)

(xi) Southgate's Mucicarmine + Ehrlich's Haematoxylin. (Humason 1962)

(xii) a. 1% aqueous Toluidine Blue. (Humason 1962).

b. 0.1% TB ... ... pH 0.5 for 10 minutes.

0.1% TB ... ... pH 1.0 for 10 minutes.

0.1% TB ... ... pH 3.0 for 10 minutes.

0.1% TB ... ... pH 4.0 for 10 minutes.

(Andersen, Møllgård and von Bülow 1970)

c. 0.1% TB in 30% Ethanol for 10 minutes. (Andersen et al. 1970)

(xiii) Deamination, 16 hours at 20°C, followed by 0.1% TB pH 1.0 or 3.0 as for (xii) b., plus Control. (Sams, Smith and Davidson 1962)

(xiv) RNase (Horn and Spicer 1964) for 2 hours, 1% TB 2 minutes. 10% Neutral Formalin fixation.

(xv) Sulphation. (Scott and Dorling 1965). 10 minutes at room temperature followed by 0.1% TB, pH 3.0 (as for (xii)b.).

(xvi) Azure A, pH extinction. (Pearse 1968)

(xvii) Aldehyde Fuchsin. (Pearse 1968).

(xviii) Aldehyde Fuchsin -AB8GX. (Pearse 1968).

(xix) HID and LID techniques with and without Peracetic Acid. (Pearse 1968).

(xx) Controls for Sialic Acid:

a. Digestion with Neuraminidase ... 17 hours. (Spicer, Horn and Leppi 1967).

b. Hydrolysis with O.1N HCl, ... 2 hours at 56°C.

c. Hydrolysis with O.1N H_2SO_4 ... 1 hour at 80°C.

all followed by 1% AB8GX, pH 2.5. Fixed in 10% Neutral Formalin. (Andersen et al. 1970)

(xxi) Geyer's Tetrazonium method. (Pearse 1968) 10% Neutral Formalin fixation.

(xxii) Hyaluronidase (4 hours), followed by 1% aqueous TB for 2 minutes and buffer Control. (Leppi and Stoward 1965). Carnoy fixation.

(xxiii) β - Glucuronidase with and without Peracetic Acid and Control. (Fullmer 1960). Followed by 1% AB8GX, pH 2.5.

B. Fluorescent techniques:

(i) Ferric Alum - Coriphosphine. (Pearse 1968).

(ii) Acridine Orange - CTAC method. RNase as before. Carnoy fixation. (Pearse 1968).

For Proteins:

(i) Millon's Reagent. (Humason 1962).

(ii) Ninhydrin-Schiff. (Humason 1962).

(iii) Heidenhain's Haematoxylin. (Humason)

(iv) a. Performic Acid - Alcian Blue, plus Control. (Pearse 1968, and Andersen et al. 1970).

b. Performic Acid -AB-PAS. (Andersen et al. 1970)

a. and b. fixed in 10% Neutral Formalin.

(v) Mercuric Bromophenol Blue, 5 µmsections. (Humason 1962) and Bromophenol Blue without Mercury.

(vi) DMAB-nitrite, 10% Neutral Formalin fixation. (Pearse 1968).

(vii) Biebrich Scarlet. (Spicer and Lillie 1961).

0.04% pH 5.0 (McIlvaine buffer).

0.04% pH 6.0 (McIlvaine buffer).

0.04% pH 8.0 (McIlvaine buffer).

0.04% pH 9.66 (Glycine/NaOH Sørensen buffer)

0.04% pH 10.42 (Glycine/NaOH Sørensen buffer)

For Nucleic Acids:

(i) Methyl Green - Pyronin Y, plus RNase Control (Humason 1962).

(ii) Methyl Green - Pyronin-CEC, plus RNase Control (Scott 1967).

10% Neutral Formalin fixation for (ii), using Revector Methyl Green and Revector Pyronin G.

For Lipids:

(i) Sudan Black B for Lipids in paraffin sections (10 µm). (Pearse 1968).

(ii) Luxol Fast Blue MBS. 10 µmsections. (Pearse 1968).

(i) and (ii) fixed in Calcium - Formol.

For Calcium:

(i) Alizarin Red S. (Humason 1962)

(ii) Modified von Kossa Method. (Humason 1962).

For Melanin:

(i) The Masson-Fontana method with the Hexamine Silver modification.

The names of dyes, solutions and enzymes will be given in Appendix II, together with Colour Index and Batch Numbers , where appropriate(p.223).

To build up a picture of the structure, contents and distribution of gland cells throughout the slug, sections were treated in a pre-arranged order so that all stains were applied to any one animal.

Frozen Sections:

A number of slugs were examined in the form of frozen sections. These were necessary, firstly as a check to see if any undue distortion had occurred in tissues that had been chemically fixed, and secondly to allow the staining of lipids which are normally lost after chemical fixation.

The cryostat used was the 'Pearse' Cold Microtome, Type H.

A small piece of slug, not exceeding 3 mm in any direction was mounted on a drop of water on a cork disc which in turn was mounted on a drop of water on a metal carriage. This was placed in a wire basket and held in liquid nitrogen vapour for a short time to ensure the animal became attached to the disc before being lowered into liquid nitrogen for 15 seconds. The Knife blade was kept cool with solid carbon dioxide and the temperature of the cabinet was maintained at -14°C to -15°C. The sections were cut at 10µm, collected on a coverslip and then allowed to dry for two to three ...

(see over).

minutes, when they were stained with the following stains for the same period of time as for paraffin sections:

1% Alcian Blue pH 2.5.

Toluidine Blue, 1% aqueous.

Alizarin Red S.

Methyl Green - Pyronin Y, and

Sudan Black B for 10 minutes.

This last was made up with 1 g of Sudan Black B in 100 ml Isopropyl Alcohol, and filtered before use. Isopropyl Alcohol was chosen as a solvent instead of 70% Alcohol or equal parts of 70% Alcohol and Acetone since they tend to dissolve small droplets of lipid. Isopropyl Alcohol, Propylene Glycol and Methyl Phosphate do this to a reduced extent (Drury and Wallingford 1967).

Once the sections were stained they were rinsed in distilled water, dried as much as possible, and temporary mounts using 50% glycerol were made, sealing the coverslip with rubber solution and nail varnish. Some sections were made into permanent mounts by dehydrating (in Alcohols or Acetone depending on the stain), clearing and mounting in Neutral Mounting Medium, and others were post-fixed, on the slide before staining, with 5% Acetic Acid in absolute Alcohol for 2 minutes.

LM.3. HISTORICAL BACKGROUND

The molluscan epidermis and associated glands have been the subjects of numerous investigations. The mucus produced by some of these glands is generally accepted as serving a number of purposes: as a lubricant - keeping the epidermis moist, pliable and free from dirt, as an 'adhesive', as an aid to locomotion - the mollusc actually moving over a trail of mucus and thus protecting the foot against sharp soil particles, as a protection against enemies inasmuch as it makes the

animal hard to grip and may even be repugnant to the predator although this is more frequently thought to be a function of the protein secretion, and also as a check to evaporation. Hunter (1964) has disputed this last function citing as evidence the work of Hogben and Kirk (1944) who found that mucus from Arion ater did not retain water at normal temperatures unless the air humidity was near saturation. However, continued evaporation from the moist skin does reduce the danger of overheating in all conditions except those of fully saturated air. Terrestrial molluscs are terrestrial only in the sense that they do not live in water; they exist on land by adopting shady, humid, environmental niches and nocturnal behavioural patterns. In a sense, the terrestrial molluscs with shells have an advantage over naked varieties in that they can reduce water loss by retreating into their shells. Another disputed function of the mucus is that it may have antiseptic properties since the molluscan body is free of bacterial and fungal growths, but Campion (1957) found this not to be the case for Helix aspersa. She found that the slime of this animal possessed proteinase thought to be involved in the breakdown of the proteinaceous epiphragm. Clearly, such a property would only be found in the shelled molluscs which produce epiphragms.

Some papers on the skin of shelled molluscs will be examined in the literature review that follows together with those on 'naked' varieties but most will be concerned with terrestrial genera since these will be exposed to approximately similar conditions as Arion hortensis and one would expect comparable structures to be evolved.

In this and previous works the glands were found to be embedded in the dermis, (composed of connective tissue and muscle fibres), below the epidermis and were usually independent gland cell units. The only multicellular glands examined were the caudal and

pedal glands. Hence the term 'gland' will only be applied to these last two cases and their individual mucous gland cells will be termed mucocytes, whilst in all other instances the term 'gland cell' will be used.

At this juncture only brief mention will be made of the other works to be considered, since they can most usefully be examined in comparison with the current research and will therefore be discussed in section LM.4 (xiv) pp 99-115.

Three papers will be described in relation to Helix pomatia. The first is by Prenant (1924) who principally studied the nature and distribution of calcium in the pallial tissue, but also described small and large mucous gland cells, which were found in the connective tissue. Prenant believed that they had a connective tissue origin, developing from leucocytes, which were free connective tissue cells.

The second work on Helix pomatia was by Roth (1929). He too found the gland cells to be subepidermal and described mucous and calcium gland cells like Prenant, as well as protein and golden yellow pigment cells. Moreover, since Roth's investigation was more extensive, covering the whole animal, he described two types of mucous gland cell, one in the mantle and the other in the sole.

Concerning the origin of the gland cells, Roth and Prenant had opposing views, for Roth envisaged the gland cells arising from the epidermis and then 'sinking' into the connective tissue where they differentiated to produce their various secretions, whilst Prenant believed they developed from connective tissue leucocytes. There is still debate about the origin of these gland cells and this will be mentioned later in the text.

A more recent work on Helix pomatia was published by Bolognani-Fantin and Bolognani in 1964. Theirs was a biochemical and histochemical investigation into the nature of the secretion produced

by the cells of the foot in active and hibernating slugs. Besides mucous cells they described calcareous, eosinophil (possibly proteinaceous - personal interpretation - but likened by them to Schneider's (1902) albumen cells), and phenolic cells. The last gland cell type was the subject of a further paper in 1965 (by Bolognani-Fantin only). The granules of these cells were found to contain di- or poly-phenol (ortho- or para-) or a di-indol, and to display a clear intense yellow coloration in fixed but unstained material.

Their work was interesting in that it gave a more detailed account of the histochemical nature of the various secretions produced by Helix pomatia, but it should be remembered that Bolognani-Fantin and Bolognani only worked on the tissues of the foot. It is clear from the text of the 1964 paper that all the cells described have, in their view, an epidermal origin agreeing with Roth, but they gave no evidence to support their statement.

Campion (1957, 1961) described eight types of gland cells in Helix aspersa: mucocytes A, B, C, & D; protein; calcium; lipid and pigment. This was a study of the whole animal and once again the gland cells were found to be present in the subepidermal connective tissue. A, B, calcium, protein and some lipid and pigment cells were found in the mantle collar and similar cells were found in the median surface of the mantle although they were smaller and differed in relative abundance. Likewise a similar situation obtained in the dorsal and lateral surfaces of the foot but in the sole gland cells C and D occurred, although it was thought that these two cell types had a common origin. Campion favoured a connective tissue origin for the gland cells and described stages in development for all cell types. She also believed the mode of discharge to be a merocrine one and that discharge could be effected by direct or indirect pressure on the gland cells induced by the contraction of the surrounding muscle

tissue, presumably under nervous control. However, in the dorsal surface some of the gland cells were seen to possess a network of fibres over the cell surface which could be contractile and might possibly be innervated.

The next work to be considered was by Elves (1961) who described the structure of the foot of Discus rotundatus and found one type of mucous gland cell to be scattered throughout the sole, although aggregated in the foot fringes, together with amoebocytes (equivalent to Barr's (1927-1928) calcic cells) both types being embedded in the connective tissue.

He also described a pedal gland, which was composed of two cell types, one being a discharged stage and the other a developing or discharging stage. Furthermore, a caudal gland was present and was composed of equal numbers of mucous gland cells and amoebocytes in the floor of the gland whereas amoeboid cells were predominant in the sides. In the roof of the gland, composed of a flap of tissue from the dorsal surface, mucus-secreting cells were predominant. However, he made no comment about the origin of the gland cells or their mode of secretion.

In examining Australorbis glabratus, Pan (1958) described four types of cellular component in the connective tissue: fibroblasts (which will be considered in the regeneration section (R.LM. 2 pp 190-191)), pigment cells, vesicular cells and mucous gland cells. A pedal gland was also present. He stated, moreover, that the fibroblasts were capable of transforming into a variety of cell types which could be taken as evidence for a connective tissue origin of cells.

The remainder of this historical survey will consider previous works published concerning other slugs.

Barr (1926) described the pedal gland of Milax sowerbii, and found it to be composed of a mass of secretory cells traversed by a duct, whose lower epidermis was elaborately ciliated. This duct could be secondarily branched. The gland produced much of the mucus on which the animal moved, but also the posterior part of the roof of the canal appeared to have an excretory function since crystalline concretions were found in it. The gland was freely suspended in the body cavity so that muscular contractions within the foot could not effect discharge, and instead Barr believed that the tenacity of the slime caused it to be drawn out of the duct as the slug moved across the substrate.

Barr (1927-1928) also described the glandular system of Arion ater var. castagnea. Similar mucous gland cells were described in the tissue of the mantle margin, foot sole and foot fringe, the aggregation of such mucous cells in the foot fringe being described as the peripodial gland. These last were found to resemble closely those of the pedal gland. In Arion this gland was attached to the tissues of the foot and discharge into intercellular spaces or directly into the gland duct could be effected by the contraction of muscles found between the individual mucous gland cells. In many other respects the gland resembled that of Milax sowerbii with its complex ciliature on the lower epidermis of the duct, but the duct in the case of Arion was never secondarily branched, did not project beyond the secretory cells at the posterior end as occurred in Milax, and did not show any evidence of an excretory function. However, unlike Milax an irregular projection arose from the posterior end of the roof of the canal in Arion and this showed mucous gland cells. 'Calcic' gland cells were found in similar positions to mucous gland cells but were also scattered throughout the deeper tissues.

Two forms of pigment cell were found in Arion ater var. castagnea, in the skin and subepidermal tissues. One was the black pigment, melanin, and the other a red pigment which produced the coloured foot fringe characteristic of var. castagnea, and which Barr believed to be an excretion product deposited in mature mucous gland cells and in the intercellular spaces.

The other intercellular gland described by Barr was the caudal gland. Its structure was much like that already described by Elves in Discus rotundatus and which is only found in the Arionidae and Zonitidae. However, Barr described the gland cells present as mucous and 'calcic' gland cells and found their distribution within the gland to be slightly different. The dorsal flap of tissue was supplied with numerous mucous and some 'calcic' gland cells whilst the floor was lined with mucous gland cells only. She found the bulk of the secretion to arise from the floor, which tended to be highly ridged in a sexually inactive slug and convex in an active one due to the presence of swollen mucous gland cells. The mucus produced by this gland was thick and sticky and used by young animals to form slime strings by which the animal could descend from one level to another. However, in mature animals it appeared to serve as a recognition mark prior to copulation.

This gland was also described in Arion rufus by Saint-Simon (1852) and his description of its general appearance was much as for Arion ater. He noted that in the sexually active period a thick globular mass of mucus accumulated in the middle of the gland and, before coupling, the two slugs moved round each other and seized the globule with their mouths and devoured it, confirming Barr's belief that it was a recognition mark.

Campion (1957) described briefly the glandular structures of two slugs, Testacella haliotidea (Drapanaud), and Milax gracilis (Leydig). In Testacella the sole gland cells were in a sub-epidermal

position, one type producing metachromatic mucus and protein and a similar type whose contents were non-metachromatic. Some larger gland cells were also found producing either a 'bubbly' metachromatic mucus or non-granular protein, which contained a yellow pigment. These various gland cells were found to occur up the sides of the foot. The mantle had a structure similar to the sides of the foot but the gland cells tended to be more numerous with some increase in the non-metachromatic type of mucus . No calcium was found.

In Milax there were sole gland cells producing granular non-metachromatic mucus and protein together with some more superficial elongated metachromatic mucous gland cells. Melanophores were found in the sole together with long, slender sub-epidermal calcium cells opening on to the sole. The sides of the foot showed large metachromatic mucous and yellow proteinaceous gland cells, but no calcium. The dorsal surface had a structure like the sides of the foot except that the gland cells on the mantle were more numerous. Furthermore, that part of the mantle lying adjacent to the foot showed smaller protein and mucous gland cells plus the occasional calcium cell.

Arcadi (1963) described the gland cells of Lehmania poirieri, a garden slug found in the United States of America. He found two and possibly three cell types, or complexes as he called them, which might be responsible for mucus production in the slug. The first type he designated the basket cell complex which was reticulate and found on the dorsal and lateral surfaces of the skin but not in the sole. Adjacent to this complex was the smaller granular cell complex which, although found in the dorsal and lateral surfaces, was most numerous in the sole. They could also be distinguished by their appearance and their histochemistry, this last being the subject of a further investigation in a 1967 paper. He believed that both types of cell contained acid mucopolysaccharides but that the granular cell complex

produced a more complex substance.

In 1965 Arcadi considered the regeneration of a portion of the skin and stated that the mucous gland cells seemed to arise from interstitial cells or fibroblasts lying deep in the connective tissue. Thus from this it would seem the mucous gland cells had a connective tissue origin, but it must be remembered that regenerated tissue may not be 'normal' and nor may the processes by which it arises. The details of this paper will be discussed in the regeneration section (R.EM.2ivpp 206).

Chétail and Binot produced a series of papers on the glandular structures of Arion rufus. The description of the pedal gland (1967) was much like that given by Barr in Arion ater (1927-1928) but they divided the gland into three regions, anterior lateral, anterior median and posterior. They also described three histochemically distinct cell types, a_1, a_2 , and b. Some a_1 cells produced a small quantity of complex acid mucopolysaccharide, whilst others contained a neutral mucopolysaccharide. a_1 cells were taken to be the less mature form of a_2, since the secretions were alike but stained more intensely in the $a_2$cells. The former cell type was found in the anterior lateral regions and the latter in the anterior median region. The b gland cells occurred in the posterior region of the gland, and resembled a_1 and a_2 but produced a different, simple acid mucopolysaccharide, lacking the lipid which was present in some $a_1$and a_2 cells. Cells a_2 only were found in a short region at the entrance of the gland. The epidermis of the gland duct showed three forms: cuboidal and non-ciliated at the edges (Epidermis 1); then two clearly defined 'lips' whose ciliated prismatic epidermis possessed nuclei arranged along the long axis of the cell (Epidermis 2); and between these two 'lips' was a median zone where the nuclei clustered towards the base of the ciliated cells (Epidermis 3). Secretion from the mature cells occurred across

Epidermis 3.

They also found four gland cell types in the sole of the foot: I, II, III and IV. Type I contained a complex mucopolysaccharide and neutral mucopolysaccharide associated with complex lipids and were most numerous in the sides and middle of the sole. Type II were like Type I but showed less intense staining and lacked heterophasic lipids, and were the most numerous cell types in the sole being found throughout the foot. Type III had granular contents of a mucoid nature associated with protein and heterophasic lipids. They were less numerous than I and II and were mainly found in the anterior region. The type IV gland cells were the largest cell type and the secretion appeared to be a mucoprotein whose mucous part was slightly acid. The protein reactions for SH, indol, pyrol and phenol groups were positive rather like the 'phenolic' cells of Bolognani-Fantin (1965), but descriptively different and they were the least numerous, being found only in the middle of the sole.

As to the development of these gland cells, Chétail and Binot found that whatever the cell type, development always occurred from the depth of the tissue to the periphery, so they would appear to favour a connective tissue origin for these cells. The pedal gland cells evolved in the same way. They also noted the presence of a zone of clear cells resembling young mucous gland cells lying in the tissues between the sole and pedal gland and they suggested these were a common stock of young cells able to differentiate into gland cells of the sole or of the pedal gland.

To study the origin of the pedal gland cells and the sole gland cells Binot and Chétail (1968) studied the embryonic development of Arion rufus and they found that the pedal gland had a double origin, with the duct arising from a single, median ectodermal invagination while the two types of gland cells arose from the differentiation of the

mesenchymatous cells, once more confirming their connective tissue origin. The gland cells a_1 of the anterior part of the gland appeared first, 28 to 31 days after laying, whilst type b, located in the posterior part, appeared later (31 to 33 days).

In the sole the epidermis originated from the ectoderm and the gland cells of the sole had a mesodermal origin, the most anterior of which differentiated first. This anterior-posterior gradient of differentiation was therefore found in both the pedal gland and the sole and applied to the ciliature also. Type I mucous gland cells originated first (28 to 31 days after laying), followed by type II (31 to 33 days). Both types III and IV originated in the 33 to 35 day stage but type III appeared first.

It should be mentioned here that the pedal glands of other molluscs do not necessarily have a similar embryonic origin. For instance, Carrick (1938) found, in Agriolimax agrestis, an ectodermal origin for both the pedal gland duct and its gland cells and Ghose (1963) showed that the canal and the gland cells of the pedal gland of Achatina fulica derived from a pair of ectodermal invaginations which only secondarily unite. Both of these are terrestrial molluscs. Chétail and Binot cite other examples, for instance, Smith (1935) on the aquatic mollusc Patella, where the whole pedal gland had a mesenchymatous origin. These different origins for the same type of gland cell illustrate another feature of the epidermal/connective tissue (ectodermal/mesodermal) controversy: that is that similar gland cell types in different molluscs may, in fact, have different origins and comparisons between such different types may simply increase the confusion. However, there does seem to be a genuine difference of opinion concerning Helix pomatia as described earlier.

In a further paper (1968b) Chétail and Binot studied the gland cells of the mantle of Arion rufus. They found two types: M_1 producing

a finely granular blend of neutral mucopolysaccharide and complex lipids; and M_2, being more numerous, larger and producing mucoproteins The mucous part was formed of complex acid and neutral mucopolysaccharides and complex lipids were also found.

A third type of gland cell was found in the groove between the mantle and foot tissues, producing complex acid and neutral mucopolysaccharides together with complex lipids; these corresponded to gland cells I of the foot.

Again they found a mesenchymal rather than ectodermal origin of these cells in an embryonic study, the M_2 gland cells appearing first (20 to 23 days after laying), a few days before the M_1 cells (day 23 to 28).

Binot (1965) found a connective tissue origin for the gland cells of Oncidiella celtica (Cuv.), although the results were obtained from a study of regeneration. This paper will be dealt with in more detail in the regeneration study (R. EM. 2 pp 206-207) later.

Further studies on the skin gland cells of Arion rufus were carried out by Wondrak using light and electron microscopic methods and he conducted a similar examination on the skin of Arion empiricorum (Fér.). Since these papers were principally concerned with the ultrastructure of the gland cells, they will be described in detail in the ultrastructure sections of this thesis in relation to the pedal gland and mantle gland cells.

The paper produced in 1967 described the mucous gland cells of Arion empiricorum where he found two distinct types in the sole, distinguishable from each other by their ultrastructure and the chemical nature of their secretion. He named the first of these the ventral sole gland cell ('Ventral Sohlendrüse') as it was distributed all over the sole; and the second type he designated the lateral sole gland cell ('Lateral Sohlendrüse'), which was found in the lateral areas of the sole below the peripodial groove. A third type of gland cell found in

Arion empiricorum was the mantle gland cell ('Manteldrüse') which was present in the mantle and presumably in the back.

In 1968 Wondrak studied the epidermis of Arion rufus (L) and described the structure of the epidermal cells, the cilia and the cell junctions. The structure of the gland cells was described in 1969 where the sole, mantle and peripodial (equivalent to lateral) gland cells were found to possess a very similar structure to those of Arion empiricorum. He also described long tube-shaped protein cells and large and small granular pigment cells. However, the principal details of this paper concern the ultrastructure of the gland cells and are more relevant to the electron microscopy sections (EM.1 and EM.2).

The most recent work was also a light and electron microscopic survey of the gland cells of Agriolimax reticulatus by Lainé (1971). The principal area studied was the post mantle region, and two types of mucous gland cells were found, both containing an acid mucopolysaccharide. They were distinguishable mainly in terms of their size, being called large and small mucous gland cells. Lainé suggested that the large mucous gland cells might develop from the smaller ones, which in turn might originate in a superficial position from a precursor in the connective tissue. After discharge these large gland cells would appear again as small gland cells. The rate of cell division within the epidermis was not deemed high enough to support their origin from that tissue. Lainé suggested that all the gland cells had a connective tissue origin similar to that described in related types, for example Lehmania (Arcadi 1963). However, as mentioned earlier (pp 27), this may not necessarily be the case.

Besides the mucous gland cells three types of granular gland cells were found in this same region, distinguishable by their various staining reactions in the Alcian Blue Tiple stain. Type (a) stained in Heidenhain's haematoxylin whilst type (b) stained in eosin. Type

(c) was the smallest and did not stain at all, appearing pale yellow. An intermediate type containing haematoxylin and eosin positive granules was also described. Lainé suggested that the granules might be proteinaceous but specific protein tests were all negative. Nor were the contents calcareous.

Type (b) was found to discharge and there was no evidence that (a) and (c) ever did so, so it was possible that they were developmental stages of (b). If this were the case it might be expected that the intermediates would be smaller and more numerous than the mature cell, and, indeed, the (c) cells were smaller than (b) but less numerous. However, (a) and (b) were the same size and it was felt that they could not be intermediates on the above grounds, but the presence of a few cells staining with both eosin and haematoxylin suggested some relationship.

Protein gland cells were found all over the surface of the body, except the foot, but they were uncommon.

Lainé also described two types of granular gland cells lying in the connective tissue of the foot just below the peripodial groove. One type was likened to the peripodial gland cells described by Barr (1927-1928) although no acid mucopolysaccharides could be identified, and the second type was found around the pneumostome and scattered among the connective tissue in most areas of the body. They stained with eosin and haematoxylin but their function was unknown.

A pedal gland was present in Agriolimax but was not described. Calcium was only found in granular clusters lying deep in the connective tissue, yet much was present in the extruded slime.

These gland cells were found embedded in the meshwork of muscle fibres but none of these fibres was actually attached to them. It was suggested that discharge of the gland cells would be effected if this meshwork were to contract, but for a local contraction of muscles,

innervation of these fibres would be necessary. However, the methods for staining nerve fibres used did not clarify the situation. The mode of discharge was believed to be merocrine.

As to pigmentation, Agriolimax was only slightly pigmented, some melanin being present in the grooves of the skin, and the granular cells (c) were pale yellow.

The ultrastructural study of the post-mantle region of Agriolimax will be examined in the electron microscope section (EM.2).

The last work to be considered was by Dyson (1965), actually on Arion hortensis, in which she considered the structure of the normal and regenerating front mantle edge. The dorsal and ventral epidermal cells tended to be cuboidal, although those nearest the free margin of the mantle were taller. They rested on a basal lamina, which also passed round the bases of the gland cells. Three separate types of cells were described, lying between the epidermal cells and extending into the sub-epidermal connective tissue: mucous (four variations I, II, III, IV), protein and calcium. Type I contained phenolic compounds and an acid mucopolysaccharide and was believed to be the mature form of type II. Similarly, type III was believed to be an early stage in the development of type I. Type IV were associated with the ventral epidermis, and appeared granular whilst the others were reticulate.

Dyson believed that the mucous gland cells were epidermal in origin since the type III cells were superficially placed. Further evidence was that the basal lamina was continuous round the mucous gland cells, whilst it did not surround the calcium cells which were believed to have a connective tissue origin. The origin of the protein gland cells was unknown.

She also noted cells containing calcium granules, cells with glycogen and two other unknown cell types in the connective tissue

of the mantle. Lastly, she likened the type I cells of Arion to the A cells of Helix described by Campion (1961) and type IV to the B cells.

The next section will consider the results obtained in the light microscope survey of the skin glands of Arion hortensis (Fér.).

LM.4. RESULTS

From the survey six gland cell types were found lying below the epidermis embedded in the connective tissue, together with a pedal gland and a caudal gland. Whether these are actually six different types of gland cell or whether some are developmental stages of others is subject to some debate.

LM.4(i). The Epidermis.

The epidermis, or epithelium, bounds the body and is composed of only one cell layer throughout. The individual epidermal cells are columnar in nature over the exposed mantle, back, sides, foot sole and foot margins. The word 'exposed' is used advisedly since at the base of the grooves and indentations of the mantle, back and sides the epidermis is not columnar but flattened. This is particularly noticeable in the case of the large peripodial groove, Fig. 2, which extends along the length of the animal and is situated between the tissues of the foot and the sides of the body. As can be seen the 'floor' of this groove is lined by the columnar epidermal cells of the foot margins, whereas the upper surface of the groove is lined by cuboidal epidermal cells which tend to be confined to this area. However, between these two types is a thin band of tissue, presumably a pavement epithelium, but the layer is so thin that individual cells cannot be distinguished.

The spherical or ovoid nuclei of the columnar epidermal cells tend to adopt a central or almost basal position and are large. They contain granules of chromatin, and occasionally a slightly larger granule which is taken to be the nucleolus. The nuclei of the cuboidal cells are also large, tending to be more ovoid than spherical, but displaying granules of chromatin and a nucleolus. In the pavement epithelium nuclei could not be distinguished easily but when detectable they tend to be very flattened and extend along the axis parallel to the body surface. Chromatin granules could not be seen.

The epidermis is ciliated around the pneumostome, the foot margins, the median region of the foot sole and the lower epidermis of the pedal gland duct. This last will be discussed in the section dealing with the light microscopy of the pedal gland (LM.4(ix) pp89-93).

The cilia of the foot margins are long, on average 6 µm, fine and closely packed whereas those of the median region of the sole are more scattered and shorter, circa 3 to 4 µm, possibly due to the effects of abrasion. The width of the median band of ciliation varies somewhat from slug to slug but is often quite wide covering about 50 to 60% of the sole in contact with the substrate. There is thus a non-ciliated area between the median band and the foot margins. The band extends from the front of the animal to the rear.

This distribution of cilia and their respective lengths agrees well with that found by Lainé (1971).

Although no obvious basal granules are seen associated with the cilia a thin band could be detected just below the outer edge of the epidermal cells (Fig. 2), and this is believed to represent the region where numerous basal granules lie adjacent to each other.

The function of the cilia is to distribute the mucus discharged from the gland cells over the body surface, and the flow of mucus induced serves to remove dirt and waste from the body surface.

No goblet cells are seen in the epidermis, only the necks of gland cells passing between the epidermal cells. The bodies of these gland cells actually lie in the connective tissue below the epidermis.

Fig. 3 illustrates the distribution of the gland cell types around the body. As can be seen they are broadly in two categories: those found in the dermis of the mantle, back and sides of the animal and those found in the dermis of the foot.

LM.4(ii). The Mucous Gland Cells of the Dorsal Surface.

There are two types in this region, designated the large, (M), and the small, (m), mucous gland cells.

(a) The large mucous gland cell has an ovoid cell body and a basal nucleus (Fig. 4), which is small, flattened against the cell wall, and usually surrounded by a limited area of cytoplasm. It shows one or two granules of chromatin but these are not very distinct. However, the nucleus is often not visible at all in these cells. The large cell body is embedded in the connective tissue and musculature of the back and sides, and the secretion of the mature cell is discharged to the exterior through a single, short, straight duct. This is illustrated in the second large mucous cell, in Fig. 4, although it does not show that the duct insinuates itself between the epidermal cells.

As mentioned previously, this gland cell is the largest type in the slug although the variation in size is considerable, ranging from 11 μmto 105.6 μmlong, and 6.6 μmto 66 μmwide. The mean size is 42.7 μmlong by 23.9 μmwide, with two modal groups, one at 22 μmlong by 19.8 μmwide and one 33 μmlong by 15.4 μmwide. Some of the size variation can be related to the difficulties involved in measuring the gland cells for it is unlikely that they will always

be sectioned through their longest axis, (that is, directly through the middle in the case of these ovoid cells), and any section taken off centre will give a reading smaller than the actual measurement. This could be counteracted by measuring the gland cell from its base to the epidermis, where presumably many of these cells will end. However, the results obtained in this manner are likely to err in the opposite direction, in that the derived measurements will probably be larger than normal since, naturally, not all cells will have developed as far as the epidermis. In all instances the 'length' of the cell is taken as being the axis perpendicular to the epidermis.

Histogram I (Volume II, p.30) shows an illustration of the type of distribution pattern seen along the length of the back of the animal. Large mucous cells are not present at the very anterior end of the body, their place being taken by smaller gland cells showing the same histochemical characteristics. The distribution along the mantle and back is constant except towards the tail where there is a rise in numbers. This occurs in the region of the caudal gland which will be described in LM.4(x) pp 93-94.

As can be seen in Figs. 4, 5 and 6 the cell contents, which occupy the bulk of the cell body and duct, have a reticulate appearance after chemical fixation. This reticulum may either reflect the appearance of the secretion within the cell or it may result from the staining of remnants of secretion attached to the collapsed cell wall. Occasionally it seems to be in parallel bands (Fig. 4), but this could be a fixation artefact caused by the flow of fixative through the cell body.

In frozen sections the gland cell appears very similar (Fig. 5), with a small basal nucleus and reticulate contents, but it always appears to contain more secretion in the frozen sections, presumably because less is discharged. Since the reticulum is present in both chemically fixed and frozen material it would seem that it is

a fairly normal feature of the cell. However, whilst the chemically fixed material shows only large mucous cells with reticular contents, in frozen sections another type of large cell is seen, containing numerous fine granules in a matrix (Fig. 5). One cell, as illustrated in Fig. 5, shows both granular and reticulate contents. It seems likely that the granular stage is a more immature one since the secretion is threadlike externally and not granular. Why these granular stages are absent in chemical fixation is not known, but it is possible that the chemicals cause aggregation of the granules.

Fig. 6 shows what is described as a large mucous cell showing 'exploded' contents, since there is a 'halo' around it of secretory material, staining just like that within the cell. This structure can also be seen discharged, still retaining its ovoid shape. What causes this "explosion" is not clear although it is probably an artefact, associated only with the larger mucous gland cells. It is seen in both chemically fixed and frozen material and it is possible that the violent contractions caused by dipping the living animal into the fixative or liquid nitrogen are responsible for the contents literally 'exploding'.

The staining reactions for the large mucous cells, M, are listed in Table II.

Table II : Staining Reactions of the Large Mucous Cells (M)

Stain	Result	Conclusion
Mallory's Triple	Blue	Mucus
Weigert's Elastin	-ve	Not elastin-like
Aldehyde fuchsin	Purple	Sulphated MPS or elastin. Non-SO_4 MPS also weakly +ve

Stain	Result	Conclusion
AF(pH1.45)-AB(pH2.5)	Purple and blue Smaller ones AB only. Purple often at apex.	(i) Strong SO_4 MPS and élastica purple. (ii) Non-SO_4 AMPS blue. (iii) Less strong SO_4 AMPS or mixed SO_4 and COOH MPS blue/purple. Have mixture of (i) and (ii)
AB (pH 2.5) - Ehrlich's haematoxylin	Blue	(i) Weakly acidic SO_4 MPS, hyaluronic acid and sialomucins blue (ii) Strongly AMPS weak or -ve. Have (i)
AB pH0.5 AB pH1.0	Blue Blue	) Only SO_4MPS) stain.
AB pH2.5	Vivid Blue	Results suggest SO_4MPS
AB-CEC 0.2M $MgCl_2$ 0.3M $MgCl_2$ 0.4M $MgCl_2$	AB+ Very pale to -ve -ve	) Indicates COOH) or SO_4 MPS (Stoward 1967)
AB (pH2.5)-PAS AB (0.2M $MgCl_2$)-PAS AB (0.5M $MgCl_2$)-PAS	AB+ AB+ -ve	) Periodate Un-) reactive MPS) containing COOH) or SO_4 groups
Methylation (Mild)	-ve	Simple acid or complex SO_4MPS -ve.
Methylation (Mild)+ Saponification	-ve	Indicates SO_4MPS, for stain restored in COOH.
Methylation (Drastic): 4 hours 12 hours	 -ve -ve	) Blocks staining of) COOH & SO_4 MPS.
Methylation (ThCl) 4 hrs. Methylation (ThCl) 4 hrs. + Saponification (cold KOH) 2 hrs. followed in all cases by AB pH2.5 and pH 1.0.	+ve	) Indicates SO_4 MPS) but may not be) methylated enough
Colloidal Iron Control Colloidal Iron-PAS	Dark blue -ve Dark blue	) Weakly AMPS stain) Strongly AMPS -ve Non-periodate reactive, weakly AMPS
PAS PAS-Diastase	-ve -ve	No vic-glycols

Stain	Result	Conclusion
Best's Carmine	-ve	No glycogen
Southgate's Mucicarmine	-ve	Epithelial mucins red but hard to get avid stain.
Toluiding blue (TB): chemically fixed frozen fixed	blue/purple β metachromasia Rose-purple and metachromasia.	Metachromatic MPS
TB pH 0.5 pH 1.09 pH 3.0 pH 4.0	-ve to pale mauve Rose purple Rose purple Rose purple	) Indicates weakly) SO_4 or COOH MPS))
TB in 30% alcohol	Bright purple	Metachromatic MPS.
Deamination + TB pH1.0 Control	-ve +ve	Indicates that metachromatic group was attached to NH_2 group which has been eliminated.
RNase +TB aq. Control	Rose purple Rose purple	Indicates cell basophilia and metachromasia not due to RNA.
Sulphation + TB pH 3.0	Rose purple	Unaffected
Azure A pH 0.5 pH 1.5 pH 2.4	-ve -ve Pale blue purple	) (i) Not SO_4 MPS, but) (ii) some SO_4 MPS with) masked azurophilia) stain only at pH 3.5) to 4.5) (iii) Many sialomucins) stain at pH 3.0 & above.
HID-AB	AB +ve	Sialomucins or hyaluronic acid not SO_4 MPS.
Peracetic acid (PA)-HID-AB	Pale AB +	Not neutral MPS or periodate reactive sialomucins.
LID-AB PA-LID-AB	Vivid AB+ -ve to pale AB	) Not neutral MPS but a) LID -ve, non-SO_4 MPS
Neuraminidase + AB Control	AB + AB +ve	Indicates no sialic acid present, but some sialomucins enzyme resistant.
Hydrolysis N/10 HCl + AB	AB +	) Not sialomucins)
Hydrolysis N/10 H_2SO_4 + AB	AB +	) Not sialomucins
Tetrazonium method for SO_4	-ve, though some pink at base	Unlikely that SO_4 present.
Hyaluronidase + 1% aq.TB Control	Purple Purple	No hyaluronic acid present

Stain	Result	Conclusion
PA-β-glucuronidase + 1% AB pH 2.5	-ve to v.pale threads	) Attacks glucuronic acid groups or non-oxidised AF +ve material
β-glucuronidase + 1% AB pH 2.5	-ve	)
Control	AB +	
Ferric Alum-Coriphosphine	Orange, especially at apex.	Sulphomucins dull red
Acridine Orange-CTAC	(i) Red but few threads (ii) -ve (iii) -ve	Indicates presence of hyaluronic acid, not chondroitin SO_4s or heparin.
Acridine orange alone	Brick red at apex	Suggests SO_4s
Millon's reagent	-ve	No tyrosine-containing proteins.
Ninhydrin-Schiff	-ve	Proteins pink if contain enough NH_2 groups, but not all reactive amines oxidised this way.
Heidenhain's haematoxylin	-ve	Indicates no COOH containing protein.
Performic acid (PFA)-AB pH 2.5	AB +ve	A+ve result shows S-S groups, but control also +ve
PFA-AB-PAS	AB +ve	
DMAB-nitrite	-ve	No tryptophan present
Bromophenol blue (BPB)	-ve	No protein
Mercuric BPB	-ve	No protein
Biebrich Scarlet pH 5.0	A few strands v.pale	) No basic protein
pH 6.0 - 10.42	-ve	
Methyl Green-Pyronin Y	Bright red at neck, remainder rose, also in frozen sections.	RNA?
Methyl Green-Pyronin Y + RNase	Lose red colour	Would seem to indicate RNA at neck
Methyl green-Pyronin (Scott 1967)	V.pale pink	) Apparently no RNA
MG-Py 2M $MgCl_2$	-ve	) Apparently no RNA
MG-Py +RNase	-ve	)
Sudan Black B	-ve	) No lipids present
Luxol Fast Blue MBS	-ve	)
Alizarin Red S	-ve	) No calcium
von Kossa	-ve	

These results are very complicated and somewhat confusing. A mucopolysaccharide (MPS), or glycosaminoglycan or glycosaminoglucuronoglycan in the new terminology (Spicer, Horn and Leppi 1967), is clearly present because of the staining reactions with Alcian Blue, Colloidal Iron, Azure A and so on. However, it is the nature of the acid mucopolysaccharide which is in doubt. The strong staining in Alcian Blue pH 2.5 and Colloidal Iron suggests the presence of weakly sulphated acidic mucopolysaccharide, hyaluronic acid or sialomucins since strongly sulphated mucopolysaccharides paradoxically stain only weakly. Mucopolysaccharides containing sialic acid do not seem to be present because neuraminidase has no effect on the alcianophilia, but it should be remembered that some sialic acids are resistant to enzyme digestion (Quintarelli and Dellovo 1965), presumably because they are bound differently and more securely to the rest of the molecule. However, acid hydrolysis does not reduce staining as it would if sialic acid units were normally present. Nor would it seem that hyaluronic acid, chondroitin 4-sulphate (old terminology - chondroitin sulphate A, Spicer, Horn and Leppi 1967; Muir 1969), or chondroitin 6-sulphate (chondroitin sulphate C) are present, for testicular hyaluronidase does not reduce staining. This enzyme splits the endo-β-N-acetyl-D-glucosaminidic residues (Leppi and Stoward 1965) present in these substances and removes their ability to stain in the Toluidine Blue. The Acridine Orange - CTAC - shows some slight red fluorescence for hyaluronic acid but this colouring could also be given by the glucuronic groups in some other substance such as the chondroitin suggested below. Hyaluronidase does not affect dermatan sulphate (chondroitin sulphate B) or any other acid mucopolysaccharides. β-Glucuronidase (from a molluscan source) however, does destroy the staining reactions in the large mucous gland cells. This enzyme acts on the terminal non-reducing glucuronic acid residue (Curran 1964), and so glucuronic acid residues

are present in the secretion, which indicates that dermatan sulphate is absent for it contains iduronic not glucuronic acid units (Spicer, Horn and Leppi 1967). From Table 43, page 253, (Spicer, Horn and Leppi, 1967), there is another mucopolysaccharide mentioned simply as "low sulphate chondroitin". This contains galactosamine as the amino sugar, glucuronic acid residues and some sulphate groups. It would presumably show weak reactions for sulphates as well as those for carboxylates, and as can be seen from Table II this is the case. For instance, a fairly weak alcianophilia is shown at pH 0.5 and 1.0, below the pK of carboxyls, indicating the presence of sulphates, but a strong reaction is obtained at pH 2.5 when presumably both groups are staining. However, this substance must lack β-N-acetyl-D-glucosaminidic residues or else staining would be lost after hyaluronidase, and the notion that a totally different mucosubstance is found in the M cells cannot be dismissed.

The results of the Alcian Blue-critical electrolyte concentration (AB-CEC) technique indicates, according to Stoward (1967), the presence of carboxylated or sulphated mucosubstances, since staining is obtained in the presence of 0.2 Magnesium Chloride but it is almost completely lost in the presence of 0.3 Magnesium Chloride. Hyaluronic acid, sialomucins and some weakly acidic sulphomucins do not stain in the presence of 0.1 Magnesium Chloride, so once again the presence of hyaluronic acid and sialomucins can be discounted.

The Azure A extinction experiment indicates that the mucosubstance involved is not strongly sulphated since no staining is obtained in very low pHs, below pH 2.0. However, it has been stated (Spicer, Horn and Leppi 1967) that some sulphated mucosubstances with masked azurophilia only stain at pH 3.5 to 4.5. The Toluidine Blue extinction results show a similar situation, that is that weakly sulphated or carboxylated mucosubstances are present and those for

the HID and LID techniques indicate that non-sulphated mucosubstances are present. The large mucous gland cells stain dually with Aldehyde Fuchsin - Alcian Blue such that the purple coloration is often around the the apex and duct of the cell, the base being blue-green while the smaller large mucous cells show alcianophilia only. Aldehyde Fuchsin is generally accepted as staining sulphates or elastica-like substances but this last can be dismissed since Weigert's elatin stain was negative. If there were to be a greater concentration of secretion and hence sulphate ions at the neck then the dual reaction could be explained.

This different reaction was also noted in other stains (Fig. 4), particularly acridine orange (without salt molarities) and Methyl Green-Pyronin Y (without CEC).

In the Methyl Green-Pyronin stain the apex is scarlet and this staining is removed by RNase. This would suggest the presence of large quantities of RNA but what purpose it would serve is not clear. Geuze (1971) noted a similar pyroninophilia in the mucous neck cells of the oesophagus of Rana esculenta, but made no comment on its nature. Furthermore, using 10% neutral formalin, recommended by Scott (1967), and Scott's CEC method this coloration is not detected. Scott found that in the absence of any electrolyte pyronin stained almost all the polyanions present in a substance and hence would stain the mucosubstance. This would explain the red obtained above. However, the effect of RNase is harder to explain since this should have a fairly specific effect. Scott found that only a small amount of electrolyte needed to be present to displace the stain from all non-nucleotide polyanions, even the amount of electrolyte present in a buffer solution would be sufficient, and since the RNase used in this work was made up in a buffer the electrolyte in this could be sufficient to displace the dye from the non-nucleotide polyanion. This tends to be borne out by the fact that no such staining is obtained when Scott's CEC technique

is applied.

However, there is clearly some difference between the substance present at the apex of the cell and that in the rest of the cell, but this need not be a histochemical difference, rather one of concentration. If the mucopolysaccharide is more concentrated in the apex and duct, the extra concentration of sulphate ions may be sufficient to give the aldehyde fuchsin and acridine orange (without electrolytes) reactions which would seem to indicate sulphomucins. Why the mucosubstances should become concentrated at the neck is not clear, and in the case of the Methyl Green-Pyronin stain the external mucus is stained both red and pink so it is not necessarily the case that the red stained mucus is more mature. However, the presence of a totally different polyanion cannot be dismissed.

The methylation-saponification sequences do not throw much light on the situation. Both carboxylated and sulphated mucosubstances are capable of being methylated, thus removing their alcianophilia, but after saponification the alcianophilia of the carboxylated compounds should be restored whilst that of sulphated compounds is not. Methylation is found to occur when using methanolic HCl at room temperature or 60°C, but 4 hours in methanolic thionyl chloride at room temperature is not sufficient to remove staining. However, saponification does not restore alcianophilia. This could be taken to indicate that only sulphate ions are present, but unfortunately this cannot be used as definite confirmation of the possibility since saponification is known to be somewhat unreliable (Quintarelli and Dellovo 1965) and the efficacy of low temperature KOH (Stoward 1967) could not be established since methanolic thionyl chloride did not affect the staining properties of the secretion.

The Sulphation technique and the use of RNase did not affect the staining of the large mucous cells. The former was not expected to work since it is normally used in the demonstration of neutral mucopolysaccharides and acid mucopolysaccharides are present. Staining after the latter indicates that metachromasia is not due to RNA. However, a deamination procedure destroyed metachromasia. It would therefore seem that the ions causing the metachromasia are attached to amine (NH_2) groups which are removed by the process.

If a low sulphate chondroitin substance is accepted as being the mucopolysaccharide in the large mucous cells, then galactosamine is also associated in the disaccharide unit (Spicer, Horn and Leppi 1967), and this substance contains amine groups. If the sulphate or glucoronic acid units were to be linked to these amine groups, then deamination would effectively cause the loss of staining.

There is an alternative to this though which is perhaps more likely: that the low sulphate chondroitin (or whatever the substance) is attached to a protein core, and when the deamination occurs these reacting groups are once more lost. The main difference between the two alternatives is that in the first case the amine groups are an integral part of the chondroitin, whereas in the second case the whole low sulphate chondroitin unit is attached to a central protein core. However, protein tests including Biebrich Scarlet for basic proteins (which would be expected in the core) were negative. It is possible that in normal circumstances the mucopolysaccharide masks the protein.

Certainly the suggestion that chondroitin sulphate may be bound to protein is not unique, for instance, Dorfman (1963), quoting work by Shatton and Schubert (1954), described the presence of a chondroitin sulphate - protein complex in cartilage and the properties

of the complex suggested that they were covalently bound. He also quoted work by Mathews and Lozaityte (1958) in which they suggest that the polysaccharide side chains are attached to a protein core. However, Bernardi (1957) suggested that the protein units did not form a core but that polysaccharide units were linked by protein bridges to form a chain. This suggested structure must also be considered in relation to the secretion within the large and small mucous gland cells, and with only histochemical techniques available it is not possible to decide which of the alternatives is appropriate.

Hunt and Jevons (1965) described a polysaccharide sulphate - peptide complex in the hypobranchial mucin of Buccinum undatum. The linkage between the peptide and polysaccharide sulphate was believed to be covalent. But Taylor (1963) on Ariolimax columbianus found the linkage to be ionic involving calcium. No comment can be made about the binding of components in Arion hortensis since biochemical methods were not employed. The conclusion, therefore, is that the secretion in the large mucous gland cells is possibly a low sulphate chondroitin substance linked to a protein core or in polysaccharide-protein chains, but it is not possible to verify this with the techniques available. The presence of some hitherto uncharted mucosubstance in these gland cells cannot be dismissed.

The mucus produced by these gland cells would function as a lubricant keeping the skin clean and moist, but when the animal is violently stimulated large quantities of it are expelled. This could be a defence reaction perhaps making the slug repugnant to its predators and certainly difficult to grip.

The mechanism of discharge for these and the other gland cells will be discussed after a consideration of the morphology and histochemistry of the other types of gland cells. Furthermore, the origin of the large gland cells will be considered after an examination of the small

mucous gland cells.

(b) The small gland cells tend to be elongated and narrow, Fig. 7 and Fig. 4, the neck not being obviously distinct from the cell body. The mean length is 36.9 μmand the mean width 6.7 μm but the range is from 13.3 μmto 72 μmin length and from 5 μmto 13 μmin width. There are two modal sizes, 40 μmlong by 5 μmwide and 34.7 μmby 5 μmwide.

The distribution of the small mucous gland cells along the mantle and the back of Arion is shown in Histogram I (Volume II, p.30). The largest numbers are found just behind the head and gradually these drop, with some fluctuations, towards the tail. In general, there are more small mucous gland cells than large ones, the exception occurring, at least in this animal, in the tail region when there are more large mucous gland cells.

The nucleus of the small mucous cell is small occupying a basal position, and whilst no bounding cytoplasm can be detected round it, it may be present. The contents of the gland cell appear reticulate, as in the large mucous gland cells and can often be seen in the process of being discharged, (Fig. 7), through a duct passing between the epidermal cells.

The small mucous gland cells tend to occupy a slightly more superficial position in the connective tissue than the large ones; that is, they lie nearer the epidermis (Fig. 4) and are found over the mantle, back and sides of the animal and also around the pneumostome.

The staining reactions of the small and large mucous gland cells are identical (Table II) except that in methyl green-pyronin Y (without electrolyte) the scarlet coloration is very rarely seen in the small cells; the typical reaction being pale to dark pink. As mentioned before this red staining could well reflect a difference in quantity not quality and clearly such a concentration of secretion is not often present in the small mucous gland cells.

It seems unlikely that the slug would possess two different types of gland cell producing an identical secretion for lubrication etc. and it is suggested, therefore, that the large and small mucous gland cells represent the same gland cell type in slightly different stages of development. The cycle that is proposed for the large and small mucous gland cells is outlined below.

The large reticulate mucous gland cells (Fig. 4) represent the mature stage just prior to, or in the process of, discharging whilst the small reticulate mucous cells (Fig. 7) represent the discharged cells, and it is these cells which will become recharged with secretion. The fine granular material described in frozen sections could be a less mature stage of the secretion, since it is not seen externally. However, in the ultrastructural investigation the secretion is seen to be thread-like (Plate 44), and why very fine granules should be seen in some instances in frozen sections is not known, unless the membrane-bound sacs are being detected. Possibly after chemical fixation or violent contraction of mature cells during freezing, these membranes are broken so that only a reticulum of secretion is normally seen.

That the large and small mucous gland cells represent one and the same type of cell is confirmed by the electron micrographs of the normal mantle tissue (for instance, Plate 42), where the mantle gland cells tend to be of a fairly uniform size showing similar types of Golgi apparatus and other cell organelles. It is not surprising that there are less discharged cells in the tissue prepared for electron microscopy because it is dissected rapidly and the fixation methods for electron microscopy are generally better, in terms of the quality of preservation, than those for light microscopy. Also the size ranges for the large and small mucous gland cells overlap considerably which might be expected if the two "types" are, in fact, one and the same.

This cycle of secretion involving the large and small mucous gland cells is, in a sense, a developmental sequence, and because few true developmental stages were seen it would appear that these cells function throughout the life of the animal. However, in the mantle and back tissue there is a type of cell, Y, which is never seen to discharge and occupies a very deep-seated position in the dermis, and it may represent a very young stage of the mucous gland cells, but it could also be a young stage in the development of the pigment/protein gland cells. Whichever type it is, it is not very numerous, not numerous enough to be taking part in the continuous replacement of mucous gland cells unless, of course, development is very rapid, or loss is very slow. These last points cannot be verified in the static picture presented by a section fixed for light microscopy. The results from the regeneration experiment carried out on the posterior part of the mantle (Section R) would suggest, but by no means confirm, a connective tissue origin for the mucous gland cells. From the tissue fixed for light microscopy, there is no evidence of cell division in the epidermal cells examined and this might be expected if the gland cells were to originate from there, even if they were only replacing the exhausted cells. In addition, 'young' cells, with large nuclei tend to occupy a position deep in the dermis and not a superficial one. However, this evidence is not conclusive, merely indicative, of a connective tissue origin of the cells. In the static situation presented by a tissue section, as already mentioned, it is very difficult to come to any conclusion concerning the origin of the gland cells.

LM.4(iii). Young Cells, Y, of the Dorsal Surface.

The young cells, Y, are found only in the back, mantle and sides of the animal, and being deeply embedded, sometimes just below the main glandular layer or between the gland cells, they are never seen

closely applied to the epidermis.

These cells are ovoid or somewhat elongated with a large spherical or ovoid centrally placed nucleus (Fig. 8), often containing a nucleolus together with granules of chromatin. However, these cells may adopt unusual shapes (Fig. 9) after chemical fixation, with cytoplasmic protuberances extending into the connective tissue, the cytoplasm itself appearing finely granular (Fig. 8), or occasionally slightly reticulate (Fig. 9). They do not possess ducts and therefore do not appear to discharge. These Y cells are found along the entire length of the animal appearing slightly more numerous at the head end (Histogram I, Volume II, p. 30); but in any one section they are not numerous.

The mean length of the cell is 16.4 µmwhilst the mean width is 16 µm,with a range from 2.2 µmto 50.6 µmlong and 2.2 µmto 48.4 µm wide. However, the modal size is 22 µmlong by 6.6 µmwide.

The Y cells were exposed to the same histochemical tests as the large and small mucous gland cells (Table II) and the results are given in Table III which follows:

Table III : Staining reactions for the Young Cells (Y)

Stain	Result	Conclusion
Mallory's Triple	Orange/pink with pink nucleus	Stains blood corpuscles, yolk, muscle orange. Could show protein.
Weigert's Elastin	-ve	No elastica
Aldehyde fuchsin	V. pale pink	Not conclusive, could show slight amount of SO_4 material.
Aldehyde fuchsin (pH1.45) & AB pH2.5	V. pale pink	
AB pH (2.5) - Ehrlich's haematoxylin	V. pale purple	Protein?
AB, low pH	-ve	NO AMPS

Stain	Result	Conclusion
AB-CEC	-ve	)
AB-PAS	Pink	)
AB-CEC-PAS	Pink	)
Methylation +AB	-ve	) NO AMPS
Meth. + Saponification + AB	-ve	)
Colloidal Iron	-ve	)
Colloidal Iron + PAS	Pink	)
PAS + Diastase	Pink	) Periodate reactive
PAS	Pink	) but not glycogen
Best's Carmine	-ve	No glycogen
Southgate's mucicarmine	-ve	No mucin
1% TB, chemically fixed	Blue cytoplasm and nucleus	NO AMPS or molecules not stacked in manner to give metachromasia or protein blocking staining.
1%aq. TB, frozen sections	"	"
TB pH 0.5	V. pale blue	As above, but low pH seems to suppress some staining
TB pH 1.09	Pale blue	
TB pH 3.0	Bright blue	
TB pH 4.0	Royal blue	
TB in 30% alcohol	Medium blue	As above
Deamination, pH1.0 TB	Rose dark blue	Removal of NH_2 groups reveals some slight metachromasia
Control	Fairly light blue	NH_2 groups suppressing staining.
RNase + TB	Blue	) No RNase causing
Control	Blue	) any staining
Sulphation + TBpH3.0	Blue	No neutral MPS
Azure A pH0.5	-ve	)
Azure A pH1.5	Pale bluish green	) No MPS
Azure A pH2.4-5.0	Pale blue	)
HID-AB	V. pale purplish grey	Indicates small amount of HID reactive subst., but oxidation has no effect so not neutral MPS
PA-HID-AB	Nucleus pale purplish	No MPS, or LID reactive substances
LID-AB	"	
PA-LID-AB	"	
Neuraminidase + AB	AB+ve	) No sialic acid
Control	AB+ve	)

Stain	Result	Conclusion
Tetrazonium	-ve	No sulphate
Hyaluronidase + TB	Pale blue	No hyaluronic acid
Control	Pale blue	No hyaluronic acid
PA-β glucuronidase + AB	-ve	)
β-glucuronidase + AB	-ve	) No glucuronic acid
Control	-ve	)
Ferric alum-Coriphosphine (Susa)	Yellow/lime green with hint of orange	Could indicate some sulphomucin but slight.
Acridine orange	Pale green	No sulphomucins
Millon's reagent	Orange/pink	Protein
Ninhydrin Schiff	-ve	Possibly indicates no NH_2 but not all reaction proteins oxidised by this.
Heidenhain's haematoxylin	Black cytoplasm and nucleus	Protein
Ehrlich's haematoxylin-Eosin	Eosinophilic cytoplasm, purple nucleus	Proteinaceous
Performic acid + AB	-ve	) No S-S groups
Control	-ve	)
Performic acid - AB-PAS	Pink	Some periodate +ve substance present
DMAB-nitrite	-ve	No tryptophan present
Bromophenol blue	Blue	) Protein present
Mercuric BPB	Blue	)
Biebrich Scarlet		
pH5.0	Scarlet	) Protein present
pH6.0	Scarlet	) but seemingly not
pH8.0	Pale red	) basic protein
pH9.66	Pale red	)
pH10.42	-ve to v. pale red	
Methyl green-pyronin Y, chem. fix.	Fairly bright pink	) Some pyroninophilic substance present
MG-PY, frozen fix.	Bright pink	) even in frozen
MG-PY + RNase	Fairly bright pink	) sections but RNase
MG-PY (Scott)	Dark pink	) and the presence of
MG-PY +RNase	Quite dark pink	) electrolytes
MG-PY 2M $MgCl_2$	-ve	) suppresses the staining
Sudan Black B	Bluish	) Indicates small
Luxol Fast blue MBS	V. pale green	) amount of lipid present

Stain	Result	Conclusion
Alizarin Red S	-ve	No calcium
von Kossa	-ve	No calcium

These results show that detectable amounts of protein are present in the cytoplasm of the young cell. The protein does not seem to be basic because of the staining reactions with Biebrich Scarlet, nor does it contain disulphide groups or tryptophan. The lack of staining in ninhydrin-Schiff is a rather puzzling result since it indicates that no amino groups are present at all. However, not all reactive proteins are oxidised by this method, which must be the case here for amino groups must be present in the protein (demonstrated by other methods) and also the deamination technique indicates their presence.

In fact, these last results are particularly interesting because slight metachromasia occurs in toluidine blue after deamination. This suggests that there is some molecule present in the cytoplasm stacked in such a way as to give metachromasia, but the amino groups normally present suppress this. The nature of the molecule is uncertain : it could be an acid mucopolysaccharide, though no other methods for mucopolysaccharides gave positive results : it does not seem to be a neutral mucopolysaccharide for, although the reaction with PAS suggests this, the sulphation technique failed to induce metachromasia. The HID technique preceded by peracetic acid would also indicate the presence of neutral mucosubstances and the results are negative. However, the slight staining by aldehyde fuchsin, HID and coriphosphine could suggest a few sulphate groups are present in the cytoplasm but they might well be attached to the protein. If some sulphate groups are present but their metachromatic effect is normally masked by amino groups then the effect of deamination ...

(see over).

would be explained. Nevertheless, some other type of ion could be causing the staining, and detectable amounts of mucosubstance, either acid or neutral, appear to be lacking unless the metachromasia revealed after deamination is an indication of the presence of such a compound. However, the small amounts of sulphate possibly present could be attached to the protein and be indicative of nothing more.

Thus the cytoplasm of the Y cells is proteinaceous, possibly containing tyrosine, is periodate reactive (but not due to glycogen), shows some weak staining for lipids and also displays pyroninophilia in all solutions except those containing electrolytes. This staining with pyronin is not due to RNA but what it does signify is unknown. One can only reiterate Scott's (1967) statement that many polyanions stain with pyronin, but the presence of an electrolyte displaces the dye from these leaving it combined with polynucleotide polyanions only.

These results give no clue to the type of cell that Y eventually becomes. It is possible that the protein/lipid substance present in the cytoplasm is a precursor of the material discharged by the mucous gland cells and that the acid mucopolysaccharide units are 'added' to this core later. The possibility of there being a protein core to the mucous gland cell secretion has already been discussed (pp44-45) and these proteinaceous cells could be precursor stages to the mucous gland cells. It is harder to visualise a system whereby polysaccharide units are interspersed between protein units if the alternating chain structure is valid. However, the effects of deamination on the mucous gland cells could also be explained if the acidic groups were attached to the amine groups present in galactosamine.

The finely granular or slightly reticulate appearance of the cytoplasm is conformable with the appearance of the secretion in some of the mucous gland cells seen in frozen sections. These granular mucous cells were felt to be less mature mucous cells (p36) and this too would

agree with the idea of their development from the Y cells and furthermore, both cell types showed pyroninophilia. Geographically they are suitably placed to be precursors, but no real intermediate stages between the Y cells and the mucous gland cells were ever detected and none of the young cells ever gave any indication of alcianophilia etc. This is not to say that a connection does not exist, it could be that the conversion to an acid mucopolysaccharide occurs very rapidly and has not been detected in the material examined.

LM.4(iv). Pigment/Protein Gland Cells

It was previously stated that the young cells Y could also be the precursors of the pigment/protein gland cells (PP). These cells are only seen in their entirety in frozen sections (Fig.10) although a nucleus is not shown in this figure. The cell, in frozen sections is ovoid or slightly elongated with finely granular cytoplasm, which displays a delicate orange/yellow colour (the matrix is coloured rather than the numerous granules, which appear only as specks), whether stained or not so this would appear to be the natural colour of the cell cytoplasm. These cells are found lying amongst the other gland cell types in the mantle, back and sides, being fairly equally distributed along the animal's length as can be seen in Histogram I (Volume II p.30).

The contents of the cell are negative to all the stains tried on frozen sections, so lipids, calcium and mucopolysaccharides can apparently be discounted; and an attempt to identify the pigment present was unsuccessful. Yellow/orange pigments in animals are likely to be carotenoids, flavines or flavones. Campion (1957, 1961) quotes carotenoids as being soluble in fat solvents, become dark blue in concentrated sulphuric acid and deep violet in iodine in potassium iodide. Flavines are usually inconspicuous, water soluble and stable in dilute acids, whilst flavones are water soluble, alcohol soluble, decolorised

by dilute acids, intensified in colour by alkalies, become green or brown in ferric chloride and form yellow or red lead salts that are insoluble.

The pigment in question is found to be soluble in fat solvents which would suggest a carotenoid, but it also becomes decolourised in concentrated sulphuric acid and was unaffected by iodine in potassium iodine. As for the tests for flavones, the pigment is decolourised by dilute acids and appears very slightly browner in the alkalies but it does not conform with the other tests. However, it is negative to all the tests for flavines, being conspicuous, insoluble in water and soluble in dilute acids. Thus the pigment could be either a carotenoid or a flavone, but it is probably a carotenoid since this pigment is quite common in molluscs, β- carotene and lutein being the most likely members of that group to be involved (Fox 1966). Fox stated that carotenoids are fat soluble polyenes which are synthesised 'de novo' only in the plant kingdom and thus the molluscs assimilate these pigments in their food and it becomes stored in the various cells of the body. It could well be that the pigment/protein cells of Arion hortensis represent such a storage cell. Interestingly, the newly hatched slug is without yellow pigmentation which would seem to confirm the suggestion that it is derived from the diet.

In normal circumstances, when the slug is allowed to move freely, without interference, the slime is colourless as mentioned in Id.p 3 and the pigment/protein cells do not appear to discharge. They do discharge in extreme conditions, for instance, when the slug is dropped into Susa, whence the slime rapidly becomes orange/yellow. Whether this is in some way a defence reaction conferring an unpleasant taste to the slime, thus deterring the predator is not clear. Graham (1957) stated that the protein secretions of certain Opisthobranchs such as Acteon, Ringula and Scaphander are actually toxic to smaller animals.

It may also be that the change from black to orange has a deterrent effect. The slime produced in times of attack tends to appear more viscous and this could be due to the protein present in it, or it could be due to a simple qualitative effect, that is, there is more present than is normal and it is easier to assess the viscosity. Campion (1961) agreed that the protein secretion was a defensive reaction but that the protein might serve to lower the surface tension of the secretion since that produced in defence was very watery. This appears not to be the case in Arion hortensis , but the amounts of protein produced by Helix aspersa were vast, and may confer a different property on the slime. Possibly the contents of these cells are discharged simply because of the violent contractions resulting from severe stimulation.

In the ordinary fixatives , especially Susa, followed by paraffin embedding, the pigment/protein cell type is not usually seen whole but as an elongated , fairly narrow 'shell', often angular(Fig.11) and perforated (Fig. 12). Outside the body, finely granular material can be seen but neither the granules nor the shells show any yellow pigmentation, due to the action of the acids in the fixative and of benzene (a fat solvent),the clearing agent, both of which affect the pigment as already described (p.55). Occasionally in Susa, the pigment/protein cells were seen whole(Fig.13) but the contents were very much contracted. The cells seemed to survive fixation in Alcoholic Bouin best although the cell contents were still rather contracted(Fig. 14). Dyson (1965) used Bouin's fixative and this could be why her protein cells generally appeared ribbon shaped and less distorted, although in other respects the descriptions are similar. In Fig.14 a nucleated pigment/protein cell can be seen and here and in other sections the nucleus tends either to be in a basal position or towards one side. In Fig.15 a partly granular, part shelled pigment/protein cell can be seen, this is presumably an effect of fixation and quite a number of these intermediate types are

present. The mean length of a 'shell' is 22.2μm and the mean width 16μm, with a range of 4.4μm to 81.4μm in length and 2.2μm to 48.4μm in width but the modal size is 22μm long by 11μm wide.

The staining reactions of the pigment/protein cell are given in Table IV:

Table IV : Staining Reactions of the Pigment/Protein Cells (PP)

Stain	Result	Conclusion
Mallory's Triple	Orange, occasionally purple or pink or all three.	Protein?
Weigert's Elastin	-ve	No elastin
Aldehyde fuchsin	V. pale pink	Some SO_4?
AF(pH1.45)-AB(pH2.5)	V. pale pink	No AMPS, some SO_4?
AB+Ehrlich's haematoxylin	-ve	No AMPS.
AB low pH	-ve	)
AB-CEC	-ve	)
AB-CEC-PAS	V. pale pink	)
Methylation+AB	-ve	) No AMPS
Methylation+ Saponification+AB	-ve	)
Colloidal Iron	-ve	)
Control	-ve	)
PAS	V. pale pink to -ve	Some slight periodate reactive substance.
PAS+Diastase	V. pale pink to -ve	" " " , not glycogen
Best's Carmine	-ve	Not glycogen
Southgate's Mucicarmine	-ve	-
TB 1% aq., frozen sections	Greenish	Protein?
TB pH 1.09	-ve	)
TB pH 3.0 to 4.0	-ve	) No AMPS
TB in 30% alcohol	-ve	)
Deamination pH 1.0 TB	-ve	No groups attached to amines.
Control	-ve	
RNase + TB	-ve	) No RNA
Control	-ve	)
Sulphation + TB	-ve	No neutral MPS

Stain	Result	Conclusion
Azure A pH range	All -ve	No metachromatic substance
HID & LID	-ve	No MPS
All enzymes	-ve	No sialic acid, hyaluronic acid or glucuronic acid present.
Tetrazonium	-ve	No SO_4
Ferric alum-Coriphosphine	Orangy/green or lime	Possibly some SO_4
Acridine orange-CTAC	All lime	No MPS
Millon's reagent	Red orange	Tyrosine present
Ninhydrin Schiff	Slightly pink	Some protein?
Heidenhain's haematoxylin	Black	Protein?
Ehrlich's haematoxylin + Eosin	Eosinophilic	Protein?
DMAB-nitrite	-ve	-
Bromophenol blue	Blue	Protein
Biebrich Scarlet		
pH5.0	Scarlet	)
pH6.0	Paler scarlet	) Protein but not
pH8.0	V. pale red	) basic protein
pH9.66	V. pale red	)
pH10.42	-ve to v. pale pink	)
MG-PY	Dark pink	) Pyroninophilic,
MG-PY + RNase	Dark pink	) except in the
MG-pyronin (Scott)	Dark pink	) presence of
MG-PY (2M $MgCl_2$)	-ve	) electrolytes.
Sudan Black B	Bluish	) Some small amount
Luxol Fast Blue MBS	-ve or v. pale blue	) of lipid.
Alizarin Red S	-ve	) No calcium
von Kossa	-ve	)

These staining reactions are very like those of the young cells Y except that deamination has no effect on the staining with toluidine blue and, in fact, only a green coloration is obtained with aqueous toluidine blue, not the blue. The PAS and lipid staining reactions are also weaker but there is still some very weak staining with

aldehyde fuchsin, weak orange fluorescence in coriphosphine and strong pyroninophilia. However, the protein reactions appear to be stronger since a brighter colouration is obtained with Millon's reagent and a pale colouration is obtained with the Ninhydrin-Schiff technique. Because of the similarity in the staining reactions of the Y cells and the pigment/protein cells it is more probable that the young cells Y give rise to the protein cells and that pigment is stored in them. The appearance of the pigment/protein cell base in Fig.14 is very like the young cell illustrated in Fig.9. It would seem that more and more protein is laid down in the young cells, resulting in the stronger protein reactions, possibly accounting for the colouration in toluidine blue. The reduction in PAS positivity and lipid staining could be due to a relative reduction in the amounts of those substances present.

In the course of development it is hypothesised that a duct to the surface is formed by the differential development of the side of the cell closest to the epidermis, and, that the nucleus becomes displaced to the side or the base of the cell where it is often bounded by some residual cytoplasm. The protein cells generally have no obvious cell neck distinct from the cell body, the cell simply tapering slightly towards the epidermis, although in Fig.15 there is a cell with a neck but this could be a fixation artefact.

The results seem, therefore, to indicate that the young cells Y give rise to protein gland cells in which pigment is secondarily stored, this last probably being a carotenoid, derived from the diet. Fox (1953) recorded the association of flavines and carotenoids with protein in many animals but did not mention flavones. However, Campion (1957) found that flavones were associated with protein in Helix aspersa.

There are many more young stages for the pigment/protein cells than for the mucous gland cells, but the latter are more numerous in the mature form. This would seem to suggest that the cell life is shorter f

the pigment/protein cells and that they need to be replenished more often. An alternative explanation is that the earlier developmental stages of pigment/protein cell formation take longer to pass through and are therefore present in the section.

It can only be conjectured that the pigment/protein gland cells originate in the connective tissue, for the youngest stages are the most deeply embedded, there being no evidence of younger stages in more superficial positions; however, this is only circumstantial evidence. Unfortunately, the regeneration experiment did not cast any light on the origin of the protein cells, for these experiments were limited by time. The mucous gland cells were developed before the protein cells and only the time of initiation of the former cells could be studied.

The mode and mechanism of discharge of these pigment/protein cells will be discussed later (LM.4(xii), pp 95-97).

LM.4(v). Melanin

The yellow pigment is not the only one present in the mantle, back and sides, since melanin is also present, and it is also in the grooves in the upper surface of the margins of the foot (Fig.1).

It is clearly visible below the epidermis and outlining the gland cells in the dermis (Fig.5). The back of the slug is grooved and there appears to be more melanin present at the base of the groove than at the top, and this agrees with the external appearance where the base of the grooves appears blacker. In sections the melanin is seen as small black granules distributed either singly or in clusters, these last being of varied form but often 'string-like' or stellate. However, there is no evidence of melanocytes in the light microscope material, and it would seem that the melanin is deposited as the melanocytes age and die. The melanin can be seen in greater detail in the mantle tissue fixed for electron microscopy (for instance, Plate 49), but melanocytes are more

common in the regenerating tissue, and will be considered at length in REM 2 (ii).

LM.4(vi). Mantle Groove Gland Cells

One last type of gland cell is present lining the ventral surface of the mantle (Fig.3). The outline of the individual cells is not at all clear and consequently their shape and size cannot be determined. This is because their contents stain very strongly and rather diffusely, thus obscuring the cell walls. The nuclei can occasionally be identified as slightly paler patches within the cells, and they tend to be central and quite large. Ducts, filled with secretion, are visible passing between the epidermal cells. The cells contain quite large granules, up to 1μm in diameter, which appear spherical. These granules are never seen externally so presumably the secretion undergoes some form of transformation during discharge.

Because these cells have rather a limited distribution they were not available for all the histochemical tests that were carried out. However, Table V gives the staining reactions that were obtained.

Table V : Staining Reactions of the Mantle Groove Mucous Gland Cells

Stain	Result	Conclusion
Mallory's Triple	-ve	-
Weigert's Elastin	-ve	No elastin
Ehrlich's haematoxylin + Eosin	Purple	Not proteinaceous but could show acid secretion since nuclei normally stain with this.
ABpH 2.5+Ehrlich's haematoxylin	Bright AB+ve	AMPS.AB displaces Ehrlich's
AB pH0.5	AB+ve	) Indicates some
AB pH1.0	AB+ve	) SO_4 groups.

Stain	Result	Conclusion
AB-PAS	Darker than AB almost indigo	) Carbohydrates) containing acidic) groups and oxidi-) sable vicinal) hydroxyl groups.
Methylation (mild)+AB	-ve	Simple or complex SO_4d MPS.
Methylation (mild) + Saponification	V. pale AB	Indicates some COOH present.
Methylation (Stoward) 4 hrs. + AB 1.0	AB+ve	This methylation not work.
Meth. (4 hrs.) + Sap. (Thionyl chloride) +AB pH1.0	+ve	" "
" " " +AB pH 2.5	+ve	" "
Meth. (6hrs.) +AB pH1.0	+ve	" "
" " +AB pH2.5	+ve	" "
Meth.(6hrs.)+Sap.2hrs. +AB pH2.5	+ve	" "
Colloidal Iron	Vivid blue	Weakly acidic MPS stain, strongly AMPS do not.
Control	-ve	
PAS	Dark pink	) Vicinal Hydroxyl
PAS+Diastase	Dark pink	) groups present but) not glycogen.
TB 1% aq.	Purple	Metachromatic MPS
TB pH 0.5	Blue/purple	)
pH 1.09	Purple	) Indicates SO_4d
pH 3.0	Rose purple	) MPS
pH 4.0	Rose purple	)
Deamination+TB pH1.0	-ve	) Metachromatic grps.) attached to amino) grps. that
Control+TB pH1.0	Purple	) eliminated.
RNase + TB	Rose purple	Indicates basophilia not due to RNA
Sulphation + TB	Rose purple	Unaffected
Azure A pH 0.5	-ve	(i) No staining at pH 0.5 if no SO_4 present
pH 1.0	Pale purple	(ii) But some SO_4 MPS masked until pH 3.5
pH 1.5	Slightly darker purple	
pH 2.4	Very purple	(iii) Many sialomucins
pH 3.4	Dark rose purple	stain at pH 3.0 & above.

Stain	Result	Conclusion
HID-AB pH 2.5	Some black, others AB+ve	Some SO_4d MPS are black. AB could indicate sialomucins or hyaluronic acid.
PA-HID-AB pH 2.5	Purplish black with some AB+ve staining though paler	Possibly indicates some neutral MPS present due to reduction of AB staining.
LID-AB pH 2.5	Pale purple to black, slight greenish tinge.	Stains most SO_4d and COOH AMPS. Some LID -ve, non-SO_4d MPS present too.
PA-LID AB pH2.5	All purplish black	Indicates some neutral MPS present since all stain blackish.
Neuraminidase	AB+ve	)
Control	AB+ve	) No sialic acid
Hydrolysis N/10 HCl+AB	AB+ve	) present.
Hydrolysis N/10 H_2SO_4+AB	AB+ve	)
Tetrazonium method	V. pale pink	Indicates some few SO_4 grps. present.
Hyaluronidase+TB	Purple	) No hyaluronic
Control	Purple	) acid
β-Glucuronidase+AB	+ve but slightly paler	) Indicates some
PA-β-Glucuronidase+AB	Pale AB+ve	) glucuronic acid
Control	AB +ve	) groups present
Heidenhain's haematoxylin	Blackish	Protein?
Performic acid + AB	Bright AB+ve	Indicates some S-S groups present.
DMAB-nitrite	-ve	No protein
MG-PY	Scarlet or dark red granules and pinkish cytoplasm	) Some poly-) anions stain
MG-PY + RNase	Bright pink	) but not RNA
MG-PY 2M $MgCl_2$	-ve	)
Alizarin Red S	-ve	No calcium
Lipid stains	Not tested	-

These results would seem to indicate that a mixture of substances is present in these gland cells, particularly when considering the combined Alcian Blue -PAS stain and the HID and LID techniques. The AB-PAS sequence shows that acidic groups and oxidisable vicinal hydroxyl groups are present in the mucosubstance, and the HID and LID techniques that the acidic groups are composed of both sulphate and carboxyl groups possibly together with some neutral mucosubstances. The return of some slight staining after saponification again indicates that carboxyl groups are present and the slight reduction of colouring after β-glucuronidase indicates that these are present in glucuronic acid units and not in hyaluronic acid or sialic acid units (since the respective enzymes had no effect). The staining in the low pH Alcian blue and tetrazonium technique indicates that some sulphate groups are indeed present and because the HID, LID and tetrazonium techniques reveal stronger coloration for these cells than for the large and small mucous gland cells it seems that more sulphate groups are present in the mantle groove mucous gland cells, assuming that stronger coloration can be interpretated to mean a quantitive variation. However, the lowest pH Azure A solution gives a negative result which is supposed to indicate that there are no sulphates, but whichever end groups are present they are stacked in such a way as to give metachromasia and it is not cellular RNA which confers the basophilia.

Since some neutral mucopolysaccharide is also present it would be expected that sulphation should confer metachromasia, but because metachromatic substances already existed in the gland cells it is not possible to decide whether sulphation had occurred.

It is suggested that the neutral mucosubstance granules are the less mature ones and that acidic groups are added later, because the material seen in the ducts (presumably ready for discharge) is always acidic in nature. On the other hand, it might represent some residue

within the cell that has undergone a change after discharge has occurred but one might expect the granules to be damaged if this were the case.

It would seem that a low sulphate chondroitin substance could be present, as opposed to chondroitin 4- sulphate and chondroitin 6-sulphate since these two would be susceptible to testicular hyaluronidase, but there would appear to be more sulphate present in the mantle groove mucous gland cells because of the stronger staining with HID and LID and with the tetrazonium technique. Again, the possibility of a totally new substance being present in these glands cannot be dismissed, for if a chondroitin substance is present it lacks the β-N-acetyl-D glucosaminidic residues normally attacked by hyaluronidase. Possibly less mature granules, in the form of a neutral mucosubstance with oxidisable vicinal hydroxyl groups are also present in this cell, but this explanation is only hypothetical and the periodate reactive groups could equally be present in the protein indicated by deamination. The application of this last technique results in a loss of staining which again suggests that the acidic groups are attached to amino groups but whether these groups form part of a protein core or chain or whether they are integrated in the galactosamine unit is not evident. The general protein reactions are negative although there is a slightly brighter coloration in the performic acid - alcian blue technique and this could indicate protein S-S groups, but this is only a subjective judgement and the performic acid could well be causing some other change. The granules stain slightly with Heidenhain's haematoxylin and Ehrlich's haematoxylin. Staining in the former can be taken to indicate protein containing carboxyl units but whether staining with Ehrlich's haematoxylin can be similarly interpreted is not known, for some other molecular configuration, such as the mucosubstance could be staining.

The comment made earlier (pp.42-43) about pyroninophilia and the effect of electrolytes on it applies here too, that is, that in any non-

electrolyte solution almost any polyanion can combine with pyronin when no electrolytes are present. It is interesting to note that the scarlet colo ration induced after the application of ordinary pyronin, is confined to the granules and the cell cytoplasm is pink which suggests that the polyanion involved in the scarlet staining is concentrated in the granule.

No calcium was found and the lipid tests were not applied to these cells.

Thus the mantle groove mucous cells contain granules composed of a sulphated and carboxylated mucosubstance, such as a low sulphate chondroitin substance. However, because of the staining with, for instance, the tetrazonium technique it would seem that more sulphate is present here than in the large and small mucous gland cells. In addition there is some periodate reactive substance present which may be a neutral mucosubstance and it is possible that the acidic material is added later to this. However, it is also possible that the periodate reactive groups are present in the protein, whose presence is indicated by the deamination results, for Kasten (1960) stated that protein might be stained by Schiff's reagent. It is also possible that the amine groups belong to a galactosamine unit and not to protein at all. Lastly, the periodate positive material could represent some residue within the cell left after discharge, but one might expect the granular structure to be damaged if this were the case and it was not.

How a neutral mucosubstance would fit into the molecule can only be guessed. Possibly neutral mucosubstances are attached to protein units (which may form a core or link units), and carboxyl and sulphate units are added later to the neutral mucosubstance. Alternatively, it may be a simple protein - acid mucopolysaccharide configuration without a neutral mucosubstance, the protein displaying the periodate reactive groups. The difficulty involved in interpreting histochemical

results is well illustrated above, for they are not entirely specific and can only indicate the types of substances and groupings present, not the actual secretion as a whole. Nor can they show the ordering of linkages within the substance.

No comment can be made about the origin of the mantle groove mucous gland cells except they are embedded in the dermis, but they are so closely applied to the epidermis that their origin could be from either.

LM.4(vii). Calcium and Glycogen Deposits

Before considering the glandular structures of the foot, two more features must be mentioned in connection with the dorsal surface.

One is the finding of deposits of calcium between the connective tissue fibres of the mantle. These deposits lie deep in the tissue well below the glandular layer (Fig.16) and stain crimson in Alizarin Red S. They are only found in unfixed frozen sections which suggests that the calcium deposits are soluble in the acids normally present in the fixatives. This in turn suggests that the calcium is deposited in the form of calcium carbonate. A region of diffuse red colouring is present round the calcium deposits indicating either that the acidity of the aqueous dye solution is sufficient to cause some dissolution, or else that some calcium phosphate is present, prior to deposition. The calcium is deposited in the form of spherical granules in fairly regular groups, almost as if they have been deposited in cells which have subsequently died, for cell walls could not be detected, mainly because of the halo of red staining material round the deposits (Fig.17). These calcium deposits are believed to represent the remnants of the internal shell (Hyman 1967).

The second finding is that of glycogen deposits, also in between the connective tissue fibres of the mantle, but also in the

tissues of the back and sides. It is detected by PAS (with diastase control) and Best's carmine. The glycogen does not appear to be deposited in actual cells but rather to be confined in the spaces between the connective tissue fibres, because after dissolving out the glycogen no nuclei or other structures could be detected. The distribution of this material is far greater than for the calcium, but an individual calcium deposit is larger than one of glycogen. These deposits are also seen in the electron microscope study of the mantle and will be discussed later (EM2.4(vi)pp 183). Here they appear to be bounded by a band of collagen but they did not seem to be cellular. These glycogen deposits are thought to be stores of excess food material which can be used in times of dearth. For instance, when humidity is low, such as in the summer and in cold weather, the slug cannot venture far in search of food and rather than starve it can draw on its reserves of glycogen, the substance which most readily yields energy supplies. This is by no means a unique finding and was described as long ago as 1885 by Blundstone in Helix and Anodonta. He found glycogen in the mantle and other regions of connective tissue in Anodonta and found it to be confined in what he called large vesicular cells. They were also seen in Helix. By treating the tissue with water the glycogen was dissolved out and a nucleus and a small amount of cell cytoplasm was visible. In Helix the glycogen was found to be particularly associated with lacunar spaces and mesenteries and Blundstone believed the glycogen maintained the 'specific gravity and nutritive quality of the blood'. Thus glycogen has been detected before, but no vesicular cells could be detected in Arion hortensis.

The next section will consider the glandular structures of the foot.

LM.4(viii). The Glandular Structures of The Foot Sole

The foot sole bears, superficially, numerous gland cells.

(a) Mucous Gland cell A

The first type of cell (A) is a mucous cell which is elongated and narrow and the bulk of the cell lies in the dermis with the 'duct' passing between the epidermal cells. The mean length of these gland cells is 20.6μm, with a mean width of 9.4μm, but the range is 9.3μm to 53.3μm long and 7.3μm to 21.3μm wide. The modal size, however, is 13.3μm long by 8.0μm wide.

They are found throughout the length and breadth of the foot sole, as can be seen in Histogram II, (Volume II.p31), although they tend not to be present at the very front edge of the foot. The largest numbers are found in the first third of the body, which might be expected in an animal which progresses on a slime trail, and the cilia of the foot spread the mucus across the whole sole. The appearance of these mucous cells is shown in Fig.18 and it can be seen from this figure that there is no distinction between the neck and cell body. Furthermore, the small basal nucleus showing some granules of chromatin can be seen. The contents of the cell may appear reticulate or finely granular, but this last stains more deeply than the reticulate material.

The staining reactions of this secretion is given in Table VI.

Table VI : The Staining Reactions of The Mucous Gland Cell A, of The Foot Sole

Stain	Result	Conclusion
Mallory's Triple	Blue	Mucus
Weigert's Elastin	-ve	No elastin
Aldehyde fuchsin (pH1.45) -AB pH2.5	AB+ve with AF+ve base	Indicates some weakly SO_4 material at base but mainly COOH
AB pH 2.5 + Ehrlich's haematoxylin	AB+ve, appear very full in frozen sections	AMPS present

Stain	Result	Conclusion
AB pH0.5	AB+ve	) Should not get
AB pH1.0	AB+ve	) staining at low pH if COOH groups only present.
AB-CEC (0.2M)	AB+ve	) Suggest COOH
AB-CEC (0.3M)	V. pale to -ve	) present or SO_4
AB-CEC (0.4M)	-ve	)
AB-CEC (0.2M)-PAS	AB+ve	) Suggests SO_4 or
AB-CEC (0.5M)-PAS	-ve	) COOH present not vic.glycols.
Methylation (mild)+AB	-ve	Shows simple acid or complex SO_4 MPS.
Meth. (mild)+Sap.+AB	-ve	Suggests SO_4 present but Sap. not always work.
Meth.(4hr.)ThCl+ABpH1.0	+ve	) Meth. not work
" " " " 2.5	+ve	)
Meth.(4hr.)ThCl+Sap.	V. pale AB, paler than	)
2hrs.+AB pH1.0	for Meth. alone.	)
Meth.(4hr.)+Sap.(2hr.)	V. pale AB+ve though	)
+AB pH2.5	darker than above.	) See text.
Meth.(6hr.)ThCl+	AB+ve	)
AB pH1.0		)
Meth.(6hr.)ThCl+	AB+ve	)
AB pH2		)
Colloidal Iron	CI+ve base	Acidic groups present.
Control	-ve	
PAS	-ve	No oxidisable vic. glycols present.
PAS-Diastase	-ve	No glycogen
Best's Carmine	-ve	" "
Southgate's mucicarmine	-ve	
1% aq. TB	Blue/purple	Metachromatic subst. present.
TB pH0.5	-ve	Metachromasia suppressed
TB pH1.09	-ve	at low pH. Suggests that
TB pH3.0	V. pale mauve	at low pH when COOH grps.
TB pH4.0	Pale rose purple	not dissociated get no metachromasia.
TB in 30% alcohol	Pale purple	
Deamination+TBpH1.0	-ve	) pH too low to
Control	-ve	) reveal staining.
Deamination+TB 1%aq.	-ve	) Metachromatic grps
Control	+ve	) attached to NH_2

Stain	Result	Conclusion
RNase +TB	Rose purple	Basophilia not due to RNA
Azure A pH range	-ve	No azurophilia, so no SO_4 present.
HID-ABpH2.5	Bright AB+ve	) No SO_4 MPS
PA-HID -AB pH2.5	AB+ve	)
LID-AB pH2.5	Vivid AB+ve	) LID-ve, non SO_4d
PA-LID pH2.5	AB+ve	) AMPS
Neuraminidase +AB	AB+ve	)
Control	AB+ve	) No sialic acids
Hydrolysis N/10 HCl+AB	AB+ve	) present
Hydrolysis N/10 H_2SO_4+AB	AB+ve	)
Tetrazonium	-ve	No SO_4
Hyaluronidase +TB	Purple	) No hyaluronic acid
Control	Purple	)
PA-β-Glucuronidase +AB	-ve	) Glucuronic acid
β-Glucuronidase+AB	-ve	) groups present.
Control	AB+ve	)
Ferric Alum-Coriphosphine	-ve	No Sulphomucins
Acridine Orange-CTAC	-ve to all treatments	No hyaluronic acid chondroitin SO_4 or heparin.
Millon's reagent	-ve	No tyrosine
Ninhydrin-Schiff	-ve	No protein
Heidenhain's haematoxylin	-ve	No protein
Performic acid-AB-PAS	AB+ve	) No S-S groups
Control	AB+ve	)
DMAB-nitrite	-ve	No tryptophan
Mercuric bromophenol blue	-ve	No protein
Biebrich Scarlet, pH range	-ve	No basic protein
MG-PY	Dark pink, rarely red	) Some polyanion
MG-PY +RNase	Dark pink, rarely red	) staining but not
MG-PY (2M $MgCl_2$)(Scott)	-ve	) RNA
Sudan Black B	-ve	) No lipid
Luxol Fast Blue	-ve	)
Alizarin Red S	-ve	) No calcium
von Kossa	-ve	)

These results indicate that a mucosubstance is present but of

a different character to that found in the mucous gland cells of the dorsal surface.

The difference between the large and small mantle gland cells and the A sole gland cells lies principally in the reactions to HID and LID, fluorescent techniques, Azure A and toluidine blue. All these are techniques which indicate the presence of sulphated mucopolysaccharides and, in all instances, negative results were obtained for the A cells. In fact, in Azure A no metachromasia is shown at any of the pHs examined and the reason for this is not known since metachromasia is displayed in toluidine blue from pH3.0 upwards, although more weakly than that by the dorsal mucous gland cells. This staining above pH3.0 is interesting because glucuronic carboxyl groups have a pK of approximately 3.1 to 3.3 and would be completely undissociated at pHs below 3.0 and hence no metachromasia would be displayed. That glucuronic carboxyl groups are present is confirmed by the effect of β-glucuronidase which destroys staining. Hyaluronidase and neuraminidase, together with hydrolysis, have no effect on staining so the carboxyl groups do definitely seem to be associated with glucuronic acid groups and not with sialic acid or hyaluronic acid, and this last is further confirmed by the negative result with the Acridine Orange -CTAC.

The LID technique, shows that LID-negative, non-sulphated mucopolysaccharides are present and the HID technique also confirms that no sulphated groups are present.

There are two unusual results, however. One is that staining is obtained in very low pH alcian blue which is supposed only to show sulphates, because of the pK of the carboxyl groups. This staining was obtained even when blotting the sections dry after staining, a technique described by Lev and Spicer (1964) who found that the pH of the water on the slide (used for washing) was sufficient to raise the pH of the dye solution to stain the carboxyl groups. Secondly, the results obtained

with the methylation -saponification method are difficult to explain. Mild and drastic methylation techniques remove the alcianophilia but if carboxyl groups are present, as is suspected, then one would expect staining to return after saponification and this did not happen. However, saponification is unreliable as a technique (Quintarelli, Scott and Dellovo 1964).

A more unexpected result is obtained with Stoward's (1967) method of methylation which does not eliminate alcianophilia (possibly because the time intervals were not long enough), but the use of the technique of methylation plus saponification leads to a reduction in staining. This was also noted by Quintarelli, Scott and Dellovo (1964) but they gave no explanation for it. Possibly the alcoholic potassium hydroxide is causing some further breakdown of the secretory product reducing the number of end groups available for combination with the dye. This could be a 'normal' effect of potassium hydroxide but in typical experimental conditions the sections are only briefly exposed to the solution because it causes detachment of sections. Using Stoward's method of cold potassium hydroxide the sections can be exposed for much longer periods, and this may cause decay of the secretion and result in weaker staining.

However, it would seem that most of the results indicate the presence of an acid mucosubstance containing glucuronic acid and from Spicer, Horn and Leppi (1967) the substance most likely to be involved is chondroitin.

At the base of the cell, around the region of the nucleus, the secretion seems to show a slightly different configuration, for it stains differently in colloidal iron and aldehyde fuchsin -alcian blue sequence. In colloidal iron this difference takes the form of a stronger staining reaction, whilst in the dual stain sequence the base of the cell shows aldehyde fuchsin staining the rest of the cell showing alcianophilia.

The reason for this is not known, the nucleus itself may even be staining thus; or there could be a residue of some slightly different material which possibly contains some sulphate groups. These groups could also be associated with the protein whose presence is indicated by deamination.

There is no evidence of any periodate positive materials in the secretion, or of lipid or calcium. None of the protein tests was positive although the results after deamination would suggest its presence, but it is possible that the protein is masked by the mucopolysaccharide groups, particularly if it forms a core. However, the amine units may be part of the galactosamine unit.

Thus the secretion appears to be chondroitin possibly associated with protein.

LM.4(viii).

(b) Mucous Gland Cell B

A second type of gland cell was found in the foot sole and in the tissue forming the lower edge of the peripodial groove (Fig.3 & 19), being named mucous gland cells B. This groove is found between the tissue of the side of the animal and that of the sole margin and it runs the length of the body (Fig.1). The mucus from the cells located in this region would lubricate the peripodial groove.

The B cells are often deeply embedded in the dermis of the sole (Fig.20) but tend to adopt a more superficial position in the region of the peripodial groove, and they are often found in clusters particularly in this last region.

Their distribution along the length of the animal is shown in Histogram II (Volume II, p31) which indicates that they are most common just behind the head, like the mucous gland cells A of the foot, and decrease in numbers before the tail.

The cells are ovoid or slightly elongated along the axis perpendicular to the surface. In this same orientation the beginnings of a duct can be observed (Fig.19), but these are never seen to reach the surface. The duct is much narrower than the cell body and within this is a large, ovoid and often central nucleus, showing dark patches of chromatin, although it was not possible to identify a nucleolus. It seemed as if the nucleus was supported by cytoplasmic bridges which in turn were attached to the cell wall, but this could well be an illusion since the whole of the cell body presented a reticulate appearance and the reticulum could be misinterpreted as cytoplasmic bridges. The reticulum is believed to reflect some internal structure in the cell, not the actual secretion, and nodules of secretory material appeared attached to this reticulum. The mean length of these gland cells is 18.1μm and the mean width 18.4μm. The range is 5.5μm to 41.8μm long and 8.8μm to 48.4μm wide. The modal size is 17.6μm long by 13.2μm wide.

Table VII gives the staining reactions of the mucous gland cells B.

Table VII : Staining reactions of the Mucous Gland Cells B

Stain	Result	Conclusion
Mallory's Triple	-ve	
Weigert's Elastin	-ve	No elastin
Aldehyde fuchsin (pH1.45) -AB pH2.5	Shadowy AB+ve	No SO_4 present
AB pH2.5 + Ehrlich's haematoxylin	Stains pale AB+ve & slightly purple in haematoxylin	Acidic MPS + some substance that stains with haematoxylin
AB pH0.5	-ve	) Suggests COOH
AB pH1.0	V. pale AB	) groups
AB-CEC 0.2M	+ve	) Suggests COOH
AB-CEC 0.3M	-ve	) groups
AB pH 2.5-PAS	AB+ve	) AB+ve in preference
AB-CEC(0.2M)-PAS	AB+ve	) to PAS in dual stain
AB-CEC(0.5M)+PAS	Pink tinge	Some slight amount of periodate substance present

Stain	Result	Conclusion
Methylation (mild)	V. pale AB+ve	Methylated for shorter time than usual.
Methylation + Sap.	V. pale AB+ve	" " "
Meth. 4hr. (ThCl) +AB pH1.0	AB+ve	)
Meth. 4hr. (ThCl) +AB pH2.5	AB+ve	)
Meth. 4hr. (ThCl) +Sap. 2hr. pH1.0	V. pale AB+ve, paler than for meth. alone	) See text
Meth. 6hr. (ThCl)	As for 4 hrs	)
Meth. 6hr. (ThCl)+Sap.	As for 4 hrs	)
Colloidal Iron	Dark blue	) Acidic groups
Control	-ve	) present
CI-PAS	CI+ with pinkish tinge	Acid groups and vic. glycols present.
PAS	Pink	) Some periodate
PAS-Diastase	Pink	) substance present) but not glycogen.
Best's Carmine	-ve	No glycogen
Southgate's Mucicarmine+ Ehrlich's haematoxylin	Purplish	Stain in Ehrlich's
1%TBaq.	V. pale purplish to -ve	Some evidence of metachromatic substance but not always.
TB pH0.5	-ve	) Metachromatic MPS
pH1.0	-ve	) present, but
pH3.0	V. pale purplish	) staining suppressed
pH4.0	V. pale purplish	) at low pH.
TB 30% alcohol	Pale purple	-
Deaminate-TB pH3.0	-ve	) Indicates NH_2
Control	Purplish	) groups present.
RNase+TB	Rose purple	) Slightly brighter
Control	Purple	) suggests RNA been) suppressing some) staining.
Sulphation+TB	Some rose specks	Brighter staining, may have SO_4d some neutral MPS.
Azure A pH range	-ve	No azurophilia
HID-AB pH2.5	AB+ve	) No sulphated MPSs
PA-HID-AB	AB+ve	)

Stain	Result	Conclusion
LID-AB pH2.5	AB+ve	)
PA-LID-AB	AB+ve but slightly darker than above	) LID -ve non-SO_4 AMPS
Neuraminidase +AB	AB+ve	)
Control	AB+ve	) No sialic acid
Hydrolysis N/10 HCl	AB+ve	) present
Hydrolysis N/10 H_2SO_4	AB+ve	)
Tetrazonium method	-ve	No sulphate
Hyaluronidase +TB	Purple	) No hyaluronic acid
Control	Purple	)
PA-β-glucuronidase +AB	-ve	) Glucuronic acid
β-glucuronidase	-ve	) present
Control	+ve	)
Ferric alum - Coriphosphine	-ve	No sulphomucins
Acridine orange-CTAC	All -ve	No sulphomucins or hyaluronic acid
Acridine Orange	-ve	No sulphomucins
Millon's reagent	-ve	No tyrosine
Ninhydrin Schiff	-ve	No protein
Heidenhain's haematoxylin	Blackish	Possibly protein but also stains in Ehrlich's.
Performic acid-AB	AB+ve	) No S-S groups
Control	AB+ve	)
Performic acid-AB-PAS	AB+ve	No S-S groups
DMAB-nitrite	-ve	No protein
Mercuric bromophenol blue	Diffuse blue in this region	) Protein?
Biebrich Scarlet pH4.3	Reddish tinge	) Protein?
Biebrich Scarlet pH5.0-10.42	-ve	))
Methyl Green-Pyronin Y	Pale pink, some reddish	) Some polyanion
MG-PY+RNase	Pale pink, some reddish	) staining but not
MG-PY (2MMgCl_2) Scott	-ve	) RNA
Sudan Black B	-ve	) No lipid
Luxol Fast Blue MBS	-ve	)
Alizarin Red S	-ve	) No calcium
von Kossa	-ve	)

These results are very like those obtained for the mucous gland cells A, seen in Table VI, particularly with reference to the unusual methylation-saponification results, lack of azurophilia, suppression of metachromasia in toluidine blue at low pH, fluorescent stains, HID and LID and Alcian blue -CEC. Moreover, hyaluronidase and neuraminidase had no effect whilst β-glucuronidase removed the alcianophilia. This suggests that the mucous gland cells contain chondroitin without sulphate groups. Also, deamination prevents staining with toluidine blue indicating that protein could be present, as already discussed with reference to mucous gland cells A or else the amines are part of a galactosamine unit.

However, there are some differences between A and B. A periodate positive substance is present in the B cells, possibly in the form of a neutral mucosubstance together with what could be some protein. These two substances were present in the mantle groove mucous gland cells (LM.4(vi) pp.61-67), and here it was suggested that they were precursor substances, acidic groups being added later to the neutral mucosubstance. Moreover, they showed staining reactions with Ehrlich's and Heidenhain's haematoxylin as do the B gland cells of the foot. Thus, the same interpretation is suggested here, that is, that the protein and neutral mucosubstance are precursors to the formation of chondroitin and the glucuronic acid groups are added later to the neutral mucosubstance, masking the protein. Alternatively the protein may be the periodate reactive substance and no neutral mucosubstance may be involved. The presence of a mixture of substances is possibly confirmed by the application of the colloidal iron -PAS sequence where a dual staining reaction was obtained. After the Alcian blue -PAS sequence, however, only alcianophilia is observed and PAS positivity is visible only when the staining of the carboxyl groups is suppressed by the addition of 0.1M magnesium chloride. The reason for this variation is unknown.

Alcianophilia is generally weaker in this cell type than in

those types described previously.

Although the presence of a neutral mucosubstance was suspected sulphation had very little effect on the mucous gland cells B, except that one or two 'spots' of rose purple metachromasia were detected and these might represent areas of less mature secretion which has become sulphated. On the basis of the tests employed it is not possible to decide whether protein or neutral mucosubstance is the periodate substance present but large amounts of protein are present as evidenced by the effect of RNase. Its application induced rose purple metachromasia throughout the cell, suggesting that there are quite large amounts of RNA present. This RNA, or probably its associated amino acids, seems to be suppressing metachromasia (Andersen, et al. 1970).

No lipid was evident in the secretion.

Because of the finding of considerable amounts of RNA and protein, and the fact that the cell is seen to possess a large nucleus but only small ducts which never seem to reach the surface, it is suggested that the B cells are an immature stage of the mucous gland cells A, and that any variation in staining between the two types can be explained in terms of the immaturity of the secretion within the B cells. Moreover, the A and B cells show a similar distribution (Histogram II, Volume II, p.31). It is suggested that as more secretion is produced the cell elongates to produce a duct (the beginnings of which are seen in Fig.19) which eventually passes between the epidermal cells, the nucleus becoming displaced to the base of the cell. By this time the secretion has matured so that the protein can no longer be detected except by deamination, and alcianophilia and metachromasia are stronger, perhaps suggesting the presence of more glucuronic acid carboxyl groups. The B cells would then quite closely resemble the A cells and a resemblance can be seen in Figs 18 & 19. Contraction of the surrounding muscle fibres would cause compression of the cell body which would effect

discharge, whilst the loss of the cell contents and possible elasticity of the cell walls would lead to the shrinkage in size which must occur to give the change in shape between B and A. Fig.18 shows a nucleated A cell which may represent an exhausted cell after discharge and possibly before recharging with secretion.

However, if the B cells represent a younger stage of the A gland cells, there are more young stages of this gland cell type present than there are for those of the dorsal surface. This might indicate that these gland cells have a shorter life than those of the dorsal surface and therefore need replacing more often. This could be related to the fact that these cells need to produce more mucus to keep the foot sole lubricated and the rapid turnover may exhaust the cells more quickly. This is possible since the mucus trail, produced by the pedal gland and these mucous gland cells, is essential if locomotion is to occur. Alternatively, or possibly also, it may take longer for the immature stage to be passed through.

One last pointer to the relative immaturity of the B gland cells comes from their close resemblance to the pedal mucocytes, a resemblance of appearance and secretion (Fig.27 and 28). This is not surprising since the gland cells must be producing mucus for an identical purpose (lubrication in locomotion) and therefore a similar secretion and cell apparatus might be expected. In the pedal gland the mucus which is discharged into the duct is metachromatic and presumably this is the mature secretory substance. This secretion has identical staining reactions to the material seen in the A mucous gland cells. The lateral pedal mucocytes themselves resemble the B gland cells closely and show reduced metachromasia due to the presence of large amounts of RNA and protein, as verified by the ultrastructure of this gland, for instance, Plate 15. This secretion collects in the cells and passes down the long ducts to be discharged into the duct proper of the pedal gland itself. Thus the metachromatic secretion seen in the A gland cells

could well be the mature mucus produced from the cell body B (equivalent to a pedal mucocyte). Occasionally in longitudinal sections prepared for the light microscope it is possible to see gland cells with cell bodies and long ducts filled with a metachromatic secretion. It is suggested that these cells have filled with the mature secretion but have not yet discharged.

Fig.20 shows what is believed to be very young stages in the development of the A/B mucous gland cells. These cells are small and spindle shaped being interspersed between the cell bodies of the A/B cells. They contain large, spherical nuclei with distinct chromatin granules but the cytoplasm shows no obvious staining reactions. These could well represent expanded fibroblasts from the connective tissue which eventually develop to give the B gland cells. However, this can only be supposition since it is difficult to produce a sequence of events from the static picture presented in a tissue section.

LM.4(viii)

(c) Mucous Gland Cell C

The last type of gland cell in the foot, C, occurs deeply embedded, often slightly below the main glandular layer (Fig.20). It is composed of a distinct cell body and an extremely long and thin duct leading to the epidermis. The ovoid cell body often has short processes leading from it rather like the pseudopodia that are seen in Amoeba (Figs.21 and 22), but whether these are natural or induced by fixation is unknown.

Fig.22 shows the nature of the duct, it is always very narrow, even as it leaves the cell body and may taper even more towards the epidermis so that at the surface it is only as wide as one of the granules it contains.

As can be seen from these drawings the nucleus is very large

(larger than for any other gland cell types), and usually centrally placed in the cell where it may occupy as much as half of the volume. Invariably it displays a distinct nucleolus and a few patches of chromatin and, because of the presence of this large nucleus and nucleolus, it would seem that this cell is very active.

It is never very numerous in any one transverse section of the sole and its distribution from head to tail is given in Histogram II, Volume II, p.31. It is most common at the front of the animal and becomes very scarce towards the tail.

The length of these C type gland cells varies between 4.4µm and 132µm (including 99µm of duct), and 4.4µm to 52.8µm wide. The mean length of these gland cells is 19.7µm and the mean width 18.5µm, but the modal sizes are 17.6µm by 26.2µm, 11µm by 13.2µm, 8.8µm by 13.2µm and 8.8µm by 17.6µm. As can be seen from these modal measurements the longest axis of the cell body is parallel to the epidermal surface.

Figures 21, 22 and 24 show the uniformly sized, spherical granules which fill both cell body and duct. Normally, they are up to 0.75µm in diameter but occasionally cells have ones up to 1µm as shown in Fig.23 whose scale is the same as in Fig.24. These larger granules stain less strongly than the smaller granules and are associated with ductless C gland cells. This lack of ducts suggests that these gland cells and their granules are less mature and not yet ready for discharge. The large granuled cell types are very deeply seated and are seen only very rarely and hence a comprehensive list of their staining reactions is not available.

The staining reactions of the common small granuled gland cell C are given below, in Table VIII.

Table VIII : The Staining Reactions of The Gland Cell C

Stain	Result	Conclusion
Mallory's Triple	Purple pink	
Weigert's Elastin	Dark purple/black	Elastin-like or some type of MPS stain.
Aldehyde fuchsin	Purple	) SO_4d groups
Aldehyde fuchsin (pH1.45) -Alcian blue (pH2.5)	Purple	) present)
Alcian blue-Ehrlich's haematoxylin	AB+ve	AMPS present
AB pH0.5	AB+ve	) SO_4 groups present
pH1.0	AB+ve	) In all cases of
pH2.5	AB+ve	) AB+ in this cell) staining always) greener.
AB-CEC (0.2 M $MgCl_2$)	AB+ve	) Indicates SO_4 or
AB-CEC (1.0M$MgCl_2$)	AB+ve	) some other non-) COOH substance) present.
AB-CEC(0.2M$MgCl_2$)-PAS	Some C cells bright pink, others AB+ve. No dual staining	) Some cells) periodate reactive) others alciano-) philic (but not
AB-CEC (0.5M$MgCl_2$)-PAS	As above.	) carboxylated).
Methylation (mild)+AB	Pale AB+ve	)
Methylation (mild)+Sap.	Pale AB+ve	)
Meth.(4hrs.ThCl)ABpH1.0	-ve to v. pale green	)
" " " ABpH2.5	-ve to v. pale green	)
" " " +Sap.2hrs. ABpH1.0	Pale green	) Methylation seems) to reduce staining
Meth.(4hrs.ThCl)+Sap. 2hrs.+AB pH2.5	Turquoise	) a little & Sap.) has no effect,
Meth.(6hrs.ThCl)+ABpH1.0	V. pale green	) except in last two
" " " +ABpH2.5	V. pale green	) cases.
Meth.(6hrs.ThCl)+Sap.2hrs. ABpH1.0	Slightly greener AB+ve	))
Meth.(6hrs.ThCl)+Sap.2hrs. ABpH2.5	Brighter AB+ve	))
Colloidal Iron	Green blue	) Acidic groups
Control	-ve	) present
PAS	Bright pink in normal size granules. Pale pink in large size granules.	)) Periodate) substance present) but not glycogen.
Control (Diastase)	Bright pink in small granules	))
Southgate's mucicarmine +Ehrlich's haematoxylin	Purplish	Substance staining in Ehrlich's

Stain	Result	Conclusion
1% aq. TB	Royal blue	) No metachromasia
TB pH0.5	Green blue	)
TB pH1.09	Royal blue	) Blue colouration
TB pH3.0	Royal blue	) somewhat suppressed
TB pH4.0	Dark blue	) by low pH.
TB in 30% alcohol	Royal blue	-
Deamination+TBpH1.0	Royal blue	) Apparently no NH_2
Control	Royal blue	) groups available.
RNase+TB	Blue	) Apparently no RNA
Control	Blue	) or associated
		) protein suppressing
		) staining.
Sulphation+TB	Royal blue	No neutral MPS
Azure A pH0.5	V. pale blue	No metachromasia
" A pH2.0	Slightly darker blue	Blue staining slightly suppressed by low pH.
" A pH3.0-5.0	Darker blue	" " " "
HID-AB	Purplish black	) SO_4d MPSs present
PA-HID-AB	Purplish black	)
LID-AB	Purplish	) SO_4d or COOHd MPSs
PA-LID-AB	Purplish	) present or elastin
Neuraminidase -AB	AB+ve	)
Control	AB+ve	) No sialic acid
Hydrolysis N/HCl	AB+ve	)
Hydrolysis N/H_2SO_4	AB+ve	)
Tetrazonium method	Pink/red granules	SO_4 groups present
Hyaluronidase+1%TB	Blue	) No hyaluronic acid
Control	Blue	)
PA-β-Glucuronidase	AB+ve	)
β-Glucuronidase	AB+ve	) No glucuronic acid
Control	AB+ve	)
Ferric Alum-Coriphosphine	Orange	Suggests SO_4 groups
Acridine Orange only	Orange	Suggests SO_4 groups
Acridine orange CTAC	1) Vivid orange	Hyaluronic acid
	2) Vivid orange	Chondroitin SO_4 & heparin
	3) Vivid orange	Heparin
Millon's reagent	Slightly pink	Some tyrosine
Heidenhain's haematoxylin	Blackish	Protein or some acidic grouping.

Stain	Result	Conclusion
Performic acid-AB	AB+ve	) Suggests there may
Control	AB+ve but greener	) be some S-S grps.
Performic acid+AB+PAS	Some cells AB+ve Some pink	Gives dual staining as in AB-CEC
DMAB-nitrite	-ve	No tryptophan
Bromophenol blue	-ve	No protein
Mercuric BPB	-ve to v. pale blue	Slight protein reaction
Biebrich Scarlet, pH range	-ve	No basic protein
Methyl Green-Pyronin	Bright pink	) Some polyanion
MG-PY (Scott)	Mauve pink	) staining which
MG-PY (RNase)	Purple pink	) unlike that in gland
MG-PY(2M$MgCl_2$)	Purple green	) cells, but not RNA.
Sudan Black B	V. pale blue/black	) Some lipid
Luxol Fast Blue MBS	Pale greenish blue	) present
Alizarin Red S	-ve	) No calcium
Modified von Kossa	-ve	)

The substance produced by these cells is quite unique in the gland cells of this slug, in that it stained with Weigert's Elastin stain. What this means is not known, but Puchtler and Sweat (1960) stated that their technique stained cartilage and some mucins as well as elastin, and it seems likely that the stain is visualising an unusual mucin. Ewer and Hanson (1945) described a Weigert's elastin positive mucoprotein in Helix aspersa but found none in Arion ater, so this type of substance has been found in other molluscs although not in the more closely related Arion ater. Ewer and Hanson described the substance as a mucoprotein and the secretion in gland cell C does give some indications of protein with the positive reaction to Millon's reagent and the weakly positive reaction to bromophenol blue. The results with Ehrlich's and Heidenhain's haematoxylins may indicate the presence of protein or perhaps some other sort of molecular grouping as discussed before (LM.4 (vi)p.65). The protein groups could be masked by the presence of acidic groups in the secretion. However, this mucoprotein, if present, is unlike that found in the M, m, and A and B mucous gland cells, for

deamination does not remove the staining and so it would seem that there are no amine groups susceptible to deamination. The results of the staining techniques show that sulphate groups are present (HID, LID, aldehyde fuchsin, Alcian blue-CEC 1.0 M $MgCl_2$, Geyer's tetrazonium technique and the fluorescent techniques) but these groups are either not 'stacked' in such a way as to confer metachromasia in toluidine blue or Azure A, or else the protein present in the secretion is suppressing the metachromasia. However, if this last explanation is correct one might expect deamination to reveal metachromasia.

What constitutes the 'mucus' part of the mucoprotein is also not known since it is not attacked by glucuronidase, neuraminidase or hyaluronidase and it is more resistant to methylation than the other mucosubstances described in Arion hortensis.

The results with methyl green -pyronin Y-CEC (Scott 1967) illustrate further the difference between this mucosubstance and the other since a purple-green coloration is maintained even after the addition of a 2M magnesium chloride solution. The greener coloration in the alcian blue also reflects this difference.

Thus, the substance is seen to be a sulphated mucoprotein, but the sulphate groups do not apparently confer metachromasia although this could be an effect of the protein present. Some lipids also seem to be associated with the secretion.

It is the results of the alcian blue -CEC-PAS which are particularly unusual. In ordinary PAS techniques the secretion is found to be PAS positive in all the C gland cells so that oxidisable hydroxyl groups appear to be present, but whether these are associated with the protein or the mucoid part of the secretion is not clear. The large granuled C gland cells show much weaker PAS positivity. In the dual technique of Alcian blue -PAS with the addition of electrolytes some of the C gland cells stain with PAS, whilst others take up alcian blue

but they never stain with both. Although the C gland cells show these two staining reactions there is no obvious physical difference between the two types of cell, so that the large granule cells cannot be associated with the periodate positive cells and the alcianophilic granules with the smaller ones. Furthermore, ducts can be seen to the surface filled with both PAS positive granules or alcianophilic granules. Yet, although these staining results clearly reflect some chemical difference between the two types of C gland cells they are never shown by any other staining techniques, and without the electrolyte addition all these gland cells stain a turquoise green with alcian blue. It is possible that in the cells staining with PAS there are only a few sites available for combination with alcian blue and the electrolytes compete with the dye molecules for these sites, thus displacing the alcian blue molecules so that only periodate staining occurs. This competition for dye binding sites is a normal feature of the alcian blue -CEC technique, but if there are only a few sites available the electrolytes may completely supplant the dye molecules. The periodate reactive substance does not appear to be a neutral mucosubstance because sulphation does not induce metachromasia and so it is not possible to identify accurately any of the components of the secretion.

The developmental stages of this secretion are uncertain though it would seem that the weakly periodate reactive large granule C cell material is the least mature stage for it has never been identified in cells with ducts. It is proposed that these granules condense in some manner, for instance, by loss of water, to give the small, more strongly periodate reactive granules. Lastly, it is suggested that the alcianophilic substance is added to the periodate reactive granules to give the alcianophilic granules but whether it is the sulphate groups that confer alcianophilia is not clear, for they certainly do not produce metachromasia.

Not only is the nature of the mucoprotein a mystery its function is also. The gland cells do not seem to be numerous enough to produce sufficient secretion to have any appreciable effect. However, the large nuclei in these cells suggest that they are very active, possibly continuously so, unlike the M/m and A/B mucous gland cells which have 'collapsed' stages represented by the m and A gland cells respectively. However, these collapsed stages may not be normal, for they are not detected ultrastructurally, the M cells appearing uniform in size and the secretion appears to be continuous also. The function of the secretion may not be for lubrication as such, since the slime produced by the pedal gland and the A/B mucous gland cells serves this function, but it may alter the viscosity of the mucus. When examining the pedal mucus on a slide only threads of metachromatic mucus could be seen, there being no evidence of non-metachromatic granules or of any non-metachromatic material at all. This suggests that the granules somehow 'dissolve' or breakdown before discharge, but this would be expected since the presence of solid granules in the mucus would seem to be of very little value without some change. It is possible that the granules dissolve on contact with mucus and this perhaps alters the viscosity of the secretion.

Young stages of these cells are never seen, except in terms of the large granule C cells and it can only be suggested that they have a connective tissue origin because of their usual deep seated position in the tissues of the foot. It is even possible that these cells function throughout the life of the animal since their nuclei always appear very large and active.

This is the last gland cell type to be found associated with the skin in Arion hortensis. The remainder of the description will be confined to the two glands found in this mollusc, namely the pedal gland and the caudal gland.

LM.4(ix)

The Pedal Gland

The pedal gland is shallowly embedded in the inner tissues of the foot, between the viscera and the sole (Figs.3 and 25). In fact, the gland duct lies in a depression in the foot, being covered by only a small amount of non-glandular connective tissue. The pedal mucocytes are embedded in the tissues of the foot, and extends for two-thirds of the length of the animal, opening by a pore at its anterior end (Fig.25). The duct tapers gradually towards the tail and could be described as a cul-de-sac. It is bounded by an epidermis which displays three cell types (Fig.26). The upper epidermis of the duct is composed of flattened cells whose nuclei are elongated parallel to the length of the cell, and are never ciliated, although the electron microscopy(Plate 1) revealed that it bore numerous long microvilli.

The lower epidermis is composed of two cell types: a central band of fairly large, vacuolated, columnar cells which occasionally appear to have basal nuclei and bear long, but widely spaced cilia; and two lateral bands of closely compact columnar cells which possess central or basal nuclei. It often appears that the central region of the lower epidermis is bounded by two densely ciliated hummocks. The cilia borne by these lateral bands are long, but not as long as those on the central cells. However, these cilia are much more closely packed, presumably reflecting the more compact cells below.

The pedal mucocytes encompass the duct although no glandular tissue is found above the upper epidermis and very few mucocytes were found below the central part of the lower epidermis. The bulk of the mucocytes form two lateral bands embracing the duct (Figs.26, 27 and 42) throughout its length and also extend for a short distance beyond the duct's end in the posterior region. The mean length of these mucocytes is 26.6µmwith a mean width of 43.5µm. The range is from 8.8µmto 6.6µmin

length and 19.8μm to 79.2μm wide. The modal size is 19.8μm long by 66μm wide, the width being taken from the axis parallel to the epidermis of the duct. The mucocytes in these lateral regions show a medium-sized nucleus which is often central, spherical and displays patches of chromatin (Fig.28). However, it is not easy to detect a separate nucleolus, although it was seen with the electron microscope (Plate 11). The cytoplasm appears reticulate but it is felt that this reticulum reflects, not the nature of the secretion, but some variation in the internal contents or a collapse of the cell walls, induced by fixation. The secretion within these cells appears homogeneous, although occasionally 'blobs' of secretion were seen to be attached to the cell walls (Fig.27).

However, the mucocytes below the lower epidermis, particularly those concentrated under the lateral ciliated hummocks, as well as being less numerous, show slightly different staining reactions. These differences are particularly noticeable in toluidine blue, alcian blue, Heidenhain's haemotoxylin and methyl green-pyronin, where the mucocytes closely applied to the lower epidermis either show metachromasia or a darker staining reaction than the non-metachromatic mucocytes of the lateral regions.

The staining reactions of the pedal mucocytes are as for those given in Table VII (pp 75-77), for the mucous gland cells B of the foot sole.

Thus, a chondroitin is present in the secretion of the pedal mucocytes, but its staining is usually suppressed by the large amounts of RNA and associated proteins present. A neutral mucosubstance may or may not be present. RNA, in the form of ribosomes associated with masses of endoplasmic reticulum, can be seen in the electron micrographs of these mucocytes, for instance, Plate 16. The possible sequence of events in the manufacture of the pedal gland secretion will be given in the ultrastructure section. Suffice it to say that these lateral mucocytes

produce large amounts of secretion which gathers in the cell, possibly at the expense of the other cellular components such as the rough endoplasmic reticulum. This accounts for the appearance of metachromatic material below the lower epidermis, for this secretion represents the mature mucosubstance prior to extrusion, when the endoplasmic reticulum bearing RNA has been reduced relative to the amount of secretion and no longer interferes with the metachromatic staining. Whether the mature cells migrate from the lateral regions to below the lower epidermis, or whether the mature secretion passes down long ducts from the lateral mucocytes (which represent the cell bodies containing the cell organelles) to collect below the lower epidermis prior to discharge will be discussed in the ultrastructural study of the pedal gland (EM.1, pp. 161-163). It is the metachromatic material which is discharged between the epidermal cells into the duct, and which is carried forward by the action of the cilia towards the anterior pore, from where the cilia of the foot sole spread the mucus over the foot. The secretion is discharged between the epidermal cells of the lateral hummocks, but more particularly between the central epidermal cells, as confirmed by the ultrastructure. The central cilia of the duct may move the mucus laterally on to those of the hummocks since presumably these more densely packed cilia would provide a more efficient 'conveyor belt' to the anterior end of the gland.

There is no evidence of any very immature pedal mucocytes, either in the light or electron microscopy, so it is possible that the mucocytes function throughout the life of the animal. Young cells may occasionally be produced to replace exhausted cells, but because of the relative rarity of this event, it may have been missed. Alternatively, the cells may be produced very rapidly and may not have been seen in the tissue examined.

However, at the very anterior end of the gland there is a group of cells which might possibly be young cells, Fig.25 shows their

position and Fig.28 shows the cells themselves. They lie in a band between the mucocytes of the pedal gland and the mucous gland cells of the foot sole, and have large spherical, centrally placed nuclei, which show patches of chromatin and a distinct nucleolus. The cell is often somewhat spindle-shaped with its long axis parallel to the foot sole surface, but it may also show rather distorted shapes (Fig.28), rather like those adopted by the very young pigment/protein cells of the dorsal surface. No ducts are present, and the cytoplasm is filled with very fine granules. Since these cells were confined to a very limited area, about $\frac{1}{3}$mm , very few histochemical tests could be carried out on them, but the results are given in Table IX which follows.

Table IX : Young Cells at Anterior End of Pedal Gland

Stain	Result	Conclusion
Mallory's Triple	Steely grey blue	?
Ehrlich's haematoxylin +Eosin	Eosinophilic	Protein?
AB+Ehrlich's haematoxylin	Purple	No AMPS
TB 1% aq.	V. pale blue tinge to cytoplasm	No AMPS
Millon's reagent	Orange	) Possibly protein
Heidenhain's haematoxylin	Blackish	) containing
		) tyrosine.
Methyl Green-Pyronin Y	Bright pink cytoplasm	) No RNA staining
MG-PY+RNase	Pink	)
Alizarin Red S	-ve	No calcium

From these results it would seem that the cytoplasm is proteinaceous, containing no acid mucopolysaccharides and no calcium. These findings would agree with the suggestion that these are young cells, further confirmed by the presence of large nuclei and the lack of ducts: but what these are the young cells of, is not known. Their position, closely applied to the pedal mucocytes and the gland cells of the sole indicates that they might be precursors to either type of cell

(since the pedal mucocytes and the A/B mucous gland cells have the same secretion), in which case it would seem that the cells have a connective tissue origin. There is no evidence of young stages sinking in from the epidermis. However, if these are precursor cells, their limited location is hard to comprehend. It is possible that mucocytes are produced here and then migrate to the other sites, but this would involve migration over considerable distances, so this would seem unlikely. An alternative explanation is that the gland cells at the front of the foot need to be constantly replenished. This could be the case if they have a faster turnover than the gland cells elsewhere and would therefore become exhausted sooner. There are more mucous gland cells present at the anterior end of the animal (Histograms I and II), and more mucus would be required anteriorly, since the animal moves over the trail of slime principally produced at the anterior end.

That these cells have some quite different purpose cannot be dismissed. They could possibly produce some substance which alters the consistency of the mucus, although such a function has already been ascribed to the C mucous gland cells of the foot, and since they lack ducts with which to convey such a secretion, this suggestion seems unlikely. One could postulate that temporary ducts may be produced but since there is no evidence to confirm or deny this, it is not possible to come to a conclusion.

LM.4(X) : The Caudal Gland

The last gland to be considered in connection with Arion hortensis is the caudal gland, another multicellular structure.

The position of this gland is shown in Fig.1, at the end of the tail. The mucocytes line a depression in the tissue of the top of the tail, protected by an 'overhang' of tissue from the dorsal surface. The peripodial grooves also meet at this point. The characteristic

outline of the gland is given in Fig.29(a) and as can be seen the tissue is very ridged.

Once again, this gland was not studied in detail because of its limited size, approximately 200μm, and in animals which are not sexually active, it is not easily visible. Even when visible, the structure of the gland is hard to discern. In Fig.29(b) the mucocytes in the right hand flap of tissue are in fact those A/B mucous gland cells of the foot fringe. The caudal mucocytes are those lying in the tissue of the inner surface. They line the inner end of the depression and the underside of the dorsal flap of tissue. These mucocytes are small, numerous and ovoid, with little evidence of nuclei, and their secretion is reticulate like that of the large mucous gland cells of the back. This secretion is metachromatic in toluidine blue, alcianophilic and stains red in methyl green-pyronin Y, a colour which owes its origin not to RNA, but to the presence of some other polyanion. It is not possible to say more than that the secretion is an acid mucopolysaccharide. The mucocytes of the caudal gland are embedded in very dense connective tissue, in which are scattered numerous nuclei, but the significance of this denser tissue is not known. The mucocytes discharge through ducts which pass between the columnar epidermal cells and the mucus passes into the depression at the top of the foot fringe. Here the cilia disperse the mucus, which, according to Saint-Simon (1852), functions as a stimulant prior to copulation.

LM.4(xi) : Pigmentation of The Sole

Finally, a brief comment must be made on the pigmentation of the sole. In the Identification section (pp.3) it was mentioned that the sole was orange. Unlike the dorsal surface, however, where the pigment is contained in protein gland cells, there is no evidence of pigment/protein gland cells in the sole, nor of pigment being

associated with any of the other gland cells. It is therefore suggested that the pigment is contained in the 'spaces' between the connective tissue fibres and musculature of the foot tissue. The appearance of the foot tissue after fixation for the electron microscope suggests that this may be so, as there is evidence of some granular material between these fibres. This material seems very distorted and damaged after fixation, which would seem to agree with the loss of colo-ration after fixation. The pigmentation of the sole is one of the characteristic features of Arion hortensis but in the newly hatched animal (Appendix I) the sole is not pigmented and appears white. The colo-ration would appear to be acquired with age, and it probably derives from the diet.

The next section will consider the mode and mechanism of discharge of the gland cells and glands.

LM.4(xii) : Mode and Mechanism of Discharge of Gland Cells and Glands

It is suggested that the mode of discharge is merocrine rather than apocrine or holocrine.

Holocrine secretion involves the discharge of the cell contents together with the cell organelles and since the whole cell is discharged the cell only functions once. Thus if this method of discharge were adopted by the gland cells and glands, nuclei and membranes should be detectable externally. This method of discharge necessarily involves the death of the cell, and there would have to be a continuous supply of young cells in the process of maturation to replace those lost. In fact, there is no evidence of the discharge of cell organelles, particularly when examining the electron micrographs and the pedal gland and mantle tissue; and little evidence of young developmental stages except in the case of the pigment/protein cells. Therefore, it would seem that a holocrine mode of discharge is not adopted by either the

gland cells or the glands.

Apocrine secretion involves the ejection of the apical portion of the cell where the product has been collected. If this mode of secretion were adopted by the various gland cells one would not expect to find the well formed ducts that are present.

For these reasons it is suggested that the gland cells and the mucocytes of the glands display a merocrine mode of discharge.

What initiates the discharge is not clear from the light microscopy. There is no evidence of a fibrous network individually encasing each gland cell, which might effect discharge by contraction, as described by Campion (1961). There are two other alternatives for mechanisms of discharge. The first is that the secretory products continue to be produced after the cell has ceased to increase in size and so the pressure of newly produced secretion will force the mucus out of the cell. However, although this might keep the skin of the animal continuously moist in normal conditions, it would not provide the large quantities of mucus seen, for instance, when the animal is under attack. This ability to vary the quantity of mucus produced suggests some form of neuro-muscular system is involved.

The second alternative relates to the fact that the gland cells and mucocytes are found to lie embedded in the subepidermal muscle and connective tissue of the body. The contraction of these muscles could directly or indirectly effect discharge. Directly, by contracting against the cell wall of the mucous gland cell thus forcing the secretion to be discharged. Indirectly, by exerting pressure on the haemocoelic fluid which in turn would push against the gland cell wall effecting discharge. Presumably the muscular contraction is under nervous control. This last mechanism seems to be more likely for the gland cells and mucocytes of the glands in Arion hortensis. The fact that the pigment/protein cells discharge after the violent contraction of the

body in fixative suggests direct or indirect pressure induced by the contraction of the body muscles is involved in their discharge. Nerves could not be detected in the dorsal surface of the slug in the light microscope material, but they were evident in the ultrastructural examination. These will be examined in the relevant section (EM2), but they were never seen to be directly attached to the gland cells, ending in the muscle fibres which lie mainly around the base of the cell.

In the foot sole, occasional large nerves could be seen fairly deeply embedded in the musculature, but in the ultrastructural survey of the pedal gland no nerves were ever seen to be associated with the gland tissue itself.

Although such attachments were never seen, it does not necessarily mean that they do not occur since only relatively small areas of tissue were examined. However, Dyson (1965) working on an Arion species and using silver impregnation methods could not detect fine nerve endings, only large ones.

It is interesting to see, in connection with the small mucous gland cells, that the lower section of the gland cell is often narrower than that part passing through the epidermis (Fig.7 and 13) which would seem to indicate that discharge might well be effected by the pressure of surrounding muscle fibres.

One other point to be considered, at least in relation to the large and small mucous gland cells, is that the cell wall must be very elastic since it undergoes considerable stretching and contraction in the cycle from large to small mucous gland cells and so on. This elasticity possibly aids discharge and if this elastic wall was under direct nervous control, then this would constitute another mechanism of discharge. However, large and small mucous gland cells were not seen using the EM, and such an appearance may be unnatural.

The next section will briefly consider the origin of the gland cells and glands.

LM.4(xiii) : The Development of The Mucous Gland Cells and Glands

The development of these cells has already been mentioned in the preceding text. As can be seen from this there is not a great deal of information available from the tissue prepared for light microscopy. However, when apparently young stages are present they occupy the depths of the connective tissue. This might perhaps indicate a connective tissue origin for the individual gland cells and those of the glands, but this is only circumstantial evidence. An embryological investigation would be the best method of establishing the origin of these cells but time did not allow for this. However, Binot and Chétail (1968a and 1968b) described the origin of these cells (both sole and mantle) of Arion rufus in an embryological study. They found the gland cells and mucocytes of the pedal gland to have a mesenchymal (connective tissue) origin, whilst the epidermis of the animal and the duct of the pedal gland had an ectodermal origin. Since the animal currently being investigated is a species of Arion also, it would seem likely that the cells in Arion hortensis have a similar origin.

Although an embryological investigation could not be carried out, a study of the regeneration of the mantle tissue was undertaken. This revealed that pigment cells might have a connective tissue origin and the possibility that the gland cells do also cannot be dismissed.

The reason for the relative lack of young cells is not known. It is possible that the newly-hatched slug has almost a full complement of gland cells which function throughout life, perhaps with the exception of the pigment/protein cells which have already been discussed (pp. 59-60)

The last unit of the light microscopy section will compare the current findings on Arion hortensis with those of previous workers.

LM.4(xiv) : Comparison of the Findings on Arion hortensis with those on Other Molluscs

A brief summary of past works was given in LM.3 (pp. 17 to 32).

No detailed comparison can be made with Prenant's work since he was principally interested in the calcium gland cells and there were none of these in Arion hortensis. This is not unexpected since Arion is not a shelled mollusc like Helix pomatia, and only possesses a rudimentary shell in the form of calcium granules (LM.4(vi), pp. 67). It is his description of the origin of the large mucous and calcium gland cells from connective tissue cells, described as young leucocytes, that is interesting, for these are comparable with the young cells seen at the anterior end of the pedal gland (LM.4(vii), pp. 91-93), and the very young B gland cells (LM.4(vii), p. 81).

The leucocyte first increased in size due to the production of large quantities of lipid, then flakes appeared within these cells; and eventually both calcium and mucus were produced from these flakes. No such pattern of development was seen in Arion hortensis, but his suggestion of a connective tissue origin of cells is comparable. Prenant described briefly the calcium found in the connective tissue of Arion rufus which was very like the calcium concretions described in Arion hortensis, being spherical, quite voluminous and deeply embedded, with no openings to the exterior.

Roth (1929) described, more extensively, the gland cells within Helix pomatia and he listed the same types of gland cells found in Arion hortensis: one type of mucous gland cell in the mantle (and presumably also in the back), and one in the foot sole together with protein, calcium and yellow pigment cells. In Helix these latter two types were separate entities whereas in Arion the pigment and protein cells were combined to give the yellow protein cell of the dorsal surface. Roth believed that the protein and calcium gland cells were related, with

the protein cells being filled with calcium granules.

Roth described the mantle mucous gland cells as being fibrous which could be equated with the reticulate contents of the M cells in Arion hortensis, as could be the granular sole mucous gland cells with the C cells of Arion.

Roth, however, favoured an epidermal origin for the gland cells in contrast to that favoured for Arion hortensis.

Concerning the work of Bolognani-Fantin and Bolognani (1964) and Bolognani-Fantin (1965), they carried out a biochemical study and found the polysaccharide fraction to contain glucosamine and galactosamine and this latter substance is a component of chondroitin which has been suggested as the mucoid substance present in all the mucous gland cells of Arion hortensis, except C.

As in Arion, these authors could find no evidence of sialic acid in the mucus of Helix pomatia, and their description of the mucous and protein gland cells resembled those of Arion hortensis, except that in Helix all these cell types were found in the foot whilst this was not so in Arion. The mucous gland cells were found to contain a sulphate radical which was only obviously found in the C cell of Arion although it was suspected in small amounts in the mantle gland cell secretion. No protein or indolic or phenolic groups were found in the mucous gland cells in Helix, whilst protein was believed to be present in Arion. However, the protein cells of Helix were found to contain tyrosine and thiol groups and whilst no protein cells were found in the sole of Arion, those of the dorsal surface contained tyrosine.

Once again calcium gland cells were found in this mollusc and not in Arion.

The most interesting point of comparison between the two molluscs lies in what Bolognani-Fantin called the 'phenolic' cell in

the sole. Her description of this cell closely resembled that of the C gland cells of Arion, although she found the contents of this cell to be negative to general protein and mucopolysaccharide tests whilst the granules of C were found to be somewhat alcianophilic and to give some protein reactions. Also, she found these cells to be naturally yellow and this was never seen in the C cells. Nevertheless, it is believed that the two types of cell could be comparable and that the secretion of C may contain a di- or poly- phenol or a di-indol, as found by Bolognani-Fantin in this cell. However, she gave no indication about what possible function this cell might have. The golden yellow pigment cells mentioned by Roth could well be the 'phenolic cells' described by Bolognani-Fantin, and both she and Bolognani ascribed an epidermal origin to the gland cells but gave no evidence to support their statement.

Considering Campion's work on Helix aspersa (1957, 1961) the mucous gland cells A are comparable with the M cells of Arion hortensis, both in their appearance and histochemical nature, although those in Helix were much larger, this probably relating to the fact that Helix aspersa is a much larger animal. The B mucous gland cells of Helix may be equivalent to mantle groove mucous gland cells since both types were periodate reactive.

No calcium gland cells were found in Arion but Campion found protein and calcium associated in one cell as in Helix pomatia. In Arion pigment and protein were associated in one cell as Campion also found in Helix aspersa. These last cells were like those in Arion, but very much larger. However, Campion described pigment only cells as well, the pigment being a flavone whereas that in Arion was believed to be either a carotene or a flavone.

The C gland cell secretion in the foot of Helix aspersa would seem to be equivalent to that of the A/B mucous gland cells in Arion histochemically and to some extent physically, and both types were found

in clusters. The D gland cells in Helix were very like the C gland cells in Arion and would also appear to be equivalent to the granular or 'phenolic' cells described in Helix pomatia by Bolognani-Fantin. Campion felt that the C and D cells could have a common origin.

As to the origin of the gland cells Campion also found few developmental stages but favoured a connective tissue origin for the gland cells describing developmental sequences for them. This could not be done for the mucous gland cells of Arion hortensis except for the spindle shaped cell, associated with the A/B cells. However, the description of the pigment/protein gland cells in both types is very similar, even to the finding of more numerous 'young' stages of protein cells. Since no calcium or lipid cells were found in Arion no comparison can be made with Helix on these points.

The mode and possible mechanisms of secretion are similar in both animals, being merocrine and possibly caused by direct or indirect muscle pressure. However, Campion also found an individual network of fibres surrounding the protein and calcium gland cells and suggested a similar situation might appertain in the mucous gland cells of the back, but there was no evidence of such a network in the gland cells of the sole.

Elves' (1961) general description of the foot sole of Discus rotundatus was much like that of Arion although 'amoebocytes', as described by him, were not found. Also he believed that some of the mucous gland cells discharged into intercellular spaces in the foot before final discharge; whilst others, those nearest the surface, discharged through ducts. Only discharge through ducts was seen in Arion hortensis.

The aggregation of the mucous gland cells in the vicinity of the peripodial grooves was noted in both animals and Elves also noticed a resemblance of the pedal gland mucocytes and the peripodial mucous

gland cells. The description of the pedal gland in Discus was much like that in Arion, except that there only appeared to be one type of epidermal cell in the lower epidermis of the duct. His description of the two states of pedal mucocytes is most interesting since a similar arrangement may be found in Arion. The first type of cell was vacuolated and found near to the gland duct and would be equivalent to the metachromatic mucocytes in Arion, whilst the second type of cell was less vacuolated and lay further from the duct, being equivalent to the non-metachromatic cells in Arion. This last type of cell was described by Elves to have long thin ducts leading to and passing between the epidermal cells of the duct. This was believed to be the case in Arion but will be discussed later (EM.1). A caudal gland was also present in Discus but whilst the description of the external characteristics of the gland and the possession of mucocytes was similar, there were no amoebocytes present in Arion, as there were in Discus. Elves suggested that the gland in Discus served the same function as in the Arionidae.

In both Discus rotundatus and Australorbis glabratus (Pan 1958) the epidermal surfaces (at least that of the foot in Discus which was the only one described) rested on a basal lamina. Such a structure was never seen in Arion even in the ultrastructural examination and there was no equivalent either to the pigment or vesicular cells of Australorbis. However, the mucous gland cells in the foot of Australorbis seem very similar descriptively and histochemically to the granular C gland cells in Arion.

In the pedal gland Pan also described mucocytes whose ducts form groups which pass towards the surface, and this is believed to be one explanation of what may happen in Arion, and certainly in some longitudinal sections this appears to be so. Moreover, Pan believed that it was the muscle fibres, which bounded the gland, that effected discharge through the mucocyte ducts to the main duct of the pedal gland. As

already discussed, this appears to be the mechanism employed in Arion also.

The pedal gland in Arion was unlike that in Milax sowerbii described by Barr (1926) in that in the latter instance the gland was free in the body cavity anchored to the foot by only a few small muscles, whilst in Arion the gland is shallowly embedded in the tissues of the foot. Moreover, the gland duct showed secondary branching which was not seen in Arion. However, the description of the lower epidermis and the arrangement of mucocytes was very much the same for both molluscs, but in Milax Barr found that the mucocytes were either granular with small nuclei (these cells representing the secretion stage), or vacuolated with large nuclei which represented the discharge state. This agrees with the description of the cells in the pedal gland of Discus rotundatus. In contrast, in Arion, the mucocytes with large nuclei were found to be the actively secreting stage.

Barr's suggested mechanism of discharge for the pedal gland, involving the cilia and the tenacity of the slime, was rather different from that proposed for Arion, necessarily so since there was no evidence of muscles fibres between the pedal mucocytes and the gland was free in the body cavity. Such a mechanism could operate to some extent in Arion, although the musculature plays a major role. One last point of difference lies in the finding of crystalline concretions in the gland duct of Milax, which were believed to be an excretory product; they were not present in Arion.

The mucous gland cells in Arion ater were described as granular by Barr (1927-1928) and possessed large nuclei, discharging through ducts or into intercellular spaces. In A. hortensis only the C gland cells were found to fit this description and they discharged to the exterior by ducts. Barr also found aggregations of mucous gland cells in the peripodial groove region, resembling those of the pedal gland

just as was found in A. hortensis.

However, she described the whole of the foot sole and foot fringe as being ciliated whereas in Arion only a median band of cilia appears along the foot sole. Nor were 'calcic' gland cells found in Arion, although Barr's description of them resembled the C gland cells, but no calcium was found in these cells. In both species calcareous granules were found deep in the connective tissue of the mantle.

Barr's description of the melanin cells in A. ater was much like that in A. hortensis, although the second pigment in A. ater var. castagnea was bright orange/red and found in globules, unlike that in A. hortensis. Barr believed this to be a waste product since it was found in the slime, as was the yellow pigment of A. hortensis.

The pedal gland of A. ater was much like that of A. hortensis which is to be expected since they belong to the same genus. However, there are three points of difference, one being that Barr found some glandular cells in the upper epidermis of the gland at the posterior end, whilst the upper epidermis in the pedal gland of A. hortensis did not appear glandular using the light microscope. The second difference was that the contents of the gland mucocytes were described as either granular or vacuolated and no obviously granular stages were seen in A. hortensis, although differing methods of fixation might result in such a disparity. It is also possible that Barr was describing as 'granules' the membranous sacs of secretion seen ultrastructurally in A. hortensis. These sacs gradually fuse to give a mass of secretion within that cell. The third difference lies in the route the secretion takes to the gland duct. In A. ater some of the secretion is discharged directly through cell ducts as in A. hortensis, but some others discharged their secretion into intercellular spaces and thence into the gland duct. Barr believed discharge to be effected by contraction of the muscles found around the cells, as in A. hortensis.

The caudal gland in A. ater was almost identical to that in A. hortensis except that Barr found some calcium glands in the tissue of the dorsal flap and this was not seen in A. hortensis. However, their presence cannot be absolutely denied in this latter species since the gland was not often seen and therefore was rarely tested for calcium. If only a few calcium gland cells are present they could have gone undetected.

Campion (1957) found calcium lacking in Testacella haliotidea, as in Arion but she found non-metachromatic mucus in the mantle gland cells which was absent in Arion. Both genera possessed metachromatic mucous gland cells in this region. The protein gland cells in Testacella were non-granular whilst those in Arion were finely granular, but a yellow pigment was associated with the former in the sides of the foot, as in the pigment/protein cells of the dorsal surface in Arion. In the sole, Campion found metachromatic mucus and protein in one gland cell type, possibly equivalent to the A/B gland cells, and also non-metachromatic mucous cells which could be equivalent to the C cells of Arion.

The gland cells of the sole of Milax gracilis and Arion hortensis were very alike, both types having granular non-metachromatic mucus and protein gland cells, together with more superficial, elongated metachromatic mucous gland cells. However, Campion found melanophores and calcium gland cells in the sole and these were not found in Arion. Yellow proteinaceous gland cells were found in the sides of the foot, rather like those of the dorsal surface in Arion, together with large metachromatic cells. This description also applied to the dorsal surface of Milax, thus making the dorsal surfaces of both molluscs very similar.

In comparing Arcadi's description of Lehmania poirieri with that of Arion hortensis the basket cell complex was found to be descriptively similar to the M gland cells in Arion, but their

histochemistry differed. The mucus was found to be intensely PAS-positive whereas that in Arion's dorsal mucous gland cell was PAS-negative. Moreover, Arcadi stated that the contents of this cell type were very soluble in buffer solutions, but this was not established in Arion since the fixation procedures adopted would 'fix' the secretion. Also, the staining reaction of this secretion to PAS could be partially reduced by exposing it to neuraminidase whilst the secretion in the M/m mucous gland cells was resistant to the enzyme and hydrolysis. The basket cell complexes were also metachromatic at a pH above 4.0 but non-metachromatic below this, whilst in the dorsal surface M cells the secretion remained metachromatic below pH 1.0. However, both cell types were alcianophilic and in both cases the ducts of these two cells were more reactive to the stains.

The second type of cell described was the granular cell complex of the sole, and again, in appearance it was much like the C gland cells of Arion. In terms of histochemistry both types were periodate reactive although the granular cell complexes were metachromatic in toluidine blue (pH7.0) and generally showed a rather complex pattern of staining in that stain, at various pHs. The C gland cells were never metachromatic. The contents of the granular complexes were resistant to extraction by buffer solutions except between pH 3.4 and 4.4 and once again this was not established in Arion. Like the C gland cells, however, the secretion of the granular cell complexes were resistant to the action of enzymes. The granular complex was also found in tissue other than the sole whilst the C cells were never described elsewhere.

Arcadi concluded that both complexes contained carbohydrates with adjacent hydroxyl groups, the basket cells containing a sialomucin and no sialomucins were found in Arion.

Lastly, his findings that the mucocytes originated from

interstitial cells in regenerated tissue tended to be confirmed by a similar study on Arion hortensis, but this will be discussed in the relevant section (R.LM and R.EM).

Chétail and Binot's (1967) description of the pedal gland of Arion rufus was almost identical to that in Arion hortensis except that they divided the gland into anterior-lateral, anterior-median and posterior regions, whereas in A. hortensis although lateral and median regions were present, no distinction could be made between anterior and posterior regions. Correspondingly, they described three types of cell in A. rufus: a_1, a_2 and b, and a_1 and a_2 can be easily identified as equivalent to the non-metachromatic and metachromatic mucous gland cells of A. hortensis respectively, even to the finding of a neutral mucopolysaccharide although the periodate reaction could be given by protein, large amounts of RNA and endoplasmic reticulum, although there were no lipids in A. hortensis. No b gland cells could be identified, however. One other point of difference was that they found no evidence of any protein in the pedal gland mucocytes even though they mentioned the large quantities of RNA. The ultrastructural survey of the pedal gland of A. hortensis (EM.1) has revealed the RNA to be present in the form of ribosomal RNA, attached to the endoplasmic reticulum, and which presumably functions to produce amino acids and proteins which might form the protein core or link units in the final mucosubstance. However, protein tests applied to this secretion in A. hortensis were generally negative, possibly because they are masked by acidic groups, which might account for Chétail and Binot's conclusion.

In A. hortensis the pedal gland presents the same appearance along its length, except for the patch of young cells at the anterior end, unlike A. rufus; but in both cases the lower epidermis of the gland duct just behind the pore was very alike with discharge mainly occurring between the central band of epidermal cells.

In this same paper, they also described the gland cells of the sole. The type III mucous gland cells of A. rufus were similar in appearance and histochemistry (as far as the two different sets of histochemical tests allowed comparison), to the C gland cells of A. hortensis but there appeared to be no equivalent in A. hortensis to the type IV mucous gland cells of A. rufus. The type I mucous gland cells were similar to the A gland cells of A. hortensis in structure but not histochemically since there was no evidence of neutral mucopolysaccharide or lipid in the latter. However, there is believed to be such a substance in the B gland cells which are thought to give rise to the secretion in the A cells.

By contrast, the type II mucous gland cells resembled the B gland cells histochemically but not physically since the former cell possessed a small basal nucleus and an apparently reticulate cytoplasm whilst the latter had a fairly large central nucleus and, although the cell contents appeared reticulate, this was believed to reflect some internal structure and not the secretion as such.

Chétail and Binot made no comment about the presence of peripodial mucous gland cells resembling those of the pedal gland as noted by Barr (1927-1928) and in the current research on A. hortensis. In the latter case the peripodial mucous gland cells were believed to reflect a concentration in numbers of the B gland cells. However, they said (1968b) that there was a type of gland cell in this region which was equivalent to the type A gland cells (being derived from the B cells), but no exact comparison could be made.

The zone of 'clear cells' seen by Chétail and Binot in the tissue between the pedal mucocytes and the mucous gland cells of the foot may be equivalent to the patch of young cells visible at the anterior end of the pedal gland in A. hortensis.

Since A. rufus and A. hortensis are so closely related, Chétail

and Binot's findings concerning the origin of the pedal gland and the mucous gland cells of the foot are most relevant. They found that the pedal gland duct and the epidermis of the sole had an ectodermal origin whilst the mucous gland cells and mucocytes had a mesodermal (or connective tissue) origin.

In considering the mucous gland cells of the mantle of A. rufus, the M_2 mucous gland cells appeared similar to the M mucous gland cells of A. hortensis, but the histochemistry of the two cell types was different. Whilst both cell types show a complex acid mucopolysaccharide and possibly protein, there was no evidence for a neutral mucopolysaccharide or lipid in the M gland cells, and no equivalent to the M_1 cells of A. rufus.

Once again these gland cells had a mesodermal origin which would suggest that the cells occupying this position in A. hortensis may originate similarly.

The ventral sole gland cells described by Wondrak (1967) in Arion empiricorum are equivalent to the B gland cells of Arion hortensis, which were found to be very like the pedal gland mucocytes, both in appearance and secretion.

Wondrak described the ventral sole gland cells as possessing a highly structured endoplasmic reticulum (EM.1, pp 122) and an identical structuring was found in the pedal mucocytes of Arion hortensis (EM.1, pp 140-142).

The gland cells occurred all over the ventral surface of the foot of Arion hortensis, like the ventral sole gland cells but with a particular concentration of them in the region of the peripodial groove. Moreover, Wondrak obtained metachromasia at pH 3.2 in the ventral sole gland cells as in the B gland cells. Similarly, the sole gland cells of Arion rufus are equivalent to the B gland cells of Arion hortensis for the same reasons.

The lateral sole gland cells in Arion empiricorum are believed to be similar to the C gland cells of Arion hortensis, although Wondrak found the former to be metachromatic at pH 2.7 and metachromasia was never found in Arion hortensis. The contents of the cell were described as membrane-bound granules which would agree with the C gland cell; and in Arion rufus the peripodial gland cells (equivalent to the lateral sole gland cells) were seen to contain granules in the duct of the cell, right to the surface, but these granules were never seen to be discharged which again agrees with the C gland cells. Thus, despite some confusion of nomenclature the lateral or peripodial gland cells are thought to be equivalent to the C gland cells and the ventral sole gland cells are believed to be like the B gland cells which are found laterally and ventrally in the sole of Arion hortensis.

Wondrak described only two types of gland cells in the sole of Arion rufus whilst Chétail and Binot described four types; this might suggest that two of the types described by Chétail and Binot are development stages.

Wondrak's description of the mantle gland cells of both these species of Arion closely resembled the M cells of Arion hortensis, and their ultrastructure will be compared later (EM.2). However, the large mucous gland cells of a A. hortensis are metachromatic in toluidine blue pH 1.0, whilst those in Arion empiricorum only showed metachromasia at pH 3.2 and above, so this indicates some chemical difference in the secretion of the two types of cell.

In Arion rufus Wondrak described protein cells which are probably equivalent to the pigment/protein cells of A. hortensis, but A. rufus showed a distinct pigment cell which contained the red pigment which gives A. rufus its name.

The pattern of ciliation described by Lainé for Agriolimax reticulatus was identical to that described in A. hortensis. The large

mucous gland cells described by Lainé appeared histochemically and structurally like the M gland cells of A. hortensis, and likewise the small mucous gland cells of both types were equivalent. Moreover, in both cases a single cycle involving both large and small mucous gland cells was adopted for both molluscs. The exact equivalence of the secretion in these glands could not be established, since relatively few techniques were available for the study on Agriolimax reticulatus.

Lainé described three types of granular cells in the post-mantle region, which appear very like the pigment/protein glands of Arion hortensis. The three types were described on the basis of three different staining reactions, but it is believed that these three types may well be one and the same kind, for in Arion hortensis, with Mallory's Triple Stain the pigment/protein cells showed quite a considerable range of staining reactions, whilst in frozen sections only one type of yellow pigmented granular cell was seen. Chemical fixation would appear to cause considerable disruption to the cell in question, and Lainé used similar fixation methods to those used for Arion hortensis. However, Lainé also described a number of granular cells in the ultrastructural investigations; and described distinct protein gland cells, so no definite conclusion can be reached.

Lainé's description of the peripodial gland cells is identical to that of the peripodial /B gland cells of Arion hortensis, although these latter showed faint alcianophilia. The reason for the faint staining is probably the relative immaturity of the secretion, there being more protein containing endoplasmic reticulum than secretion.

No equivalent to the scattered granular connective tissue cells (LM.3, p 30) was found in Arion hortensis. Lainé did not describe the gland cells of the sole or the pedal gland, but she did comment, however, that the staining reactions of the sole glands cells of

Agriolimax reticulatus were like those of type III as described by Chétail and Binot (1967).

The description of melanin in both animals was very similar.

Lainé found the mode and mechanism of discharge and the origin of the gland cells to be similar to that in Arion.

Dyson's (1965) description of the type I and III gland cells agreed well with those of the M and m cells respectively, in the current investigation of Arion hortensis, and in both cases they were believed to be stages of one cell type. There was some variation in measurements, but this is to be expected when different fixatives were employed. However, type II cells were not detected. The type IV gland cells were equivalent to the mantle groove mucous cells, but Dyson noted that their contents appeared spindle shaped rather than granular after formalin fixation.

Dyson also described cells in the connective tissue which closely resembled the young pigment/protein cells, Y, together with calcium deposits (shell remnants), which she found to be membrane-bound. The protein gland cells were fundamentally similar, except that besides tyrosine, she also detected one or more of cysteine, cystine and methionine. The methods for S-S groups were negative in the current work. She failed to note the presence of the yellow pigment in the matrix of the protein cells. However, she found some calcium gland cells in the mantle and these were not detected in the current work. Dyson used a more extensive range of calcium tests in her work so one might expect more adequate results. The calcium granules were in a protein matrix and it is possible that some of the protein cells described in this thesis could be calcium/protein cells rather than pigment/protein cells; alternatively pigment, protein and calcium may be found in the same cell. Some of the calcium cells she showed resembled the young pigment/protein cells (Y) of the present work.

Roth (1929) and Campion (1957) found protein and calcium to be associated in one cell.

Dyson was also able to describe glycogen deposits in the mantle, as in the present work.

Another point of difference was that Dyson described the mantle epidermal cells as resting on a basal lamina but one was not found here, even when using the electron microscope. It is possible that what Dyson interpreted as a lamina was the band of muscle and collagen fibres which were often present below the epidermis.

The main disagreement was that Dyson believed the mucous gland cells to have an epidermal origin, whilst in this work they were believed to have a connective tissue origin. In both cases, the reasons for the relevant decision were tenuous. Dyson believed the type III cells to be young stages in the formation of type I and since these were superficial in position they were deemed to be epidermal in origin. In this work the small mucous gland cells were believed to represent discharged M cells since secretion was seen passing from their ducts. Dyson believed that her view was substantiated by the presence of a basal lamina round the bases of the mucous gland cells - but as mentioned before this feature was not detected in the current work. She did not know where the protein cells originated from, whilst here they seemed to be from the connective tissue. However, Dyson believed the calcium gland cells had a connective tissue origin and since some of the protein cells in the current work could contain calcium, there is some agreement here. The embryological investigations of Binot and Chétail (1968a and b) on another species of Arion suggest that all the gland cells have a mesodermal origin, and the regeneration study, (R), to be described later would seem to confirm this.

In conclusion, large (M) and small (m) mucous gland cells were described in the dorsal surface of Arion hortensis, which were believed to be one and the same sort of cell, together with pigment/protein cells. Gland cells were also found to line the groove between the mantle and the sides of the body. In the sole were found A and B mucous gland cells, which once again were believed to be the same type of cell, with a particular concentration in the region of the peripodial gland cells. Together with these cells were found C gland cells. Two major glands were also described, the pedal gland and the caudal gland.

As can be seen from the comparison of the results of the current work with those of past workers, some types of cell were found to be comparable, whilst others were quite different. However, there are sometimes differences of opinions concerning one particular mollusc, notably the findings of both Chétail and Binot and Wondrak on Arion rufus, and Dyson with the current work. It can also be difficult to make comparisons when different terminologies and histochemical tests are used by various authors, particularly if their work is of some age.

The ultrastructure of the pedal gland will be considered in the next section.

EM1. ELECTRON MICROSCOPY OF PEDAL GLAND

EM1 (1) INTRODUCTION

As already stated, the pedal gland was selected as the tissue to be examined because it represented a well defined and limited area for study, and its ultrastructure had not been investigated. Fixation for electron microscopy had to be very rapid, to prevent cell autolysis, and also very good, so that the cell structure could be distinguished easily and accurately without the presence of artefacts.

EM1 (2) METHODS

(i) Dissection of Material

Before fixation could occur the gland had to be extracted from the body tissues. This was accomplished by placing the living slug into the first fixative solution (glutaraldehyde) and cutting off the last third of the body. The back was then opened and the body organs removed. The shell of skin remaining was placed in fresh fixative solution and trimmed so that the foot sole tissue was exposed for dissection. This tissue was cut down to a strip, nowhere more than 2mm in diameter, embracing an imaginary line down the centre of the sole (the region of the pedal gland). This in turn was cut into pieces approximately 1mm long so that the resultant blocks (six or seven in number) of pedal gland tissue were no more than 2mm wide and 1mm long. Consecutive blocks were placed individually into labelled pots so that the position of any part within the gland could be established. Moreover, when the strip of tissue was dissected out of the foot sole it was cut so that the diameter of the tissue at the anterior end of the gland was less that that of the posterior end. In consequence the anterior face of each block was narrower than the posterior face, allowing the blocks to be embedded in an exact manner, so that sectioning always proceeded from the anterior to the posterior face. The whole dissection took between three and five minutes

to complete.

(ii) Solutions

Before the fixative solutions were made up it was necessary to establish the correct molarity for the phosphate buffers in which the solutions are made, so that the tissue fluids and fixative should be, as far as possible, isotonic, in the hope that this might prevent excessive shrinkage or swelling of tissue components in the fixative.

Lockwood (1963) gives the concentration of tissue fluids of certain animals, and although he gave no measurements for Arion hortensis, he did state them for Helix, a terrestrial gastropod mollusc (like Arion hortensis) occupying the same sort of ecological niche. He stated that the blood concentration of Helix may vary from 0.47Δ^{o}C to 0.2Δ^{o}C during a shower of rain, and since the specimens of Arion hortensis from the indoor vivarium were not exposed to shower conditions, the concentration of the blood of Arion hortensis was taken to be about 0.47Δ^{o}C.

The usual Sörensen phosphate buffer has a molarity of 0.135 in the Maunsbach recipe for 1% Glutaraldehyde (p149 Sjöstrand 1967), where it is stated that the buffer has a tonicity of 300 milliosmols. It was then possible to find the concentration of this solution in Δ^{o}C using the graph produced by Millonig and Marinozzi (p292, 1968) which converts the concentration of a solution, expressed in terms of Δ^{o}C, to the molarity of monovalent ions. Thus the buffered glutaraldehyde solution produced by using the Maunsbach recipe has a concentration of 0.575Δ^{o}C.

The molarity of phosphate buffer necessary to give an approximately isotonic solution with the body fluids of the slug is therefore expressed as

$$= \frac{0.135 \times 0.47}{0.575}$$

$$= 0.1104$$

This means that phosphate buffer solutions of 0.1M will be

almost isotonic with the body fluids of the slug and a 3% solution of glutaraldehyde and a 1% solution of osmium tetroxide were made up in 0.1M phosphate buffer in a range of pHs, 5.5, 6.1 and 7.3. The schedule outlined below was used for fixation regardless of the pH of the solutions but there appeared to be no difference between the results, so after this preliminary run only solutions of pH 6.1 were used. After fixation, the tissue has to be dehydrated (in alcohols) and transferred via propylene oxide to TAAB embedding resins (composed of 1 part Accelerator to 5 parts MNA to 45 parts DDSA to 50 parts resin).

S1 Long Schedule for Fixing and Embedding Pedal Gland Tissue

1. Prefixing in 3% glutaraldehyde in 0.1M phosphate buffer, pH 6.1. 4 hours. Agitated.
2. 0.1M phosphate rinse, pH 6.1. Left in refrigerator overnight.
3. Post-fixing in 1% osmium tetroxide in 0.1M phosphate buffer, pH 6.1. 1 hour. Agitated.
4. Rinse and wash in 0.1M phosphate buffer pH 6.1. 1 hour. Agitated.
5. 50% Alcohol for 15 mins. 2 changes. Agitated.
6. 70% Alcohol for 15 mins. 2 changes. Agitated.
7. 80% Alcohol for 15 mins. 2 changes. Agitated.
8. 95% Alcohol for 15 mins. 2 changes. Agitated.
9. Absolute Alcohol for 30 mins. 2 changes. Agitated.
10. Absolute Alcohol/Propylene oxide, 2:1, for 15 mins. Agitated.
11. Absolute Alcohol/Propylene oxide, 1:2, for 15 mins. Agitated.
12. Propylene oxide for 30 mins. 2 changes. Agitated.
13. Propylene oxide/resin, 2:1, for 90 mins. Agitated.
14. Propylene oxide/resin, 1:2, overnight. Agitated.
15. Pure resin (TAAB Embedding Medium) for 24 hours. 2 changes. Agitated.
16. Embed tissue in TAAB resin in embedding capsules and place in 60°C oven for 60 hours.

After the blocks had cooled they were trimmed and sectioned on

a Reichert Ultramicrotome, using glass knives. Sections showing silver or silver-gold interference colours, 600 to 900 Å thick according to Sjöstrand (1967), were used and mounted on uncoated Athene 200 copper grids. These ultrathin sections were stained for 15 minutes in 5% uranyl acetate and 15 minutes in Reynolds' lead citrate (Reynolds 1963). At the same time as these thin sections were taken off the block, adjacent thick ones (1µm and ½µm) were cut and mounted onto glass slides where they were stained for 15 seconds with 0.5% Toluidine blue in 10% acetone and 1% borax. They were then permanently mounted and could be used for orientating the ultrathin sections, which were examined and photographed using a Philips EM 200 at 60Kv. The film used in the microscope camera was Recordak Microfile, Type 5669, which was developed in Kodak D19 for 5 minutes at 20°C with regular agitation. The negatives were printed onto Kodak paper K2 or K3, depending on the degree of contrast in the negative, and developed in Kodak D163 for 4 minutes.

Several other staining and embedding techniques were used on the pedal gland tissue and these are listed below.

Schedule 2 differed from Schedule 1 in the use of alcian blue in the glutaraldehyde solution, a technique derived from that given by Behnke and Zelander (1970). It gave better tissue preservation and contrast, particularly enhancing the staining of the nucleus and the mucosubstances.

S2 Alcian Blue - Glutaraldehyde Schedule

1. 0.5% Alcian Blue 8GX (ICI Ltd.) in 4% Glutaraldehyde in 0.1M phosphate buffer pH 6.1 for 2 hours. Agitated.
2. Wash in 0.1M phosphate buffer, pH 6.1, for 1 hour. 1 change. Place in fresh buffer and leave in refrigerator overnight.
3. to 16. as for S1 (pp 118).

After this technique the ultrathin sections were stained for 15 minutes in 5% uranyl acetate and 10 minutes in Reynolds' Lead citrate.

Schedule 3 gives the method used for deamination pedal gland tissue prior to preparation for electron microscopy. For this schedule the pedal gland tissue of one slug was divided into three groups.

S3 Deamination techniques

Group 1: Tissue treated as for Schedule 1, omitting stages 3 and 4.

Group 2: Deamination group. Stages 1 and 2 as for Schedule 1.

3. Tissue treated with nitrous acid, (6 g Sodium nitrite in 35 ml distilled water together with 5 ml of glacial acetic acid), pH 3.95, for 6 hours at room temperature. Agitated.
4. Rinse tissue briefly in buffer solution.
5. 50% Alcohol for 15 mins., 2 changes, agitated.
6. 70% alcohol in refri-gerator overnight.
7. to 14. as in the same stages in Schedule 1, except that left in stage 14. for $3\frac{1}{2}$ hours.

15. and 16. as before.

Group 3: Control group. Stages 1. and 2. carried out as for Schedule 1.

3. Tissue placed in water whose pH adjusted to 3.95 by NHCl for 6 hours at room temperature. Agitated.
4. to 16. as for group 2.

The following schedule is:

S4 A Rapid Schedule for Treating Tissue with Pepsin

Divide tissue from one gland into two groups.

1. Fix in 3% Glutaraldehyde in 0.1M phosphate buffer, pH 6.1, for 2 hours. Agitate.
2. Wash in 0.1M phosphate buffer, pH 6.1, for 30 minutes. Wash in 0.1M phosphate buffer, pH 6.1, for 30 minutes. Agitate.
3. One group of tissue placed in pepsin solution (Pepsin 1:2500 powder, BDH suppliers). 0.2% Pepsin in 0.1NHCl, pH 1.6 to 1.7, for 1 hour at 37°C.

Second group of tissue (Control) placed in O.1NHCl for same time and temperature. (Lev and Gerard 1967).

4. Wash in distilled water. Agitate.
5. Stain in 2% uranyl acetate for 5 mins. Agitate.
6. Rinse in distilled water.
7. Dehydrate in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 95% alcohol for 5 minutes each. Agitate.
8. Absolute alcohol, 2 changes for 5 minutes each. Agitate.
9. Absolute alcohol: Propylene Oxide, 2:1, for 10 minutes.
10. Absolute alcohol: Propylene Oxide, 1:2, for 10 minutes.
11. Propylene Oxide. 2 changes for 10 minutes each.
12. Propylene Oxide: Resin, 2:1 for 30 minutes.
13. Propylene Oxide: Resin, 1:2 for 30 minutes.
14. Resin. 2 changes for 30 minutes.
15. Leave in resin overnight.
16. Embed.

The last schedule, which gives better contrast, is:

S5 A Rapid Schedule for Staining Tissue in The Block, using Uranyl Acetate

1. Fix in 3% Glutaraldehyde in O.1M phosphate buffer, pH 6.1, for 2 hours. Agitate.
2. Wash in O.1M phosphate buffer, pH 6.1, for 30 minutes. Agitate.
3. 1% OsO_4 in O.1M phosphate buffer, pH 6.1, for 1 hour. Agitate.
4. Rinse in distilled water 5 minutes. Agitate.
5. Stain in 2% aqueous Uranyl acetate for 5 minutes. Agitate.
6. Rinse in distilled water for 5 minutes. Agitate.
7. Dehydrate in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 95% alcohol for 5 minutes each. Agitate.
8. Absolute alcohol, 2 changes for 5 minutes each. Agitate.
9. Absolute alcohol:Propylene oxide, 2.1, for 10 minutes. Agitate.

10. Absolute alcohol:Propylene oxide, 1:2, for 10 minutes. Agitate.

11. Propylene oxide, 2 changes for 10 minutes each. Agitate.

12. Propylene oxide:Resin, 2:1, for 30 minutes. Agitate.

13. Propylene oxide:Resin, 1:2, for 30 minutes. Agitate.

14. Resin, 2 changes for 30 minutes each. Agitate.

15. Leave in resin overnight. Agitate.

16. Embed.

Following treatment in Schedules 4 and 5 the ultrathin sections were stained for 15 minutes in 5% uranyl acetate and 10 minutes in Reynolds' lead citrate.

These then were the various ways in which the tissues were prepared for electron microscopy and the results will be given after a brief historical survey.

EM1 (3) HISTORICAL BACKGROUND

Wondrak's papers on the ultrastructure of the sole gland cells of Arion empiricorum and Arion rufus will be considered before reporting the present findings.

Wondrak's papers have already been mentioned briefly in the historical review relating to the light microscope section, and as stated before the paper produced in 1967 described the mucous gland cells of Arion empiricorum. He found two distinct gland cell types, the ventral sole gland and the lateral sole gland, whose gross appearance and secretion differed as described in LM.3 pp 28 .

The ventral sole gland cell possessed a basal cell body which lay deep in the connective tissue; and also a twisting gland duct whose upper region was attached to the neighbouring epithelial cells by a zonula adhaerens and septate desmosomes. The free surface of the gland duct often bore microvilli and most of the organelles were situated in the cell body. The most unusual feature was the presence of an extensive

tubule-filled, rough endoplasmic reticulum. These tubules were believed to radiate from a central point, except at the ramifications, whilst in tangential sections the tubules were seen as circles. The nucleus of the ventral sole gland cell was very rich in chromatin (of a coarse, granular structure), and surrounded by a distinct, wide perinuclear gap. This gap contained the same inner tubular structure as the endoplasmic reticulum. Extracisternally, electron dense, membrane bound granules (about 0.5µm to 0.8µm wide) were found, they seemed to represent stages in the formation of the secretion product. Nerve endings with synaptic vesicles were often found close to the gland cell body. What role the tubules in the endoplasmic reticulum played in the formation of secretion was not known.

The lateral sole gland cell had a form rather like the ventral ones but it was fortified in the epithelium with 'homogeneous contact zones'. Another point of difference concerned the structure of the rough endoplasmic reticulum, since no tubules were seen and the ribosomes showed various enlargements depending on their functional state. The cell nucleus was also rich in coarsely granular chromatin, and its outer membrane was bounded by ribosomes. During the secretion cycle extracisternal granules were formed, being membrane bound, but appearing less electron dense and larger (1µm to 2µm) than in the ventral sole gland cells. They were innervated in the same way as the ventral cells and, again, he did not know the function of the lateral sole gland cells but believed the highly developed, closely packed membrane system of the endoplasmic reticulum suggested that protein was a major component of the secretion.

The mantle gland cells were also described by Wondrak but this will be considered in relation to the ultrastructure of the mantle of Arion hortensis (EM2 (3) pp166-167).

The sole gland cells of Arion rufus were described in a paper

(1969) and they had a very similar structure to those of Arion empiricorum, save that the small tubules of the endoplasmic reticulum were approximately 0.02μm in diameter, and that the width of the endoplasmic reticulum tubules was very constant (about 0.15μm to 0.2μm), except at the points of ramification. He described stages in the formation of the secretion and suggested that the ingestion phase was characterised by clearly distinguishable gland cell bodies showing surface invaginations. Extracisternally, electron-dense, membrane-bound granules were seen, with a diameter of about 0.5μm and a 'spongy' structure. Also extracisternally, less electron-dense structures were seen which were not membrane-bound but which showed a very regular shape. The dark granules were never seen to be extruded, but there were many cells whose homogeneous, only slightly electron-dense contents, were extruded through their ducts.

The peripodial gland cells (equivalent to the lateral sole gland cells of Arion empiricorum) resembled those of the sole in their outward appearance but the structure of the endoplasmic reticulum was again rather different, being of a type known to be associated with protein secretion, and lacking tubules. These cells were innervated and, once again the ingestion phase was characterised by surface invaginations. Membrane-bound granules of 1μm to 2μm diameter were seen lying extracisternally during the secretion process as well as non-membrane bound structures of slight electron density. Membrane-bound granules were seen along the neck as far as the free surface, but the extrusion of granules could not be seen.

Wondrak's description of the mantle gland cells of this species will be considered in the section concerned with the ultrastructure of the mantle of Arion hortensis (EM2 (3), pp167-168).

Wondrak (1968) also described the epidermal structure of Arion rufus and found the central part and edges of the sole to be ciliated just as in Arion hortensis (LM4(i)). He described two types of

epithelium which covered most of the body surface: the first type was termed by him a striated epidermis, and the second was ciliated. The striated epidermis was composed of cells, with irregularly shaped nuclei, bearing microvilli which in turn bore fine fibrils. Below the free surface of these epidermal cells lay an organelle-free layer of cytoplasm which was traversed by a 'terminal web' (after Palay and Karlin 1959). The epidermal cell was crossed from apex to base by bundles of fibrils which divided at one end, and the cell organelles lay in the cylinder thus formed.

Numerous vesicles were found in these cells, lying between the free surface and the nucleus and most of the mitochondria appeared in the supranuclear region, embedded in endoplasmic reticulum. Apocrine secretion was described as occurring in the dorsal surface of the mantle from cells resembling the striated cells, and during secretion the microvilli were lost and the epithelium became crenellated as small vesicles were 'pinched off' from its surface.

The ciliated cells were cylindrical, or sometimes, in the case of the foot sole, conical. The inner ends of these cells were deeply indented and a basal lamina passed between these indentations. He found each cilium to sit on a pedestal but otherwise to show the typical 9+2 internal structure, and to possess a striated root. He sometimes found the free cell surface to display invaginations and the cytoplasm below to contain vesicles. Fibrils were present traversing the cell from apex to base, and the organelles were surrounded by this fibrous cylinder. The nuclei appeared as in the striated cells and above these lay mitochondria and many smooth-walled vesicles which contained granular structures of medium electron density. The endoplasmic reticulum was only slightly developed in these cells. The Golgi complexes were found to lie near the nuclei and the cisternae contained material of varying electron density.

Wondrak described a zonula adhaerens, an intermediate zone and a zonula septata between neighbouring epidermal cells but some lateral walls showed a macula occludens (desmosome) instead. Furthermore, these lateral walls showed contours, which interlocked with corresponding ones from the neighbouring cells so that the cells were anchored together.

EM1 (4) RESULTS

(i) The Epidermis of the Pedal Gland Duct

The light microscopic structure of the epidermis has already been described LM.4(i) pp 32-34. Unfortunately, very few micrographs were obtained of the upper epidermis of the duct because it was necessary to trim the tissue blocks, before ultrathin sections could be taken, to ensure that there was no pure resin/tissue resin boundary.... for this could cause the knife to vibrate and produce knife marks. This trimming meant that the upper epidermis was usually sacrificed since it was the tissue at the edge of the block.

However, Plate 1 shows part of the upper epidermis and as can be seen it is a rather complex and apparently rather disorganised layer. The various components seem to be very loosely packed but whether this reflects a normal situation is not known. It is possible that fixation has caused disruption of the tissue and that the spaces are not natural.

Two epithelial cells can be seen in the lower right quadrant of the micrograph. They are ovoid in shape with the longer axis approximately parallel to the width of the duct. Their nuclei are also somewhat elongated in this plane, and show peripheral and central patches of chromatin. One of these patches is larger than the others and may represent a nucleolus although its structure cannot be discerned. The nuclei are large in relation to the rest of the cell,

occupying about half of its volume.

The cytoplasm is seen to contain mitochondria and small vesicles of secretory material of medium electron density. Golgi complexes and endoplasmic reticulum can not be distinguished but this is probably because of the low magnification and poor quality of the print. What this secretion might be is not known since it was not detected at the light microscope level, for it appears to be produced in very small quantities. However, Barr (1927-1928), described the presence of some secretory cells in the posterior part of Arion ater (p. 22).

The outer (apical) surface of these upper epidermal cells is covered with numerous, long, fine microvilli, approximately 1.5µm to 1.75µm by 0.09µm. As can be seen from the cross-section through the ends of a group of microvilli in the lower left quadrant of the print, they are very closely packed. Their function is presumably an absorptive one but what they can be absorbing is not known unless it is to increase the viscosity of the secretion by removing fluid.

The appearance of these microvilli is not constant, for in Plate 3 the upper epidermis of another slug's pedal gland can be seen and here the microvilli are short, on average 0.39µm and relatively broad, 0.11µm. Also, they are quite scattered over the cell surface and not densely packed. Again, the reason for this variation is not known. It might represent a variation along the length of the gland since a survey of the upper epidermis was not possible due to the difficulties already mentioned, or else it could reflect a seasonal variation.

Basally, the epidermal cells of the upper epidermis are flanked by connective tissue cells, collagen and small bundles of muscle fibres, and muscle fibres are located in the extreme edges of the upper surface of the gland duct.

Plate 2 shows the same area as Plate 1, but illustrates the connection between the upper epidermis of the gland duct and the ciliated

hump of the lower epidermis. This connection is made by intermediate epidermal cells, which are very like those of the upper epidermis although consistently smaller. They are covered with microvilli but these are neither so numerous nor so long, 0.6μm to 0.7μm by about 0.12μm wide.

None of the microvilli appeared to show any internal fibrillar structure, such as was described by Sumner (1966) in the microvilli of the digestive gland cells of Helix aspersa, or by Ovtracht (1967) in the multifid gland of Helix pomatia.

Plate 3 shows the right ciliated hump of the lower epidermis. This tissue has been treated according to Schedule 5, and the contrast within it is rather better.

The cilia show the typical 9+2 arrangement of fibres, and in transverse section their diameter is, on average, 0.22μm, about twice the diameter of the microvilli of the upper surface of this same tissue. Their length cannot be ascertained since they are cut obliquely, but it is much greater than that of the microvilli.

Microvilli also intermingle with the cilia on the hump, and they are of yet another size, 0.64μm long by 0.13μm wide with apparently no internal structure. In shape they appear to be somewhat club-shaped with the narrower end abutting on to the epidermal cell edge. These measurements are comparable with the other measurements given from Plate 3 but not from Plates 1 and 2 because these are from a different animal. In some cases it seems that the cilia and microvilli alternate with each other on the humps, but in others it seems that there are a few microvilli side by side. Of the six epithelial cells visible in Plate 3, five show a mixture of cilia and microvilli and one cell shows microvilli only. In any one cell section (except Cell 3), the number of microvilli and cilia is approximately equivalent although there may be a slight preponderance of one type or other. This pattern is

repeated in montages of the gland lumen in other animals. The exception is cell 3 which bears microvilli only, and it would seem therefore that this cell has an absorptive function of some sort, whilst the other cells function to absorb and to conduct the secretion forwards in the gland duct.

Plate 9 shows a cell neck(or duct) from one of the mucocytes to the surface, lined with microvilli. The microvilli are possibly involved in absorbing some substance from the secretion prior to discharge, or else are absorbing something from the lumen.

The other structures within the epidermal cells will be illustrated in Plates 5 and 6 which show the same cells but at a higher magnification. It can be seen that the ciliated cells appear to taper basally, whilst the microvillar cell does not, but appears slightly more expanded at its base.

Plate 3 also shows the area where the epidermal cells unite with those cells below them which form the rest of the gland. The cell bases appear fairly electron-dense and to the left of the plate the area appears to be fibrous. Plate4 shows a higher magnification of the fibrous region which could be part of a basal lamina underlain by a nerve, but since there is no evidence of such a basal lamina elsewhere, this interpretation must be in doubt.

Cells 2, 3 and 4, as discussed earlier, are seen in higher magnification (x 24,620), in Plate 5, where it is possible to see the typically structured cilium, with its 9 paired outer fibres and the two central, single fibres from which radiate electron-dense bands. The outer membrane of the cilium in Arion is confluent with the epidermal cell membrane and appears to be ridged longitudinally but the size of these ridges is variable, sometimes being quite long. This was seen in the cilia of all the slugs examined but whether this ridging is

natural or induced by fixation is not known. A dark transverse basal plate is seen at the base of the cilium just a short distance above the main part of the basal body which is embedded in the apical tissue of the epidermal cell. The internal fibres of the cilium appear to end on or close to this plate, as is usually found. The basal body in this animal is somewhat elongated but tapers slightly at each end. Its central core is encompassed by an electron dense 'cylinder'. From the inner end of the basal body, a single unbranched rootlet passes into the depths of the epidermal cells. These rootlets taper as they pass inwards and bear transverse bandings of a more electron-dense nature. This banding cannot be seen clearly in Plates 5 and 6 because of the very intense staining induced by the use of uranyl acetate in the block (Schedule 5). The greatest distance these rootlets extended was 1.9µm and they did not appear to terminate in association with any particular cell structure. They could act as some type of 'anchoring' mechanism.

At this magnification the microvilli still appear to have no obvious internal structure and are somewhat club shaped as described above. The outer membrane appears slightly wrinkled.

Immediately within the upper surface of the ciliated cell, there is a band of tissue which is quite uniform and of medium electron density and only the basal bodies and rootlets of the cilia lie in it. It is equivalent to the 'terminal web' described by Wondrak p.125 and is absent in the microvillar cells.

Numerous mitochondria are visible in the upper regions of the mixed ciliated and microvillar cells. Mitochondria are also present in the microvillar cell but they are not so numerous and tend to be more scattered in the cytoplasm. The mitochondria in both cases are of a typical structure and are elongated, up to 1.4µm long and 0.35µm wide in some places. The diagonal cristae are fairly numerous but it is not possible to count them accurately because of oblique sectioning which

blurs the image, but there may be up to about ten in any one mitochondrion. One or two electron-dense intramitochondrial granules are seen within the matrix, and these are believed to represent areas containing bivalent cations.

The mitochondria contain energy, in the form of ATP and hence a concentration of mitochondria associated with the ciliated cells suggests that these mitochondria are supplying energy to the cilia for movement.

A rather unusually shaped mitochondrion is seen in Plate 6, and possibly this indicates that the mitochondrion is cup-shaped at one end, the angle of sectioning giving the appearance of a bifurcation within it. It could be in what is known as a dividing phase.

The cell cytoplasm contains small, electron-dense particles which could represent ribosomes, polyribosomes, or glycogen. This last is possibly more likely because of the aggregation of these particles. The glycogen would serve as a reserve food or energy supply, and Hyman (1967) found glycogen to be distributed throughout the tissues of pulmonates, constituting a food reserve.

The nuclei of the epidermal cells are centrally or basally positioned and are usually elongated along the axis at right angles to the upper cell surface. Plate 6 shows such a nucleus, although it is rather irregular in outline with an extension. It is bounded by a double membrane whose two surfaces are separated by a gap of varying width. It shows quite clearly a nuclear pore, 730Å in diameter, which apparently penetrates the nuclear membranes, the latter being linked to form the wall of the pore. The diameter of this pore is just within the range of sizes, 500 to 750Å, given by Toner & Carr (1968) whilst Lainé (1971) gave a diameter of 500Å for nuclear pores of the M mucous gland cells of Agriolimax. However, when examining the pore using a x10 eyepiece, it appears to be blocked by an electron dense band, and this diaphragm has been described many times, for instance by Watson (1955), Fawcett (1966)

and Toner & Carr (1968). It is through these pores that exchanges between the nucleus and the rest of the cell are believed to occur (Fawcett 1966).

The nucleus appears typical with a moderately electron-dense granular nucleoplasm together with electron-dense patches of chromatin. Some of these patches are scattered and isolated within the nucleoplasm but there is also a band of chromatin closely applied to the inner nuclear membrane and it is continuous round the nucleus except in the regions of the nuclear pore. Why some chromatin aggregates at the membrane is not known, it could indicate faulty fixation but it might reflect some natural feature within the cell. The number of isolated patches varies from cell to cell and none of the epidermal nuclei seen in Plates 5 and 6 appears to have nucleoli visible in the plane of sectioning. No Golgi complexes or endoplasmic reticulum are evident in any of the ciliated/microvillar cells examined, but their presence can not be dismissed. Most of the slugs examined were fixed using Schedule 1, and by this method the cells of the lower epidermis tend to appear very dense and the internal cellular structures cannot be easily detected. Only material treated by Schedule 5 showed these structures well and even by this method Golgi complexes and endoplasmic reticulum could not be seen in these cells.

In the cell bearing only microvilli the cytoplasm is granular (either ribosomes or glycogen) and contains some mitochondria which are scattered in the cytoplasm. However, there is a Golgi complex present in this cell, closely applied to the upper edge of the nucleus. It is composed of four cisternae containing a substance of medium electron density and to one end of these cisternae are a number of vesicles containing a similar substance. The nature of this secretion is unknown. There is no evidence of an endoplasmic reticulum but it may be reduced.

At the upper edges of the junctions of the epidermal cells

there is a small indent and space between two abutting cells. The depth of this gap varies somewhat but is on average about 4600Å, whilst Wondrak (1968) found a depth of 2000Å in Arion rufus. Ovtracht (1967) noted a depth of 5000Å in the multifid gland of Helix pomatia, and Lainé one of 2000Å to 8000Å in Agriolimax reticulatus. Thus the results on the pedal gland in Arion hortensis resemble quite closely those of the multifid gland of Helix pomatia which is interesting since they are both internal glands. The inner third of this indent has a thickened electron dense desmosome applied to it and this whole region is termed the zonula adhaerens. The width of the zonula adhaerens is approximately 408Å at the thickened base but widens at the cell surface to 1265Å. Wondrak found this width to be 200Å, whilst Ovtracht noted one of 100 to 180Å and Lainé one of 350 and 1000Å.

Below this zonula adhaerens is a zonula septata or septate desmosome. One such structure is seen in Plate 6, where three intercellular bridges are clearly visible linking the two cellular membrances and the distance between the two cells is reduced to only 163Å. The distance that is covered by the zonula septata is not known because of oblique sections, but it seems from the lateral membranes of the ciliated/microvillar cells that the cell boundaries meander considerably and may show 'bloated' regions where the two adjacent membranes are widely separated. Whether this separation represents a normal feature or is induced by fixation is not known.

Plate 7 illustrates a zonula septata between an epithelial cell and an interstitial cell or part of a mucocyte. The double structure of the unit membrane can be seen and the distance separating the two is 108Å. The distance between the two electron dense bands of the unit membrane is about 32 Å. The intercellular bridges linking the unit membranes appear to be composed of two parallel electron dense bands (using a magnifying eyepiece), but this is not clear.

In Plate 6 there is a large area between the epidermal cells which contains some mitochondria, thin fibres and some membrane-bound vesicles whose appearance varies depending on the density of the central core. This latter may be pale to very electron-dense, bounded by a pale region. The whole 'cell', if it is one, is membrane-bound, and its nature is unknown.

Plate 8 illustrates the region between the two epithelial humps in the lower epidermis. The variation in ciliation between the central and lateral bands of the lower epidermis as described in the light microscopy section (LM.4(ix) pp 89) is evident here. Parts of the two ciliated humps can be seen encompassing the central band. In the central region the epidermis is quite distorted and the whole area is filled by parts of mature mucocytes, discharging or about to discharge. The mucocytes contain individual or coalesced sacs of secretion and these sacs may be seen whole or ruptured in the lumen. There are no obvious epidermal cells present in this central region, but there are a few scattered cilia present which are attached to reduced amounts of cytoplasm which presumably are parts of distorted epidermal cells. Hence the number of cilia present is a reflection of the number of epidermal cells present: they are closely packed in the humps where the cilia are very numerous and very scattered or reduced in the central band. No microvilli are evident in the central region, but this might be because of the low magnification. Two small nuclei are present below the gland masses in the central region and these are probably epidermal nuclei displaced by the secretion. This would agree with the finding of few, and basally situated, nuclei in this central region in the light microscopy LM.4(ix) pp 89). More will be said concerning the secretion (pp154-156) and the mechanism of discharge (EM1.(4) (ii)d. pp161-163) later. The central band is not always quite so disrupted as it appears in Plate 8 and this is the only difference that is seen in the pedal gland along its

length, since the patch of young cells at the anterior end of the gland were not seen with the electron microscope. In the regions of the gland closest to the gland pore (and extending inwards from that point for about one-third of the length of the gland) there is evidence of more discharging cells. Further along the gland, discharging cells intermingle with the epidermal cells and consequently less disruption occurs and more cilia are present (Plate 9).

Plate 9 also shows that the base of the cilium is encased in a small cytoplasmic sheath which is equivalent to the pedestal described by Wondrak (1968).

The same plate illustrates the last feature to be discussed in relation to the lower epidermis. This is the presence of long, thin microtubules within the epidermal cells. These microtubules have a rather specific orientation, lying parallel to each other and passing from the apical region of the epidermal cell towards the base and at right angles to the apical surface. It is more usual for such tubules to lie parallel to the surface but Wondrak noticed a similar organisation of microtubules in the foot sole epidermal cells of both Arion empiricorum and Arion rufus. He found that the epidermal cell organelles lay within these tubules; and also stated that, at least in Arion rufus, the bundles of fibrils divided at one end. Hubendick (1958) also described fibrils in the adhesive epithelium of the mollusc, Acroloxus lacustris (Müller), and found them to run perpendicularly from cell base to cell surface, but their function was not discussed. Schmekel (1971) described bundles of fine tonofilaments in the middle region of the mucous cells comprising part of the nudibranch genital system.

The tubules are particularly noticeable around the ducts of the discharging mucocytes, and where the surface of the epidermis has been cut superficially the tubules appear to be converging on one point. They seem to be associated with the epidermal cells of the central discharging

region. There is no evidence of their presence in the ciliated hump illustrated in Plates 4, 5 and 6, which had been treated as for Schedule 5. These humps, when treated according to Schedule 1, appear to be very dense and the presence of microtubules can not be established. It does seem from the evidence that the microtubules are confined to the epithelial cells of the central region, but are often not visible here when the amount of secretion present is great and the epidermal cells are distorted and displaced. It is possible that they provide some support to the ducts which pass between the epidermal cells. A certain degree of rigidity may ensure that the duct remains open, and this could be necessary if discharge is effected by contraction of the muscles of the foot, as is believed. Movement may result in some of the ducts being closed if they lack some form of support. However, such an interpretation can only be supposition. Microtubules are not visible in Plate 8, possibly because of the low magnification.

The next section will describe the ultrastructure of a 'typical' pedal mucocyte.

EM1 (4)

(ii) The Pedal Mucocyte

(a) Structure

Plate 10 shows a fairly typical mucocyte, but it should be borne in mind that these cells undergo changes during the development of secretion, which will be discussed in the next section. This tissue is treated with uranyl acetate in the block and, as can be seen, good definition is obtained although the nuclei stain rather darkly.

The components of the mucocyte can be seen to be a nucleus, rough endoplasmic reticulum, Golgi bodies, mitochondria and sacs of secretion.

The nucleus is laterally positioned close to the cell boundary

possibly being displaced by secretion, for sometimes the nucleus adopts a central position. Plate 11 shows another interphase nucleus at higher magnification. This material has been treated with alcian blue in the block and once again the chromatin is stained very strongly. In order to obtain a picture of the nucleus that was not too dark it was necessary to under-develop the print, hence the rest of the cell contents appear very pale. As can be seen the nucleus is quite regular in shape being slightly longer along one axis (7.75μ by 6.65μ). This ovoid shape is fairly typical, but the long axis is usually slightly longer in relation to the short axis than is indicated here.

The double membrane around the nucleus is visible although not so clear as in the epidermal cell seen in Plate 6, and nuclear pores can not be discerned. There could be several reasons for this, two depending on technique and two depending on actual structural differences. The first two involve accuracy of focus and obliqueness of sectioning. The two structural differences are that the outer nuclear membrane is bordered on its external surface by ribosomes and the perinuclear space (between the two membranes) is filled with small tubules. Although the structure of the perinuclear space is much like that in the endoplasmic reticulum, its width is much less than the ER cisternae, varying from about 350Å to 450Å, whilst the cisternae of the reticulum is on average 1350Å in this plate (in regions which are neither oblique nor ramified). The similarity of perinuclear space and endoplasmic reticulum was noted as early as 1955 by Watson, and the endoplasmic reticulum is believed to be an extension of the nuclear membrane. However, the space may be narrower, and the tubular structure not in evidence (Plate 12). The reason for this is not known, but it may reflect some other functional state. Within the double nuclear membrane is the nucleoplasm, chromatin and nucleolus. The nucleoplasm consists of a pale matrix with more electron-dense material (diffuse chromatin?) in it, and contains the

patches of chromatin. As can be seen in Plate 11, these patches are numerous, many more than are evident in the epidermal nuclei (Plate 3). This is possibly related to the fact that the former nuclei must be very active in the secreting cells. There is a slight tendency for the chromatin patches to be arranged nearer to the nuclear membrane, although there are some central patches. However, chromatin is not present in an almost continuous band around the inner membrane in these nuclei, as occurs in the epidermal nuclei. Again it is not clear whether this tendency to gravitate towards the membrane is due to fixation or whether this is a natural feature. One is inclined to think that it is a natural feature because the chromatin is not aggregated into large clumps, as might occur in bad fixation.

A nucleolus is evident in Plate 11, being displaced to one end of the nucleus and towards the nuclear wall. It is not membrane bound and the two areas that comprise the nucleolus are visible. The very densely staining central core is termed the nucleonema whilst the less dense outer region is the pars amorpha.

Plate 13 shows the only example of a binucleolate nucleus found in the pedal mucocytes. This could mean that it is a rare occurrence or that possibly the nucleus has been sectioned in an unusual plane. It might represent a longitudinal section of the nucleus showing a bipolar arrangement of nucleoli, whilst those nuclei showing only one nucleolus have been sectioned in a transverse plane.

The nucleus is invariably embedded in masses of endoplasmic reticulum (Plates 11 to 13), but as more and more secretion is produced it begins to encroach on the nucleus and displace or reduce the reticulum.

It is evident in the above plates that the endoplasmic reticulum bears ribosomes externally and is filled with tubules internally. The system is envisaged as being composed of stacks of

ramifying membrane-bound cisternae as illustrated in Fig.30.

Evidence of these ramifying cisternae can be seen in Plate 14. The changes in orientation of tubules within the ramifications would result from the meeting of two or more cisternae at any one point, the cisternae being at perhaps 90° to one another. Since the cisternal tubules are believed to be distinctly orientated the different profiles of tubules would be expected when two cisternae meet. The orientation of the tubules will be discussed later (pp 140-142). In this highly structured endoplasmic reticulum the cisternae are fairly constant in width, on average 1100Å, although some parts appear wider, (up to 1700Å), but this difference is never great except at points of ramification of the plates. The narrowest measurement between the walls of the endoplasmic reticulum is 570Å. These measurements fall into the range of 300Å to 3000Å described by Goldblatt (1969). At the points of ramification the measurements vary considerably, the one on Plate 15 measuring about 3700Å. The variations in width, excluding the ramifications, could be explained in terms of obliqueness of sectioning.

The walls of the endoplasmic reticulum can be discerned in some places and it can be seen to be a typical unit membrane, but only 53Å wide. This measurement agrees well with the 50Å given by Goldblatt (1969).

The distance between each cisterna in the 'stack' is fairly constant, averaging 460Å in the areas where the membranes can be discerned for accurate measurement, and this is much narrower than the 1500Å quoted by Goldblatt. Ribosomes line the external surface of the cisternal walls, and although their outline is not absolutely clear, their size would seem to be fairly constant. Plate 16 shows the ribosomes more clearly since the Alcian blue/Glutaraldehyde technique causes the chromatin and the ribosomes to appear more electron dense. They are typically not entirely spherical, but tend to have a longer axis at

right angles to the membrane of the endoplasmic reticulum. The average length of the long axis is 218Å, but it can be as much as 263Å; whilst the width is, on average, 143Å. Toner and Carr (1968) quoted an average diameter of 150Å which agrees with the width of the above ribosomes, but indicates a considerable extension of the axis perpendicular to the reticulum membrane. The reason for this is not known, but it might reflect a condition of increased activity. The measurements show there is some size fluctuation even on the same cisterna. Plate 16 also shows some regions of the endoplasmic reticulum which have been sectioned superficially so that a mass of ribosomes can be seen. These ribosomes are fairly evenly spaced, sometimes seeming to be arranged in alternating rows, but not in rosettes. The distance between the ribosomes varied, being on average 95Å. Some areas of the endoplasmic reticulum wall are not covered with ribosomes, this could be where 'blebs' (pp. 149) are about to form.

The arrangement of microtubules within the cisternae appears to be highly organised, although this is not clear in the micrographs because of oblique sectioning. They are very closely packed and their diameter constant. However, they are flattened in one direction so that the average length of the long axis is about 250Å as opposed to 185Å along the axis at right angles to the long axis. Wondrak (1969) found these tubules to be about 200Å wide in Arion rufus, quite similar to that in A. hortensis. Because of this uniformity of size and shape, it is possible that these tubules are rather rigid in construction or turgid. The walls of the tubules appear to be of a single layer, not the trilaminar structure of the unit membranes, and this will be considered later (143-146).

Wondrak (1967, 1969) proposed that the arrangement of ER tubules in Arion empiricorum and Arion rufus (ventral sole gland cells and sole gland cells respectively) is as illustrated in Fig.31. The

tubules are arranged radially from an approximately central point when seen in circular profile (transverse section). According to his diagrams the individual tubule is shaped rather like a cul-de-sac with the 'blind' end at the central point. He made no comment about the relationship of the ribosomes to these tubules and did not know what function they might serve.

In Arion hortensis it has already been stated that the cisternae are in the form of plates and not cylindrical. One reason for this suggestion is that circular profiles are never seen. This could mean that the cisternae are very rigidly oriented so that their longitudinal outlines are always presented in section, but this would seem to be rather unlikely. However, if 'plates' of cisternae are accepted (Fig.30), then no matter whether sectioning is longitudinal or transverse, the long cisternal profile will be seen. The presence of such plates tends to be confirmed in Plate 17 where wide expanses of endoplasmic reticulum can be seen, representing tangentially sectioned plates. Thus Wondrak's interpretation must be modified for the pedal gland mucocytes of Arion hortensis. Since the form of the endoplasmic reticulum is considered to be different then Wondrak's proposed organisation of the internal tubules must be altered since the arrangement illustrated in Fig.31(ii) would not fit into the plate system.

There are two possible alternatives for the organisation. The first is a modification of Wondrak's, illustrated in Fig.32. The tubules would still represent small 'cul-de-sacs' passing from the membrane to an approximately central point within the cisterna. This arrangement would give the plates of circular profiles seen in Plate 17 when sectioned tangentially. This type of arrangement would seem to be borne out in Plate 18 where there is a distinct 'line' down the centre of the cisterna where the two rows of tubules meet. Not all the tubules meet in the centre of the cisterna, some penetrate two-thirds of the way across

and the tubules opposing these extend for only one-third of the width, as in Fig,33. This would suggest that either the arrangement of tubules is not quite so orderly as indicated in Fig.32, or else that the appearance results from oblique sectioning. However, there are instances in a number of micrographs where some tubules extend from wall to wall. These can be seen in Plates 18, 19, 20 and 21. Such tubules would be impossible in either Wondrak's proposed arrangement or in that shown in Fig.32. It would seem therefore, that the tubules are less orderly, extending for varying lengths across the cisternae, although in the majority of cases two tubules would appear to meet centrally.

An alternative construction would be the presence of tubules within the endoplasmic reticulum which run from wall to wall. If these tubules were to adopt a shallowly 'S'-shaped or wavy configuration then the difficulties already mentioned might be explained. They would appear as in Fig.34(i). Sectioning cisternae containing ranks of these tubules would give the appearance of two tubules leading from an approximately central point, or from any other point. The two pieces would in fact be of two separate parts of adjacent 'bent' tubules, and the angle of sectioning would give the appearance of two short tubules meeting centrally, as illustrated in Fig.34(ii). The angle of sectioning would have to be very precise to give the appearance of a whole tubule (Fig.34 (ii), section angle 2), and such an event would be rare. This is the case in the micrographs shown, where only one or two instances are visible in any one Plate.

Unfortunately, it is not easy to draw the construction accurately since it must be seen in 3-D. With a closely packed system of slightly bent, wall to wall tubules arranged in plate-like cisternae, the difficulties which have been mentioned can be eliminated.

The principal problem remaining is the relationship of ribosomes to the tubules within the cisternae. Wondrak makes no mention of

this. It is a difficult problem because the tubules and ribosomes never appear with very clear outlines. In Plate 18 there is one region where there appears to be a 1:1 ratio of ribosomes to tubules, but in other counts the number of tubules to ribosomes is always more in the ratio of 1.2 to 1. However, because of the relative lack of areas where counting is possible these figures cannot be taken as absolute. Since the ratio of tubules to ribosomes is very close it seems likely that there are ribosomes at both ends of the tubules rather than one ribosome at one end only, as shown in Fig.35. When a clear picture of tubules and ribosomes is seen, Plate 18, the ribosomes are apparently placed directly over the tubules as illustrated in Fig.35 and not over the adjacent walls.

The two functions attributed to the endoplasmic reticulum are the separation of protein (produced on the ribosomes) from other cytoplasmic components, and its transport. The arrangement of ribosomes over the tubules might suggest that proteins formed on the ribosomes under the influence of m-RNA and t-RNA in the cytoplasm might pass through the ER membrane into the tubules. The protein would then effectively be separated from the rest of the cytoplasm (Fig.36).

However, it is not easy to see how the protein could be transported in such a system when the tubule walls would be acting as barriers, unless the protein can move between the membranes - these latter are not typical unit membranes as has already been mentioned (p140).

One alternative to the nature of the tubules is that perhaps the 'walls' of the tubules are protein 'strings' and it was for that reason that the deamination and pepsin schedules were applied (Schedules 3 and 4). After deamination the tubules appear rather more distinct although they have contracted slightly away from the cisternal walls (Plate 22). The relative clarity could be due to the removal of free amino acids, which might be somewhat electron-dense thus reducing the contrast between the walls and contents of the tubules. It is also

interesting to note that the secretion appears to be considerably damaged and this would agree with the histochemical findings (LM.4 viii pp. 90) where metachromasia is lost after deamination, suggesting that the metachromatic groups are attached to protein. Plates 23 and 24 show the effect of pepsin. Again the secretion has been damaged, but in this instance the structures within the cisternae of the endoplasmic reticulum have also been very damaged. Some outlines of the tubules are visible (Plate 23) but the contents are very disorganised. However, this is also true of the other cell components, except the nucleus and the ribosomes. It seems likely that the pepsin is destroying all the protein within the tissue. Because of the damage to the tubules it is quite possible that the tubule walls are proteinaceous and not of the lipid/protein type ---- this finding would agree with the earlier mention that the walls of the tubules were of a single layer (pp. 140). The formation of cylinders of protein from the ribosomes would appear to be a rather unlikely proposition, but past workers have found such structures in the endoplasmic reticulum and believe them to be secretory products. De Martino et al. (1969) described tubular structures within the endothelial endoplasmic reticulum in the glomerular capillaries of the rhesus monkey and nephritic man, but they did not appear like those in Arion hortensis. They suggested that the tubular system might originate from the organisation of secretory products, and this could provide some backing for the suggestion that the tubules in the endoplasmic reticulum of Arion might be secretory products.

A further suggestion that the tubules in the endoplasmic reticulum might be secretory products was reiterated by Valeri et al. (1971) working on the rough endoplasmic reticulum of the pars intermedia of the hypophysis of Bufo paracnemis (L). Not all the cisternae contained tubules and the workers found that animals kept in darkness for three to four days lost these tubules and they believed that this

was evidence for the secretory nature of the tubules. The tubules appeared as in Fig.37, unlike those in Arion hortensis, but this could still be evidence that the tubules are of a secretory nature.

Spitznas et al. (1971) described, in some of the inner fibroblasts of the rabbit sclera the presence of two types of structures. The first was composed of two bands, running parallel to each other and to the cisternal membrane. These bands were divided up into electron dense and electron translucent units as shown in Fig.38 (i). Fig.38 (ii) shows the second type of structure in the rough endoplasmic reticulum, namely cross striations produced by alternating dark and light zones with a periodicity of 200Å. They were positioned at right angles to each other or were slightly tilted towards the limiting membranes of the cisternae. This latter description and measurement fits quite well with the tubules in A. hortensis, but those in Arion are clearly cylindrical. Transitions between the two types of structure could be seen in one cisterna of the sclera. Spitznas et al. believed that these structures were the result of an accumulation of synthesised substances in the cisternae of the reticulum and that these substances crystallise to form the inclusions. This interpretation was based on a suggestion made by Fawcett (1964) that proteins crystallise if they exceed a certain concentration in the endoplasmic reticulum. Thus it would seem that the tubular structures in the ER may result from the crystallisation of proteins when present in excessive quantities.

Lastly, Chandra (1968) described undulating tubules in the endoplasmic reticulum in the pathologic tissues in human cell lines. He proposed two possible arrangements of the tubules as shown in Fig.39. He believed that these inclusions were not viral but represented 'morphologic manifestations of pathologic changes occurring in affected cells' ..... a slightly different interpretation from that of the other workers.

From these results it seems possible that the tubules within

the endoplasmic reticulum of Arion hortensis could result from the crystallisation of large quantities of protein. The results with pepsin may add some weight to this interpretation. If this is so, it is interesting that a similar structure was found in the ventral and sole gland cells of Arion empiricorum and Arion rufus, and nowhere else. This would seem to suggest that this pattern of crystallisation is unique to the type of protein produced by these cells, and may even be characteristic of one type of secretion in the Arionidae.

A second type of endoplasmic reticulum is occasionally seen in the pedal gland, and is illustrated in Plate 25. The cisternae are more distended and the internal tubules are shorter and very disorganised, such that 'spaces' are present between the pieces of tubules, only short cylinders or their circular profiles being visible. Ribosomes still line the walls externally in a fairly orderly manner. Moreover, in the central patch of disorganised endoplasmic reticulum the walls of the cisternae appear to have broken down and the contents of the ER and the secretion are confluent. Because of the presence of these pieces of tubules, it is believed that the highly structured and the disorganised cisternae represent one and the same type of endoplasmic reticulum, the disorganised reticulum representing an exhausted stage in a dying cell. This interpretation tends to be confirmed by the presence of a myelin figure, which is believed to be produced in dying tissue when normal membrane systems break down. Mitochondria and Golgi complexes were also present in this mass, Plate 26. The mitochondria appear normal but the Golgi complex is also very distended which is unusual, and could indicate cell disruption.

Nuclei are hardly ever seen in association with these disorganised masses, the only example photographed is seen in part in Plate 34; it is rather elongated and considerably flattened in comparison with those of 'normal' mucocytes. Moreover, the nucleoplasm appears more electron dense than in the other cells and this could

reflect some change in its physical state. If the mucocytes are continuously producing secretion as is believed, then eventually one would expect the cell to become exhausted and therefore some evidence of cell decay might appear.

Associated with the endoplasmic reticulum are Golgi complexes, mitochondria and sacs of secretion. Plate 10 shows the Golgi complexes at low magnification. They seem to be scattered throughout the mucocyte and not just centred around the nucleus, although a spatial relationship between the nucleus and Golgi complex has been established in chick pancreas and the cells of plasma cell tumours (cf. Zeigel and Dalton 1962).

The Golgi complex is composed of stacks of parallel, flattened cisternae, some of the cisternae being very narrow and others very distended. Such a system is illustrated in Plate 27 between the cisternae of the endoplasmic reticulum.

The Golgi complex is generally accepted as displaying polarity in the production of secretion (Favard 1969; Novikoff and Shin 1964). There is a forming face which receives materials from the endoplasmic reticulum, and a mature face where Golgi vacuoles are separated. Smooth surfaced vesicles containing protein separate from the endoplasmic reticulum and fuse with the Golgi cisternae, supplying both extra membrane and protein. Membrane-bound sacs containing secretion are produced on the mature face. Thus, there is a progression in the production of secretion across the Golgi complex. The appearance of the Golgi complex, in Plate 27, is not quite so straightforward, for there would appear to be two mature faces, although larger vesicles are present on one face. The probable explanation for this lies in the generally accepted shape of the Golgi complex (Favard 1969), that is, that the cisternae are somewhat concave as shown in Fig.40. If this concave arrangement of disc-like cisternae were to be sectioned tangentially as

indicated in Fig.40 then the appearance would be rather like that seen in Plate 27. A more typically shaped complex can be seen in Plate 28, where there is one forming face and one mature face apparent. The cisternae are usually elongated and flattened centrally, but become distended at their ends as they fill with secretion. The number of cisternae per stack varies somewhat but it is usually between four and seven. Moreover, the diameter of the cisternae of two individual Golgi complexes appears to vary considerably, as can be seen by comparing Plates 27 and 28, but this could be due to the angle of sectioning.

In the complex seen in Plate 27 the narrowest distance across one cisterna is on average 160Å, widening at its ends to about 785Å. However, this latter figure is rather misleading since the ends may be hardly distended at all or vastly more distended. The sac labelled Sac.1 in Plate 27 represents a Golgi cisterna on the mature face which has filled with secretion but as yet has not separated from the complex. Sac.2 has filled with secretion, is separating from the complex and now represents a single sac of secretion.

As said before the membranes of the complex are smooth and do not appear to display the trilaminar unit membrane. Instead they seem to be a single structure measuring 53Å across - identical in measurement to that of the endoplasmic reticulum, but the latter membrane was believed to be a unit membrane. However, Beams and Kessel (1968) and Favard (1969) state that the Golgi cisterna has a unit membrane and oblique sectioning may give the appearance of a single structure.

Numerous vesicles can be seen in the region between the endoplasmic reticulum and the ends of the cisternae, they are of medium electron density rather like the contents of the endoplasmic reticulum, but not tubular, and are bound by smooth membranes. It is not possible to determine the nature of these smooth membranes but again their width is in the region of 52Å. The vesicles are spherical with an average diameter of about 480Å.

The secretion within the cisternae appears principally electron-translucent but containing the threads of material of medium electron density, and these appear to be somewhat aggregated in Sac.2. More will be said concerning the secretion later (pp154-156). The thread-like material is only clearly evident in the distended ends and vacuoles of the Golgi complex, it is not clear in the very narrow portions of the cisternae.

There is no evidence of vesicles budding off the endoplasmic reticulum in Plate 27, and in Plate 28 a group of four vesicles can be seen between the endoplasmic reticulum and the Golgi complex, but again no direct connection can be seen. In Plate 29, however, there is some evidence of a connection between these and the endoplasmic reticulum. The vesicles seem to be pinched off the endoplasmic reticulum and appear as if on short stalks (in Fig.41). Essner and Novikoff (1962) noted a similar event in hepatoma cells. In addition, it would appear that the ribosomes are lost in that part of the ER cisterna which is budding, as seen in Plate 29 but more clearly in Plate 28. This lack of ribosomes in endoplasmic reticulum adjacent to Golgi complexes was noted by Zeigel and Dalton (1962). The vesicles thus formed provide membranes and proteins to the Golgi, necessary since both are lost at the mature face.

It is evident that the Golgi complex 'packs' the secretion into membrane-bound vesicles, but it is believed that the secretion also becomes modified within the complex. Most of the evidence for this has come from other workers' radioautographic investigations. The proteins for the secretion are supplied by the endoplasmic reticulum, but carbohydrate units appear to be added in the Golgi complex ... thus the glucuronic acid units of the pedal gland secretion would be added here. When pure protein secretions are involved the Golgi complex may act as a packaging system, concentrating the secretion, as for instance in zymogen granules of the pancreas (Zeigel and Dalton 1962). The principal

radioautographic work was conducted by Neutra and Leblond (1966a) using glucose-H^3, on the goblet cells of the rat colon. Labelled material was found in the Golgi complexes only five minutes after the injection was administered, but none of the other cell organelles or secretion was labelled. After twenty minutes the Golgi complexes were still labelled, as were nearby mucigen granules; and after forty minutes the granules were labelled but the Golgi complexes were clear. Thus a whole complex or its contents would appear to be replaced in less than forty minutes. This illustrates firstly that carbohydrates are added at the Golgi level; and secondly, the rapidity of turnover of the secretion. Even more interesting were the results obtained when these authors applied peracetic acid-β-glucuronidase to the sections before labelling. They found that the radioautographic reactions in the Golgi complex and mucigen were largely removed, although there was some scatter over the rest of the cytoplasm. From the effect of this enzyme it seems that the secretions of the rat colon and the pedal gland of Arion both contain glucuronic acid groups, and therefore it seems very likely that the carbohydrate units are added in the Golgi complex of Arion as well. Labelling would be lost from the Golgi complex as already described. Normally the monosaccharides necessary to produce the carbohydrate, under the influence of synthetases and transferases, would be derived from the diet. This latter point was illustrated by Bennett (1970) who injected rats with galactose-H^3 and found it to be incorporated in the Golgi complex of the duodenal columnar cells after $2\frac{1}{2}$ minutes. He suggested that the rapidity of labelling indicated that the galactose passed straight to the Golgi complex where it combined with the protein moiety.

In a second paper (1966b) Neutra and Leblond repeated the labelling with glucose-H^3, together with galactose-H^3 and followed these by enzyme digestions. Again the Golgi region was the only one showing signs of synthesis of complex carbohydrates. Both Hollande (1966) and

Ovtracht et al. (1969) were able to isolate mucopolysaccharides in the multifid gland of Helix pomatia. Moreover, Jennings and Florey (1956), working with mammalian stomachs and intestines, and Lane et al. (1964), on colonic goblet cells, found that labelled sulphur, S^{35}, was incorporated into the secretion at the level of the Golgi complex. Thiéry (1969) went further, however, and demonstrated, in the goblet cells of Brunner's gland, the presence of mucopolysaccharides in some of the vesicles between the endoplasmic reticulum and the Golgi complex. This showed that the linking of carbohydrate to protein could begin to occur in these vesicles which will eventually fuse with Golgi cisternae. However, the Golgi complex was also found to function in the synthesis of the secretion. No mucopolysaccharide was ever found in the endoplasmic reticulum.

Many other examples could be cited to illustrate the involvement of the Golgi complex in the synthesis of mucoid substances.

One last mention will be on the work of Rambourg et al. (1969) working with the rat. Again they found complex carbohydrates in the Golgi complexes, but they used staining methods on the ultrathin sections and found a gradient of staining across the complex, so that the most dense staining was on the mature face. This is further evidence for the polarity of the Golgi complex, and also for the presence of carbohydrates within them. They also mentioned the finding of acid phosphatases and other hydrolases in some Golgi complexes and suggested that these could be part of the lysosomal system within the animal. Thus, the Golgi complex appears to be involved in the synthesis of muco- or glyco-proteins in many actively secreting cells, but may also elaborate a carbohydrate moiety for the lysosomal system.

The rapidity of turnover within the Golgi complex has already been mentioned. It is possibly illustrated by the relative amounts of endoplasmic reticulum and Golgi complex within a mucocyte, the volume of

the endoplasmic reticulum being much greater than that of the Golgi complex, Plate 13. As intimated earlier, it is believed that new membranes are supplied to the Golgi complex by the vesicles from the endoplasmic reticulum. However, other workers have suggested alternative origins: from the nuclear envelope, and the cell membrane. For instance, Flickinger (1969) showed, in Amoeba proteus, that the production of Golgi complexes was dependent on the nucleus, since no Golgi complexes could be identified in an enucleate animal after five days. On the introduction of a nucleus, Golgi complexes began to appear after 15 minutes. Since no normal sized complexes were seen in the cytoplasm immediately after renucleation he assumed that none had been transferred with the nucleus, and so replication from other such complexes could be eliminated. However such replication may occur in normal animals. The source of the new membranes was thought to be the endoplasmic reticulum, as in Arion, since the Golgi complexes in renucleates contained a dense substance rather like that in the endoplasmic reticulum of enucleated and recently renucleated cells. Also direct continuity between the Golgi cisternae and the rough endoplasmic reticulum could be seen in some renucleated amoebae. This continuity was absent in normal specimens and was not a feature of other cells. For these reasons he believed that the rough endoplasmic reticulum would also be involved in the maintenance of existing Golgi complexes.

Daniels (1964), however, believed the Golgi complexes derived from the plasmalemma in Pelomyxa illinoisensis, the giant amoeba. He found that the plasmalemma vesicles, derived either by pinocytosis or phagocytosis, flattened or invaginated to form the Golgi complexes in both experimental and normal animals. The reason for his deduction was that the plasmalemma in Pelomyxa was fringed and material very like this was found lining the pinocytotic vesicles on the forming face of the Golgi complex. Flickinger commented on this, noting that fringe

material was also evident on the plasmalemma of Amoeba proteus, but the forming Golgi complexes of renucleates contained material like that in the rough endoplasmic reticulum. He suggested that the fringe material was actually formed in the Golgi complex and transferred in vesicles to form part of the plasmalemma. This illustrates another problem of working with the static images provided by micrographs, and that is the establishment of the direction of movement of the vesicles involved. Bonneville and Weinstock (1969) made a similar deduction for the origin of the plasma membrane of the absorptive cells of Xenopus, prior to metamorphosis.

Kessel (1971) noted small 'blebs' from the outer layer of the nuclear envelope of the embryonic cells of the grasshopper. These blebs separated from the membrane to become vesicles which fused to produce an endoplasmic reticulum cisterna which then began to produce small vesicles as in the other cells, which contribute to the Golgi cisterna.

The conclusion seems to be that the Golgi complex may originate from the nuclear membrane or the endoplasmic reticulum, the latter being under nuclear control.

Whaley (1966) suggested that the nuclear origin of the complex may be confined to embryonic cells such as illustrated by Kessel (1971), but that the endoplasmic reticulum may take over after this. No relationship of Golgi with nucleus is evident in the pedal mucocyte of Arion, but all the animals dealt with were adult.

After a consideration of the Golgi complex, the next organelle to be considered is the mitochondrion.

Plate 29 shows such an organelle and the double membrane encompassing it can be seen, being about 79Å wide. The inner membrane is believed to be convoluted to form the cristae which pass across the mitochondrial matrix, but such a relationship is not visible in Arion. In Plate 29 these cristae do not appear to be uniformly organised in any one

direction, but this could be an effect of the angle of sectioning. There is some evidence, from other sections, that they are diagonal. The cristae have an average width of 149Å but they vary along their length, often appearing somewhat dilated. It is not possible to ascertain the number of cristae present because of lack of clarity, but they seem to be fairly numerous, up to about 14 in number. The inside of the cristae appears pale like the material between the inner and outer membrane, but the matrix of the mitochondrion is quite dense. It is elongated in one plane and has rounded ends, but it may also be bent with the contour or the cell organelles. The sizes of the mitochondria also vary, but this could reflect differences of sectioning angle as well as possible size variations. The longest specimen measured is 1.44μm and the widest 0.25μm.

They lie, very closely applied to, and between the cisternae of the endoplasmic reticulum, but occasionally they can be seen near the nuclear membrane or the secretion sacs. The mitochondria may be very numerous in any one place, for instance in Plate 30, where there are about fourteen in an area of about 64μm². The actual number may be slightly less than this because a bent mitochondrion could be sectioned through more than once. This large number of mitochondria suggests an area of very high activity, as might be expected in a secretory cell.

Within the matrix are a number of almost spherical, very electron-dense particles. Their size varies from about 250Å to 440Å in diameter. Pasquali - Ronchetti et al. (1969) found calcium and magnesium to be present in these regions together with some unspecified component thought to be lipid. However, their function within the mitochondrion is not known.

The last component within the pedal mucocyte is, of course, the secretion sac. Plate 10 shows several of these sacs, which result from the filling of Golgi cisternae. The size of these sacs varies as do their

contents. As in Plate 10 some sacs appear palely electron-dense with some denser threads in them, whilst others appear principally electron-translucent with some thicker, darker threads present, but both types discharge. Plates 31 and 32 show the secretion at higher magnifications (and stained with alcian blue in glutaraldehyde, which enhances the mucoid secretion). The darker and paler secretions are clear and it seem that the darker secretion is formed by the aggregation of the nodular thread-like material within the matrix and the secretion may even appear as a reticulum (Plate 32). The variation in aggregation may reflect some biophysical difference between them. The sacs closest to the Golgi complex appear to be the mainly electron-translucent type with some electron dense threads (Plate 27).

This variation in aggregation is quite usual, no matter which schedule of fixation is used, but the Alcian blue technique makes the secretion more electron dense. The darker material is often, but not always, found in coalesced sacs of secretion, but both types can be seen in the gland lumen. As more sacs of secretion are produced by the Golgi complex, these sacs begin to coalesce, possibly due to pressures within the cell, and the separate membranes rupture to allow the secretions to mingle. Such a coalescence of mucous sacs was absent in the mucus of the genital tracts of the nudibranchs (Schmekel 1971). However, not all the membranes rupture before the secretion is discharged into the gland lumen as can be seen in Plate 8. It is possible that in the extreme situation caused by dissecting the live animal, some membrane-bound sacs may be discharged which usually are not. Freeman (1966) saw some mucous droplets discharged still membrane-bound, whilst others coalesced, just as in Arion hortensis, and also described the mucus as being stringy and granular.

The nature of the membrane limiting the sacs is not very clear, due to obliqueness of section, but in Plate 32 it would seem to be a

typical unit membrane, suggesting again that unit membranes must be present in the Golgi complex.

(b) Distribution

Having considered the components of the pedal mucocyte, a brief mention will be made of their distribution round the lumen and of the tissue they are embedded in.

The distribution of the mucocytes is shown in Fig.42. The mucocytes lie in two lateral bands, sometimes extending above the upper epidermis of the lumen, as can be seen in Plate 1, and also lying below the ciliated hummocks (Plate 10). They are generally very closely packed together. The main area of discharge is, as has already been mentioned, in the central 'well' between the two hummocks, Plate 8.

The mucocytes are surrounded by the muscular tissue of the foot, as in Plate 13, where a mucocyte in the depths of the gland is almost completely surrounded by muscle fibres. Two connective tissue nuclei and their surrounding cytoplasm are also visible. These latter nuclei are a little more irregular in outline, much more so than those of the pedal mucocytes. The chromatin is present as isolated patches within the nucleoplasm as well as in a fairly thin band round the inner membrane. The details of the cytoplasm are not evident.

The individual mucocytes tend to be separated by very small muscle fibres and collagen fibres in some intercellular substance, and can be seen in Plate 10. Plate 33 shows some of these collagen fibres in the intercellular matrix and as can be seen the fibres are very closely applied to the outer surface of the mucocyte. This region is not clear in the micrograph because of the granularity of the print, but some of these fibres are of considerable length and the banding on the fibres can also be seen. Their width is very constant, at about 155Å. The collagen fibres would presumably provide some form of strengthening for

the tissue.

The last point to be considered in relation to the pedal mucocyte is its innervation. In all the investigations on the gland the only evidence of nerves associated with the mucocytes is seen in Plate 10, where a small nerve can be seen. The only other nerve to be seen is in Plate 4, immediately below the lower epidermis of the lumen. The possibility that these nerves may be involved in discharge will be discussed later (pp 161-163). Wondrak (1967, 1969) described vegetative nerve endings closely applied to the gland cell bodies of the ventral and lateral sole gland cells of Arion empiricorum and Arion rufus. These nerves contained synaptic vesicles, which sometimes appeared electron translucent and in other instances electron dense. Some of these light and dark vesicles can be seen in Plate 4 in the nerve just below the lower epidermis.

The last pages have considered the pedal mucocyte, the tissue it is embedded in, and has discussed the production of secretion. This production can be summarised as follows. The protein moiety is produced on the ribosomes lining the walls of the endoplasmic reticulum under the influence of the messenger RNA from the nucleus, the units for the protein being supplied by the transfer RNA. By some unspecified mechanism the protein units pass into the endoplasmic reticulum which results in their separation from the rest of the cytoplasm. Transport of proteins may also occur in the reticulum. The tubules within the ER may represent some permanent structure or else they may reflect the protein itself. Small vesicles bud off from the cisternae and represent transition elements between the endoplasmic reticulum and the Golgi complexes. The walls of the cisternae from whence budding occurs are smooth, having lost their ribosomes. The vesicles are membrane-bound and contain small amounts of protein. Whether modification of the secretion begins in these vesicles, as suggested by Thiéry (1969), is

not known. These vesicles fuse at the forming face of the Golgi complex to produce new cisternae for this organelle. There is a stepwise progression of these cisternae across the complex towards the mature face and during this time the secretion is modified, since the mucopolysaccharide fraction is added at this level. At the mature face a full secretion sac is produced and this gradually becomes displaced into the cytoplasm. As more sacs of secretion develop they begin to coalesce until the whole cell is filled with secretion and is ready to discharge into the lumen between the ciliated humps of the lower epidermis. The secretion is then carried forward on the cilia to the anterior pore of the gland where it is dispersed over the foot sole.

During the development of the secretion, the mucocyte undergoes some changes in appearance. Plate 34 shows a fairly young specimen, containing a large nucleus with scattered patches of chromatin, some being applied to the inner membrane. There are relatively large amounts of rough endoplasmic reticulum and mitochondria are numerous, suggesting that there is high energy consumption within the cell, which is to be expected since the cell is actively secreting. Golgi complexes are quite numerous and associated with the mature face are a few sacs of secretion, which occupy approximately 20% of the cell. The patches are isolated, usually being somewhat peripheral in position. However, three groups of sacs are found quite close to the nucleus. Plate 13 shows a similar young mucocyte, but with the secretion sacs beginning to coalesce. Plate 10 shows a slightly more developed mucocyte and the nucleus appears to be smaller and to have been displaced to one side, but this could be misleading since the angle of sectioning may be giving a false impression. The single and coalesced sacs occupy some 70% of the cell in this section, the secretion tending to occupy the two poles of the cell. The endoplasmic reticulum forms a peripheral layer and a central patch. Mitochondria, apparently fewer in number, lie between the

cisternae of the endoplasmic reticulum, and the Golgi complexes are generally between these cisternae and the sacs of secretion.

Plate 35 shows a mucocyte whose secretion has almost filled the central region of the cell. The endoplasmic reticulum appears mainly to be confined to the circumference of the cell and the Golgi complexes are also peripherally placed between the ER and the sacs of secretion. It would seem from these plates that as more secretion is produced the amount of endoplasmic reticulum and the number of Golgi complexes and mitochondria are reduced, together with their displacement to the periphery of the mucocyte. However, a positive statement about this change in quantity cannot safely be made because the point of sectioning within the cells cannot be determined accurately. It is possible that most of the endoplasmic reticulum and other cell organelles become displaced to the base of the cell, only a small quantity of ER etc. being left round the periphery of the upper reaches of the cell. Sectioning across the upper reaches of a cell would then give the impression of a large amount of secretion and few organelles as indicated in Fig.43, which could well be the case in the pedal mucocyte,but it is possible that some reduction of volume of cell organelles also occurs.

Plate 36 shows a higher magnification of the type of cell illustrated in Plate 35. The majority of the cell is filled with secretion, and the endoplasmic reticulum and mitochondria are pressed to the periphery of the cell.

The mature mucocytes are seen in Plate 37 where the secretion completely fills them, only remnants of endoplasmic reticulum appearing round the cell walls. These are the cells which discharge into the gland lumen. However, again they could be the ducts of cells which have been sectioned, as illustrated in Fig.43.

Other workers have noted the compression of cell organelles during the process of secretion. Hollmann (1963) observed that as

secretion was produced in the goblet cells of the rat intestine, the nucleus, mitochondria and endoplasmic reticulum became pressed at the base of the cell, so much so that their limits were hardly discernible. Likewise, Freeman (1966) noted that the Golgi cisternae were compressed to the lateral margins of the cell or between the mucus and the nucleus. Similarly, the endoplasmic reticulum cisternae became laterally or basally compressed and the other cell organelles were contained in minute amounts of cytoplasm between the mucous droplets.

The change in proportions of the cell components and secretion would account for the non-metachromatic and metachromatic mucocytes that are seen in the light microscope investigation (LM.4 viii pp 90-91). The non-metachromatic cells are those which contain large amounts of endoplasmic reticulum and relatively little secretion, since the protein present in the reticulum would obscure any metachromasia. However, as soon as the amount of secretion increases in relation to the endoplasmic reticulum then the cells will begin to show it. The cells below the centre of the lower epidermis are completely filled with secretion and hence strong metachromasia is apparent in this region. After treatment with RNase the suppressing effect of the protein associated with it is lost.

(c) <u>The Origin of the Pedal Mucocytes</u>

There is no evidence of very young stages in the development of the mucocytes, the youngest type being that seen in Plate 34 where large quantities of endoplasmic reticulum are present, and this would seem to be a quite highly developed cell. Nor is there any evidence of cell division in either the epidermis or the connective tissue. It is possible that development could occur from the numerous connective tissue cells, such as illustrated in Plate 13 but no evidence is available for this. Chétail and Binot's (1967) findings on the genesis of

the pedal gland in the embryo of Arion rufus would seem to be relevant to that in Arion hortensis since they both belong to the same genus. They found a dual origin for the gland, the epidermis being ectodermal and the mucocytes being mesodermal. Therefore, it is possible that when new mucocytes develop they do so from connective tissue cells. Since it is believed that the mucocytes fill and discharge continuously, replacements are probably only required when the mucocytes become exhausted, and this has not been detected.

(d) Mechanism of Discharge in the Pedal Mucocytes

Since there is no evidence of cell division or of very young mucocytes it would seem that the cells may secrete and discharge continuously or cyclically throughout the life of the animal. The lack of cell division seems to confirm the suggestion, proposed in the light microscope section (LM.4 xii pp 95-97) that discharge is not holocrine. Further confirmation is provided by the absence of cell organelles in the gland lumen, which might be expected if discharge were holocrine, or possibly even apocrine.

The disruption of the central epidermis in Plate 8 might suggest that apocrine secretion could occur. On the other hand it may be the result of severe contraction during dissection. However, the disruption is not always so great as can be seen in Plate 9 where the ducts appear quite well formed, and this would be evidence against apocrine secretion.

This leaves the merocrine mode of discharge, which has already been discussed in the light microscopy (LM.4).

In this method of discharge the mucocyte would continue to produce secretion for several cycles, filling with secretion, discharging and filling again. It is therefore suggested that as the mucocytes fill with secretion the endoplasmic reticulum and other organelles

are possibly greatly reduced but are not destroyed. During secretion the apical membrane of the discharging cell is disrupted, Plate 9. The mucocyte could begin to secrete again after discharge. This type of secretion corresponds to the Extrusion Type IV described by Kurosumi (1961).

It is also possible that secretion and discharge occur simultaneously, and in this way there might be a hydrostatic pressure developed within the mucocyte that may aid discharge.

Freeman (1966) found that goblet cells were capable of secreting several times in their life, but that after prolonged stress they were found en masse in the gland lumen. However, he believed the mode of secretion to be apocrine in this case because some organelles were seen externally. Schmekel (1971), describing the genital tract of nudibranchs, found the mucous gland cells to have a limited life, whilst the protein gland cells functioned throughout life.

It seems that the secretion that is produced by the mucocytes passes into long ducts through which it is discharged. Such ducts were seen by the light microscope (pp 91) and this would agree with the suggestion illustrated in Fig.43 that most of the cell organelles come to lie basally or peripherally. It can be further illustrated in Fig.42 which is a tracing from a print of a ½µm section stained with toluidine blue. In this figure large amounts of metachromatic material can be seen around the 'well', but most of the cell bodies are arranged some distance from this region. Three nuclei are illustrated with a mass of secretion below them. It would seem that this secretion is the cells' contents prior to discharge, but their nuclei still look quite large and active, suggesting that the mucocytes do indeed recycle or continuously secrete.

Further evidence of long ducts was seen ultrastructurally as in Plate 38, where some of them have been cut transversally. The

secretion within them is evident, and collagen lies in the intercellular space possibly acting as a strengthening component. Fig.44 shows the position of these ducts in relation to the lumen. They are presumably from cell bodies which lie either above or below the plane of the drawing.

There is little evidence to refute the suggestion made in the light microscope section (LM.4 xii pp 95-97) that discharge is induced by direct or indirect pressure on the mucocytes, caused by the contraction of the muscles of the foot. These can be seen embracing the region of the pedal mucocytes, Plate 13, and also passing in between the mucocytes, Plate 10. The evidence of direct innervation of the mucocytes is slight, the only example being on Plate 10, where a small nerve is closely applied to a mucocyte. Because of this one example the possibility of direct nervous control cannot be dismissed but it would seem to be rare.

The last section on the pedal mucocyte involves a comparison of these results with those of Wondrak.

EM1 (5) COMPARISON OF RESULTS

The ultrastructure of the pedal gland has not been described before, but Wondrak described the ventral sole gland cells of Arion empiricorum and the sole gland cells of Arion rufus, both of which are believed to be comparable to the B gland cell in Arion hortensis. In turn the B gland cell is believed to be like the pedal mucocyte, so a comparison between these sole gland cells and the pedal mucocyte is possible. The descriptive details of these gland cells have already been given briefly in the historical background (pp 122-124). In the ventral sole gland cell of Arion empiricorum Wondrak (1967) the nucleus was found to be basal, whilst in Arion hortensis it tends to be centrally positioned in the cell body, unless large amounts of secretion are present when it becomes basal. In both animals a zonula adhaerens and septate desmosomes were present between adjacent epidermal cells and the gland cell ducts were

lined with microvilli. The Golgi complexes and endoplasmic reticulum were similar, but a different interpretation has been placed on the organisation of the ER tubules in Arion hortensis. Moreover, it was suggested that these tubules might result from the precipitation of protein in the event of an excess being present in the ER cisternae, whilst Wondrak made no comment about their nature.

In both animals the nucleus is rich in chromatin and showed a fairly wide perinuclear gap, containing the tubules characteristic of the endoplasmic reticulum.

Wondrak described nerve endings close to the gland cell bodies, whilst in Arion hortensis there was only evidence of one such alliance. It is possible that discharge of the ventral sole gland cells of Arion empiricorum is under direct nervous control whilst in Arion hortensis discharge is believed to be effected by direct or indirect pressure caused by the contraction of the muscles of the foot.

The sole gland cells of Arion rufus (Wondrak 1969) were much like those of Arion empiricorum, so the points of comparison with Arion hortensis are similar. However, Wondrak found the gland cell bodies to show surface invaginations and vesicles between the outer membrane and the endoplasmic reticulum. This he believed characterised the ingestion phase of secretion. No such finding was made in Arion hortensis. The various stages in the appearance of the secretion within the Golgi complex in Arion rufus were similar to those in Arion hortensis, but the secretion within the still flattened cisternae was more electron dense in A. rufus.

The epidermal cells of A. rufus were similar to those of the lower epidermis of the pedal gland duct in A. hortensis, although cells bearing both cilia and microvilli were not described in the former species. The other difference was the presence of vesicles in the epidermal cells of Arion rufus, together with invaginations of the free cell surface, but neither were found in Arion hortensis.

The next section will deal with the ultrastructure of the posterior part of the mantle in Arion hortensis. Such an investigation is necessary before regeneration of the mantle tissue can be examined.

EM2. THE STRUCTURE OF THE UNDAMAGED MANTLE

The structure of the normal mantle must be considered before examining the regenerating tissue.

EM2 (1) DISSECTION OF MATERIAL

The material was dissected out as for the pedal gland (EM1 (2) (i) pp 116-117), except that it was the tissues of the foot that were cut medianly and the skin trimmed so that only a small piece of posteriorly positioned mantle tissue remained. This area was selected as the area to study regeneration and therefore was used in the investigation of the normal tissue.

EM2 (2) METHODS

The solutions used for fixing and embedding the pedal gland (EM1 (2) (ii) pp 117-118) were also used for the mantle. Schedule 5 (pp 121-122) was used on this tissue, but with a modification in the alcohol series since only 30%, 50%, 70%, 80%, 95% and absolute alcohols were used. After this treatment, the ultrathin sections were stained for 15 minutes in 5% uranyl acetate and 10 minutes in Reynolds' lead citrate.

EM2 (3) HISTORICAL BACKGROUND

Wondrak (1967, 1969) and Lainé (1971) have described the ultrastructure of the mantles of Arion empiricorum, Arion rufus and Agriolimax reticulatus.

Wondrak (1967) described the mantle gland cell in Arion empiricorum as sac-like in shape with a duct which was less conspicuous than those in the sole gland cells, and which connected with the surface. The rough endoplasmic reticulum was less extensive than that in the sole gland cells and lacked the inner tubules. The most striking

feature he noted was the presence of large areas of Golgi complexes (composed of sizeable cisternae) and large vacuoles in the cytoplasm. The basal region of the cell membrane was invaginated, the invaginations being closely associated with the membranes of the endoplasmic reticulum. The gland cells were seemingly not innervated. The upper surface of the mantle epidermis bore microvilli, and septate desmosomes were present between the epidermal and gland cells.

The structure of the striated epidermal cells of A. rufus, covering the mantle, has already been described in EM1 (3) pp124-125, in connection with the pedal gland. Occasionally, however, cells were seen with a crenellated surface, whilst others were irregularly 'domed'. Apocrine secretion of Extrusion Type 2 (Kurosomi 1961) was believed to occur from these cells.

The mantle gland cells of Arion rufus were like those of Arion empiricorum, except that there were cisternae of smooth endoplasmic reticulum lying between the secretion vacuoles. The cell organelles were all basally situated, the rest of the cell being filled with secretion. Invaginations were again present around the cell base: long, tubular protein glands could be seen, with extensive rough endoplasmic reticulum (lacking internal tubules). In one form the ER showed wide cisternae containing a substance of medium electron density. In another form it was composed of spherical, ribosome covered vesicles with less dense contents. The Golgi complexes occupied only a limited volume and the cisternae and vesicles sometimes contained material of similar electron density to that in the endoplasmic reticulum with wide cisternae. Globular bodies of varying electron density, membrane-bound granules, and multivesicular bodies were also present in the cytoplasm. In another functional state the above mentioned spherical cisternae of the ER were present and the centre of the cell was filled with a homogeneous granular aggregate, which was seen to be discharged. Two types

of pigment gland cells or two stages of one type were present in the tissues of the dorsal surface, conferring the red brown colouring to the animal. Wondrak found them discharging in extreme conditions and suggested they had an excretory function.

In his 1968 paper Wondrak described the cell junctions, involving a zonula adhaerens, an intermediate zone, a zonula septata and a macula occludens. However, he did not state which of these were present in the mantle, although he commented that in many parts the epithelial cells lay so close together that there were no intercellular spaces.

Lainé (1971) described three types of mucous gland cell in the mantle of Agriolimax reticulatus, together with five granular gland types or areas and deposits of melanin.

The mucous of the M1 gland cell lay in a single mass and was generally electron-translucent with some electron-dense strands, clusters of spots and spots linked by faint threads. The main feature of this cell was the large numbers of Golgi complexes present, with associated vesicles, which lay close to the nucleus and restricted to the inner end of the gland. The walls of the Golgi cisternae were often ruptured, but it was not known if this was a fixation artefact. The single layered cell membrane was indented so that it resembled a pinocytotic membrane. Some large, irregularly-shaped, membrane-bound spaces lay just within the membrane, some containing slightly electron-dense material. A nerve was found close to one of these gland cells, but no other evidence was available to indicate whether or not they were directly innervated.

The M2 mucous gland cell was like M1 in terms of the cell organelles present, the appearance of mature secretion and the size, number and geometry of the Golgi complexes, but a series of varying contents could be seen in the Golgi cisternae. These ranged from electron-translucent material at the forming face to electron-dense (which became dispersed) at the mature face.

The M3 mucous gland cell was filled with membrane-bound

globules of slightly granular mucus. The other organelles lay around the periphery of the cell and the cell membrane was invaginated, especially close to the Golgi complexes.

Some intermediates between M2 and M3 were found, containing Golgi complexes as in the M2 cells but the secretion was globular, although not so regularly patterned as in the M3 variety.

Granular gland cell, G1, contained dark granules scattered in a sponge-like ground substance (which did not fill the area in which it lay). The matrix and granules were composed of fine fibrils. These gland cells occupied a superficial position in the skin, apparently with an opening to the exterior but the granules were never seen in the slime and their function was unknown.

The G2 gland cell was found below the mucous cells, close to the musculature and contained ovoid granules, which lacked membranes and were composed of grains and fibrils centrally, surrounded by an electron-transparent halo. Lainé believed that these were the calcium gland cells.

The G3 gland cell was common and lay in the muscle tissue. The nucleus was stellate, being surrounded by a thin cytoplasmic layer, and the remainder of the cell contents were widely separated by a finely granular ground substance. It was not possible to establish whether the ovoid granules were membrane-bound since they were surrounded by an area of electron-dense material. The interior of the granule was filled with fine grains and some fibrils, but occasionally the centre was free of contents. Lainé suggested that the G2 gland cell might derive from G3 by the concentration of fine grain material centrally, leaving an outer halo. The remaining cell cytoplasm present in G3 would become concentrated between the halos and then be displaced to one side.

The G4 cell contained many closely packed membrane-bound granules, some having more electron-dense areas centrally. The cytoplasm was considerably restricted. This cell was again found superficially but no granules were evident externally. However, only one

example of this type was seen and Lainé suggested it might be the neck of an undescribed cell, and that the granules could be proteinaceous.

The G5 area was well defined, but only seen once. It was situated between the M1 and M2 cells. The cell membrane was convoluted and basally there was a darker, sponge-like area, composed of small parallel tubules. The rest of the cell was filled with electron-translucent material. The nature of the G5 area was unknown and since no Golgi complexes or nucleus could be seen its glandular nature was in doubt.

The epidermis of the mantle was grooved, those cells in the groove being cubical or squamous, and the others columnar. The cell junctions meandered considerably and displayed a zonula adhaerens and a septated desmosome. The cells were separated at the outer edge by a gap, and the cytoplasm next to the zonula adhaerens was more dense than elsewhere. Numerous mitochondria were present between the cell surface and the nucleus, and Golgi complexes lay close to this last mentioned organelle. Microvilli, with attached fine strands, were present on the outer surface of the epidermal cells. No basal lamina was present. Granules of melanin lay in melanocytes just inside the epidermis.

The next section will describe the ultrastructure of the mantle in Arion hortensis.

EM2 (4) RESULTS

(i) The Epidermis of the Mantle

A similar problem to that encountered in the pedal gland (EM1 (4) (i) pp 126) occurs with the mantle epidermis, that is, that the edge of the mantle is usually at the perimeter of the section and therefore may be damaged.

Plate 39 shows a low power picture of the mantle epidermis, not of good quality. The epidermal cells are columnar, approximately 14µm

long and 3μm wide, although the width varies somewhat because the cells taper slightly towards their bases and because complex interdigitations are present between cells. These are present in Plates 40 and 41 and the junctions can be seen to be composed of a zonula adhaerens and a septate desmosome. At the apical point of conjunction between two cells there is a gap, on average 3295Å long, less than in the pedal gland epidermis (EM1 (4) (i) p 133), and 555Å wide, although in one instance a width of 2350Å was recorded. The large desmosome in the zonula adhaerens, noted in the pedal gland epidermis (EM1 (4) (i) p133) is not so apparent in the mantle. The septate desmosomes can be seen at higher magnification in Plate 41, although they are generally obliquely sectioned. The width of the unit membrane of each cell is 53Å (as found in the pedal gland pp139), the gap between the two groups of membranes is 116Å and the width of the intercellular bridges is 105Å.

Intercellular spaces are present between the lateral unit membranes, Plate 39, and are of varying sizes. It is not known whether these spaces are present naturally or whether they are the result of fixation.

The epidermal nuclei fill 40 to 50% of the cell volume, and are elongated along the axis perpendicular to the cell surface. Their size varies somewhat, possibly depending on the angle of sectioning, but is on average 7μm long by 2μm wide. Isolated patches of chromatin are present in the nucleoplasm, 3 to 7 patches in this section, together with a thin, interrupted band of chromatin around the inner nuclear membrane. The distance across the double membrane is about 235Å. A single nucleolus appears to be present in each nucleus but its structure cannot be discerned. Typical nuclear pores are present, measuring on average 94Å in diameter (less than in the pedal gland p.131).

The cytoplasm of these cells is rather electron-dense and so details of organelles are not easy to determine. Numerous mitochondria are present between the cell nucleus and the cell surface and this would

suggest an area of high energy requirements. They are on average 4250Å long and 2730Å wide with a double membrane of 163Å encompassing them. The direction of the long axis varies but it is often perpendicular to the cell surface (Plate 40). Also in this plate, some cristae are visible, but the number cannot be established. However, in Plate 40 two mitochondria are arrowed which appear to be in cytoplasmic projections from other cells, and in these the cristae run diagonally and number at least ten. In this same Plate there is a small amount of rough endoplasmic reticulum present, whose cisternae are about 300Å wide and there are also some free ribosomes present in the cytoplasm. A Golgi complex, in a supranuclear position, is present in Plate 39, but the number of cisternae cannot be clearly established (possibly six or seven can be counted). The distended cisterna at the mature face contains an electron translucent substance, as do the vesicles present in the supranuclear cytoplasm. It is possible that they derive from the Golgi complex since there is no evidence of pinocytosis in the apical membrane. The nature and function of this secretion is unknown.

The epidermis of the mantle is ridged, as can be seen, to some extent, in Plate 39. The microvilli on the ridges measure on average 5700Å in length by 2100Å wide, whilst those in the hollows measure on average 8150Å long by 1330Å wide. In a set distance there are twelve "hollow" microvilli to six "ridge" microvilli but this could be the result of tissue being stretched on the ridge. In Plate 40, fine strands of material appear to radiate from the surface of the microvilli in the hollows. In Plate 39 some material can be seen associated with the "ridge" microvilli but this could be remnants of slime or a very short band of fine strands, hardly projecting beyond the level of the microvilli. It is not known whether these strands are branched and it is not easy to ascertain their length since only parts of them can be seen in any one section, but they would appear to be in the region of

1.43μm. Lainé (1971) described a similar arrangement in the mantle of Agriolimax reticulatus and found the strands to be 1.3μm. She related this finding to the "fuzzy coat" described by, for example, Toner and Carr (1968) in the human small intestine. Wondrak also noted its presence in Arion rufus.

The function of these microvilli and strands is not known, but the presence of microvilli clearly increases the surface area of the mantle considerably for the absorption of some substance. It is possible that some oxygen is absorbed here to supplement that taken in through the pneumostome, and the slime trapped between the microvilli would supply a moist atmosphere for this, or at least might reduce the desiccating effect of the air to some extent.

No internal fibres are present within the microvilli but a "terminal web" is present below them, although it is not as evident as in the lower epidermal cells of the pedal gland (EM1 (4) (i) p.130).

Lastly, the epidermal cells do not appear to rest on a basal lamina (Plate 39) and Lainé (1971) found a similar lack in Agriolimax. However, there is a layer of muscle below the epidermis, which Dyson (1965) may have seen and identified as a basal lamina. This muscle may aid in the discharge of secretion.

EM2 (4)

(ii) The Mantle Mucous Gland Cells

The mucous gland cells are long and narrow, tapering somewhat towards the apex, and no obvious neck is visible (Plate 42). This in in contrast to the appearance of these cells, M and m, after fixation for light microscopy (Figs.4 and 7), where they appear ovoid and occasionally have a neck. Moreover, the size of the mantle gland cells in the micrographs is fairly constant, circa 47μm, except where the cells have been sectioned oddly. Presumably this variation in appearance is the result of the different methods adopted in dissection, fixation and embedding.

As can be seen the bulk of the cell is filled with sacs of secretion, with patches of cytoplasm between (Plate 42). The nucleus and other cell organelles are basal, but in the above Plate the structure of the nucleus is not distinct, whilst in Plate 43 a transverse section through the base of a mucous gland cell shows it more clearly. Nucleus (1) has a fairly regular outline, being ovoid in shape but nucleus (2) is more irregular, with small projections into the cytoplasm. The nucleus appears slightly elongated along the axis perpendicular to the cell surface (Plate 42). Nucleus (1) displays the double membrane, of 765Å width, but because of the poor quality of the micrograph no nuclear pores are visible. Plate 44 shows a nucleus at higher magnification and typical pores are present, measuring on average 523Å in diameter. The width of this nuclear membrane is less regular than that in Plate 43, varying from 469Å to 1065Å. This last measurement is high and may indicate that fixation is inadequate (although the other cell organelles appear to be well fixed) or that the angle of sectioning is at a tangent to the membrane. Between the double membranes is an electron-translucent material with slightly more dense fibres within it, and only one or two ribosomes appear to be attached to the outer membrane. The chromatin is scattered in patches throughout the nucleoplasm, together with a fairly thin band around the inner nuclear membrane. A typical nucleolus can be seen in Plate 43.

Some endoplasmic reticulum is visible in Plate 43 but it is quite unlike that in the pedal gland, only occupying a small volume of cytoplasm and being composed of quite narrow cisternae (250Å to 400Å). Plate 45 shows more detail of the reticulum and it can be seen that it usually adopts a peripheral position, between the cell boundary and the Golgi complexes. Because of poor focus it is not possible to discern any invaginations of the cell surface contacting the endoplasmic reticulum, as described by Wondrak (1967, 1969). In this Plate the ER cisternae are rather more distended, from 340Å to 1720Å, and are ribosome covered, suggesting that protein is produced at this point. They

contain an electron-translucent substance with more dense threads within, as between the nuclear membranes (pp174), but no small internal tubules are present. The ribosomes are elongated along the axis perpendicular to the endoplasmic reticulum membrane and measure on average 198Å long by 126Å wide. Unlike the pedal gland (EM1 (4) (ii)a. p149), blebs are not apparent from the ER.

The reduction in the volume of endoplasmic reticulum (as compared with the pedal gland) suggests either that there is less protein in this secretion or else that the rate of turnover is much greater in the mantle gland cells. In contrast, the Golgi complexes occupy a much larger volume of the cell cytoplasm than in the pedal gland. Plate 46 shows this well, as it represents a transverse section through the basal cytoplasm and nucleus of a mantle mucous gland cell. Plate 45 is a magnified print of one area of Plate 46, and the Golgi complexes can be seen. They are composed of stacks of cisternae, up to about nine in any one, which appear either as almost flat layers or else are curved considerably. Each cisterna is much longer than its counterpart in the pedal gland, measuring up to 2.4µm. They are on average 180Å wide on the forming face and 145Å apart, whilst on the mature face they are 540Å wide and 180Å apart. In parts these last appear even wider, but this is believed to be the result of oblique sectioning since no membranes can be discerned. At the forming face the cisternae are filled with a substance of medium electron density but as they move towards the mature face this becomes much more electron-dense. Plate 47 illustrates the stages of secretion production. The cisterna, filled with the very dense substance, begins to increase in size as some electron-translucent material is "added" to it. The very dense substance begins to disperse within this, so that at first there is a dark, irregular band surrounded by translucent material which in turn contains some electron-dense strands, presumably derived from the dark mass. Eventually the dense substance becomes evenly

dispersed throughout the electron-translucent material such that a fine reticulum is formed (Plate 47) and this is the mature secretion. The very dense material never completely fills the almost mature cisternae, for there is always a band of medium electron density between it and the double membranes, and this resembles that in the less mature cisternae. It would seem that some substance is concentrated within the cisternae before becoming dispersed in an electron-translucent material. Apparent vesicles are present at the ends of the cisternae and contain a substance of medium electron density, often with a slightly paler centre. The word "apparent" is used since they could also represent a transverse section through the tips of the cisternae themselves (Plates 44 and 45). It is possible though that they are supplying material to the complex, since they are of a similar density to the cisternae of the forming face. Where this substance is derived from is not known, although it is presumably from the endoplasmic reticulum (as in the pedal gland, EM1 (4) (ii)a.148-149). However, if this is the case the material must undergo some slight modification within the vesicles, since the material in them is more dense than that in the endoplasmic reticulum. Possibly a situation like that described by Thiéry (1969) arises where some mucopolysaccharides were detected in the vesicles before they reached the Golgi cisternae. The vesicles are on average 580Å in diameter, somewhat wider than the less mature cisternae, which might suggest that they are indeed vesicles and not cross-sections of the cisternae. However, since membranes are not detectable round these, they could be oblique sections and would therefore be of larger diameter. Thus, the nature of the "vesicles" can be established here.

The mechanism for producing the secretion is possibly the same as that outlined in the pedal gland mucocyte (EM1 (4) (ii)a. pp.149-151) but no "blebs" and no vesicles, between the ER and the Golgi complex, are discernible. This could be because the survey was shorter in this case

and less material was examined, but, on the other hand, it could indicate that the mechanisms are different. It is not easy to envisage an alternative sequence of events, unless material diffuses out of the ER, or unless the ER is not involved in the production of this secretion. The secretions, which are produced by the mantle mucous gland cells and the pedal gland mucocytes, are quite different, since that produced by the mantle cells is possibly a low sulphate chondroitin, and that by the pedal gland, a chondroitin. The difference in appearance of the two secretions within their respective Golgi complexes might reflect the difference in their structure. The denser material in the cisternae of the mantle gland cells may contain sulphate and carboxyl groups in the mucopolysaccharide moiety, whilst the less dense material contains only carboxyls. Protein appears to form part of the mantle gland cell secretion as well, since deamination prevents any staining (LM4 (ii) pp.44), although it must be remembered that the amine units could belong to the galactosamine portion of the chondroitin rather than to protein.

Another difference between the pedal gland mucocytes and the mantle gland cells lies in the nature and number of mitochondria. Those in the pedal gland are considerably elongated, numerous and have a dense matrix containing intramitochondrial granules. In the area visible in Plate 46, only two can be seen, much fewer than in the pedal gland and they are only slightly elongated. The cristae are not clearly discernible in the pale matrix and intramitochondrial granules appear to be lacking. Plate 45 shows one of these mitochondria, apparently between two ER cisternae, which measures 4320Å by 2700Å as against an average of 1.4µm long by 2500Å wide in the pedal gland mucocytes. The double membrane around the mitochondrion is about 180Å across. The reduction in their numbers suggests that the level of energy consumption is less in the mantle gland cells than in the pedal gland, but whether this indicates a slower rate of turnover is not known.

As mentioned earlier (p.174) the sacs of secretion fill the bulk of the cell and it is believed that secretion and discharge occur simultaneously since one gland cell can be seen to be discharging whilst still containing apparently active Golgi complexes. Discharge occurs through the slightly tapering neck which in turn passes between the epidermal cells. The membranes of the secretion sacs rupture at the surface to release their contents. Secretion and discharged fragments of membranes are seen externally but complete membrane-bound sacs (visible in the pedal gland duct) are absent.

Most of the cell organelles are basal but some extend peripherally for a short distance up the lateral walls of the mucous cell, Plate 42. Plate 43 shows a transverse section through the region above the nucleus and the peripheral arrangement of organelles around the secretion is evident.

Two other features must be mentioned in connection with the mantle mucous gland cells. The first is seen in Plate 42, where the bases of the mantle mucous gland cells appear rather different. Only one or two examples of Type II are detected, the majority being Type I, which has been described in detail above. The contents of II appear much the same as in I in relation to the nature of the mature secretion and the relative proportion of endoplasmic reticulum to Golgi complexes. However, the Golgi cisternae seem rather distended and are considerably curved in some areas so that only parts are visible (Plate 42). It is not thought that these two types represent different gland cells because of the similarities involved, but that they are different functional states, since in both cases almost mature secretion, (in the form of a dense substance in an electron-translucent matrix), can be seen. However, this cannot be substantiated by the static images provided by the electron microscope.

The second feature is seen in Plate 39, where a gland cell containing very electron-dense sacs is present. These contain a

homogeneous substance, some being very dark, others appearing slightly less dense. There are electron-translucent spaces between the sacs, suggesting that some contraction of contents has occurred in fixation. This type of cell is also very rare and the cell base, presumably containing the organelles, has never been seen. It appears to taper considerably towards the epidermis, but is never seen to discharge. The nature of this cell is unknown and cannot be related to any cell described in the light microscopy section, but since its appearance is rather unnatural it may be an unusual fixation artefact.

No obvious large and small mantle mucous gland cells (M and m) are visible in the tissue fixed for electron microscopy which seems to confirm the suggestion that they are one and the same type of cell (LM4(ii)p.47). Possibly the more violent contractions induced when fixing for the light microscope may cause exceptionally large amounts of slime to be discharged from some cells so that they appear much smaller.

EM2 (4)

(iii) <u>The Pigment/Protein Cells</u>

Plate 48 shows part of a pigment/protein gland cell, the cell base, with nucleus, and the opening between the epidermal cells being absent. However, the considerable size of the cell is apparent, measuring 46.8µmlong by an average of 18.7µmwide. The bulk of the cell is filled with a very finely granular secretion of medium electron density.

The structure around the edge of the cell is unusual and complex. It is believed that this is rough endoplasmic reticulum in a fairly dense cytoplasm since the paler regions appear to contain fine granules of a similar size to that in the gland cell body, and there seem to be ribosomes around these paler regions and in the cytoplasm. It is not possible to discern any other cell organelles in this Plate, but it seems unlikely that the secretion is discharged directly from these

cisternae. The alternative explanation is that this is a very complex outer membrane, but a "honeycomb" would be most unusual and one would expect to see invaginations in the boundaries if a highly convoluted system of membranes is involved. Unfortunately, a higher magnification print of this feature is not available.

The nucleus of a similar pigment/protein cell is visible in Plate 49, and since it is embedded in a fairly large area of the "honeycombed" tissue described above, it would indeed seem that this is cell cytoplasm with organelles rather than a complex membrane. It is probable that this is a section through the base of a cell, since the nucleus was found to be basal in the light microscope work (LM4 (iv) p. 56). The nucleus has a fairly irregular outline, is elongated and the chromatin is mainly present in narrow patches around the inner nuclear membrane, although there are a few isolated patches in the nucleoplasm, which in turn is quite granular. No nuclear pores are detectable at this magnification, but the average distance between the two membranes is 330Å. The space between the double membranes is filled with a finely granular substance, of medium electron density, in a translucent matrix (Plate 48). The cisternae are rather varied in shape from long and narrow, to rather more bloated profiles. Some appear almost spherical in outline but these could be sacs of secretion from the Golgi complexes, the magnification being too low to establish conclusively whether or not they are covered with ribosomes. The narrowest cisterna visible in this section measures about 660Å and this is at the periphery of the cell.

In this same cell there is a localised accumulation of spherical or ovoid 'vesicles' of varying size, which may represent a Golgi complex, but the quality and magnification of the print is not good enough to establish this point. The 'vesicles' are filled with a substance like that in the ER, but do not appear to be surrounded by

ribosomes, perhaps indicating that they are part of a Golgi complex or are the mature secretion sacs 'leaving' the complex. If this accumulation is a Golgi complex then the secretion does not seem to undergo any obvious change in appearance and the complex may be a 'packaging system' only. Presumably, the membranes of the sacs rupture to discharge their contents into the main cell body, since the mature secretion is not membrane-bound. No mitochondria could be identified. The pigment found associated with these cells in the light microscopy (LM4 (iv) pp.54-55) is not detectable, possibly because of the fixation techniques used. In examining the frozen sections, it was noted that the colour appeared in the matrix and not in the fine granules.

Young pigment/protein cells (Y) are not obviously present in this tissue, but the nucleus and associated cytoplasm described above (p.180), which was assumed to represent a cell sectioned through its base, could equally be a section through a young cell. Without an examination of serial sections it is impossible to distinguish between these two alternatives.

Since only a very small amount of normal mantle tissue has been examined with the electron microscope, young cells may have gone undetected since they are not numerous.

EM2 (4)

(iv) Melanin

Only a brief mention will be made of melanin in this section since more developmental stages are evident in the regenerating tissue R.EM. pp200-201). In the mature mantle black melanin granules, in a membrane-bound cytoplasm, are present but there is no evidence of nucleated melanocytes. The granules are, in some instances, almost completely spherical with an average diameter of ½µm, and the cytoplasm is electron-translucent with small, dark granules within. The mature

melanocytes (apparently lacking nuclei) take the form of ovoid or string-like bodies of varying size and Plate 49 shows one of these latter projections emanating from one of the ovoid bodies. This agrees with the description of stellate bodies in the light microscope section (LM.4 (v),p.60). There is, in Plate 49, an intrusion from a melanocyte into the protein cell already described. The granules within this body are called pre-melanin granules because they are of medium electron density and have not yet condensed to give the mature granule. Their average diameter is 7170Å.

The melanin is distributed laterally and basally round the gland cells and for some depth into the connective tissue, but none is present in the epidermis - this agrees with the light microscope evidence (LM.4 (v) pp 60).

EM2 (4)

(v) The Connective Tissue

Although the gland cells discharge between the epidermal cells, a large proportion of each cell lies in the sub-epithelial connective tissue. This is composed here of muscle fibres, connective tissue cells and collagen in some intercellular matrix (Plate 42). Contraction of the surrounding muscles may cause discharge, and they could be equivalent to the network of fibres (possibly contractile) described by Campion in some dorsal gland cells in Helix aspersa. The muscle fibres apparently surround the cell from immediately below the epidermal layer, to their bases. The collagen presumably has some strengthening role in the tissue, and its presence is well demonstrated in Plate 43. The fibres are fine and darkly staining in this section but striation is not visible at this magnification.

EM2 (4)

(vi) Glycogen

Plate 49 shows the last feature to be mentioned in the mantle tissue, and that is the fairly large granular area at the bottom of the print which is believed to be glycogen, because it occupies the same position as the deposits found in the tissue fixed for light microscopy. For a confirmation of this, however, it would be necessary to treat a comparable block with diastase to see if it is removed. The glycogen is in the form of electron-dense aggregations of material in an electron-translucent matrix. The aggregations are unequally distributed in the delimited area but this is probably a fixation artefact. The deposit appears to be membrane-bound and around it is a band of material, approximately 3000Å wide, which could well be collagen, sectioned transversally so that the fibrous nature is not detectable. Certainly the material in the band and in the general connective tissue of the mantle (indicated in the Plate) have a similar electron density, and in one region fibres do seem apparent. Thus it seems that the glycogen deposits are bounded by a band of collagen, the fibres of which may be quite specifically orientated. Whether these regions are cellular cannot be established, since only parts are visible, but in the brief scanning of tissue (without photography) no nuclei are detectable. It was suggested earlier (pp. 68) that the glycogen represented a reserve food store in the animal. The deposits all tend to be very large, like the one shown, and they are quite numerous in the depths of the mantle tissue.

In this investigation, there is no evidence of the calcium granules described in the light microscope work (LM.4 (vii) p67) as shell remnants.

EM2 (4)

(vii) Discharge of Gland Cells

For the reasons discussed in the light microscope survey (LM4 (xii) p.95-97) and for the pedal gland (EM1 (4) (ii) pp161-163), discharge is believed to be merocrine at least in the case of the mantle mucous gland cells. Using the electron microscope, they would also appear to be merocrine since the gland necks are well formed and no organelles are seen externally.

It would also appear that discharge and production of secretion occur at the same time, at least in the case of the mantle mucous gland cells, since discharging cells could be seen whilst apparently still active Golgi complexes are present in the basal cytoplasm. In very mature cells, containing much secretion, the cytoplasm may be displaced and reduced somewhat, as illustrated in Fig.43 concerning the pedal gland, but this cannot be confirmed with only micrographs as evidence. Schmekel (1971) found the protein gland cells of nudibranch genital organs to be merocrine, discharge and production of secretion occurring simultaneously.

As has already been mentioned, the abundance of muscle fibres around the gland cell bodies suggests that they may be involved in effecting discharge, for contraction would exert pressure on the cells forcing the mature secretion out. The stronger the contraction, presumably the greater is the amount of secretion discharged. There is probably some discharge even when the muscles are at rest, because of the hydrostatic pressure built up within the cell as more and more secretion is produced. The small mucous gland cells (m), described in the light survey (LM.4 (ii) b.p.46-48), may result from severe contractions of the muscles forcing out exceptionally large amounts of secretion, resulting in the "shrinkage" of these cells if they have contractile walls. Large and small mucous gland cells are not obviously detectable

in the ultrastructural survey.

There is much more evidence for nerves in the mantle, possibly associated with gland cells, than for the pedal gland ..... for instance, in Plates 43 and 48. The nerves can be seen closely applied to, but not actually connecting with a pigment/protein gland cell in one case and a mucous gland cell in the other. The nerves could control discharge either by synapsing with the gland cells themselves or with muscle fibres.

EM2 (4)

(viii) Origin of the Gland Cells

There is no evidence for the origin of the gland cells in the material examined, for they are all at a fairly advanced stage containing large sacs of secretion. This would seem to suggest that the gland cells function throughout the life of the animal. Even young pigment/protein cells appear to be lacking, although these were detected in the light microscope survey, but this could be a reflection of the small amount of tissue examined.

EM2 (5) COMPARISON WITH OTHER WORKS

The details of Wondrak's and Laines investigations on the molluscan mantle have already been given (EM2 (3) pp166-170), and in general only points of obvious contrast or similarity will be considered here.

The description given by Wondrak for the mantle gland cell of Arion empiricorum (1967) was much like the mucous cell in A. hortensis, particularly in connection with the appearance of the Golgi complexes. There are two points of difference, however, for Wondrak described invaginations of the basal cell membrane, (connected with the endoplasmic reticulum) which were not seen in A. hortensis, and also he saw no

associated nerves. These last have already been described in A. hortensis (pp. 185).

The similarity between the mantle epidermis and mucous gland cells in A. rufus (Wondrak, 1967, 1968) and those in A. hortensis was considerable, but the epidermal cells were only slightly domed and no apocrine secretion was seen to occur from them. Also invaginations in the mucous gland cell base were present in A. rufus together with cisternae of smooth endoplasmic reticulum, and these were absent in Arion hortensis.

The pigment/protein gland cell of A. hortensis resembles one form of the protein cell described by Wondrak in A. rufus in that the central region was filled with a homogeneous granular aggregate which was seen externally. Golgi complexes were comparatively rare.

Melanin was found in both species, but not suprisingly the reddish pigment which gives A. rufus its name was missing in A. hortensis.

Lainé described three types of mantle mucous gland cells in Agriolimax reticulatus (1971), the M2 cell most closely resembling the M cells of A. hortensis, in terms of the nature of the Golgi complexes and their contents. There tended to be more cisternae in the complexes of A. reticulatus, 8 to 15, whilst there were only about 9 in A. hortensis but they were longer in the last mentioned species. Also, the Golgi cisternae were more closely packed together in A. hortensis, 144Å to 180Å as opposed to 300Å to 400Å in Agriolimax.

However, the secretion of M (Plate 42) appeared like that in M3 as described by Lainé. However, she found some intermediates between M2 and M3, so the M cells of A. hortensis may be equivalent to her M2/M3 gland cells, since these two may be functional stages of the same cell.

There was no equivalent to the M1 cells or to the five granular areas described by Lainé, and although she believed one to be proteinaceous, it did not resemble the pigment/protein cell of Arion

The melanin deposits found in both genera were similar in appearance and distribution (although being much less in Agriolimax), but melanocytes were found by Lainé in the adult mantle and this was not the case in Arion (not even using the light microscope). There was evidence of melanocytes in the regenerating tissue of Arion hortensis.

The mantle epidermal cells of both Arion and Agriolimax were very similar, including the presence of a microvillar layer, although the size of the gap between the two cells on their outer surface differed, possibly because of the different fixation techniques employed.

After this brief examination of the ultrastructure of the normal mantle, the next section will consider the regeneration of that tissue, particularly in relation to the mucous gland cells.

R. REGENERATION OF THE MANTLE

R.D. Damaging of the Tissue

A constant area of tissue, from the midpoint of the posterior mantle edge inwards in an extended slug, was damaged using a hot, bent wire. This wire was heated to red heat, the glow was allowed to subside and then it was placed on the animal's back for two seconds. The size of the wound was approximately 2.5mm long by 0.5mm wide.

R.M. Maintenance of Regenerating Slugs

After damaging, the animals were returned to the vivarium (CM.pp. 4-6) and kept in those conditions until they were sacrificed at the following intervals after wounding: Days 0, 1, 6, 8, 11, 15, 19, 22, 25, 27, 29, 32, 35, 39, 42 and 46.

R.LM.1. Fixation for Light Microscopy

For the light microscope survey the whole slug was immersed in Lillie's 10% Neutral formalin for 23 hours and washed in running tap water for 2 hours. Dehydration, clearing (in toluene) and embedding (leaving in one of the wax baths overnight) was accomplished in the usual manner. Sections were cut at 6µm and 10µm and stained with 1% aqueous toluidine blue, PAS with diastase, mercuric bromophenol blue, Sudan black B (in 70% alcohol) and Alcian blue -CEC (0.2M and 0.5M $MgCl_2$) using the methods listed in LM2 pp.11-16 .

R.EM.1. Fixation for the Electron Microscope

The damaged tissue only was dissected out for fixation in glutaraldehyde and osmium tetroxide and embedding in TAAB resin, using Schedule 5 (EM1 (2) (ii) pp. 121-122) with the modifications given on pp. 166 for the normal tissue (EM2 (2)).

Instead of considering the historical background at this

juncture, it will be given after the light and electron microscope results for this survey, since it is considered that they will be more useful here.

R.LM.2 (i) Results from the Light Microscope Survey

Tissue fixed immediately after wounding shows a 'V'-shaped area of damage, the edges of the wound appearing rather ragged. It penetrates through the epidermis and glandular layer into the connective tissue below. There is evidence of slime around the edges of the wound, together with some tissue debris.

The appearance of the wound 24 hours after damage is shown in Fig.45. The area is still approximately 'V'-shaped and penetrates into the connective tissue. However, it is no longer ragged in appearance for the wound is completely covered by a layer of nucleated cells which appear to derive from the epidermis of the surrounding undamaged tissue. At the edge of the wound the epidermal cells, instead of being columnar, are extended laterally and appear quite flat. There is considerable 'piling up' of epidermal cells in the wound region so that the sheet covering it is two or three layers deep. It is interesting to note that whilst the normal, ovoid nuclei near the edges of the wound have their long axis perpendicular to the cell surface, those at the base are parallel to it and gradations between these two are visible up the sides.

In the connective tissue below the epidermal cell sheet there is a mass of cells. Some of these are possibly phagocytes containing melanin or else melanocytes (this cannot be established because the contents are so dense). It is perhaps more likely that they are phagocytes removing cell debris. Others are quite big (about 35μm) with large central nuclei and a somewhat reticulate or granular cytoplasm, which is faintly metachromatic but also contains glycogen.

Fig.46 shows the wound six days after damage, and the epidermal

cells, which completely cover the wound, appear flattened or almost cuboidal. Below this is a band composed mainly of phagocytes filled with melanin (or possibly melanocytes) and some cell debris. This area constitutes the demolition zone, and this zone plus the layer of cells below this constitutes the blastema. This lower layer appears to be composed of spindle-shaped bodies and connective tissue fibres. The spindle-shaped bodies could be fibroblasts or myoblasts. Their nuclei are large and almost centrally placed in some instances and terminal in others. Patches of chromatin are evident, and some of the cells contain melanin granules but do not appear to be as densely filled with pigment as the 'phagocytes' in the demolition zone. It could be that these cells are producing pigment for future deposition around the gland cells. They will be described in the ultrastructural investigation (R.EM.2 pp. 196-197). The tissue of the blastema appears to be very loosely packed and disorganised at this time, and below it can be seen the undamaged mantle tissue containing glycogen deposits (EM.2.4.(vi) pp. 183). Fig.46 is drawn from a thick section (1µm) taken from tissue fixed and embedded for an electron microscope investigation and the clarity is much greater, since individual cells are well preserved. However, since the tissue is embedded in resin it could only be stained with toluidine blue (EM1 (2) (ii) p. 119), so histochemical results are limited to stating that there is no evidence of metachromatic gland cells in the wound area, and the cytoplasm of the fibroblasts/myoblasts stained blue.

It is interesting to note that most of the above cells are orientated with their long axis perpendicular to the epidermal surface (Fig.46).

Pan's (1958) description of the fibroblasts of Australorbis agrees with the above quite well in that he found them to be spindle-shaped or fusiform, occasionally having several branched processes. These processes were attached to or ended on nearby fibroblasts thus

forming a loose meshwork of connective tissue - rather as is seen in the ultrastructure of the regnerating tissue (R.EM.2.(ii) a. pp. 19). The fibroblast had an ovoid nucleus, which was fairly rich in chromatin, and a little cytoplasm which was mainly at the poles of the cell. Various amounts of brown-black pigment were seen and he thought that the fibroblasts might transform into pigment cells, as well as other types.

Day 8 appears much as Day 6 but the cells containing glycogen seem to be absent from the blastema, presumably since their contents have been used as an energy supply.

By Day 11 the number of melanin-bearing phagocytes has dropped considerably, and the distinction between the demolition zone and the rest of the blastema, made after six days, is not applicable here. The inner part of the blastema appears to be composed of a very densely staining connective tissue with fibres encompassing the differentiating cells. The epidermis is well-formed although only cuboidal and not columnar as in the normal mantle.

Days 15 and 19 resemble Day 11 tissue closely and Fig.47 shows the Day 19 tissue. The only point of variation lies in the epidermal cells of the Day 19 regenerate. Here some 'piling up' of cells is still apparent. This illustrates a difficulty of regeneration studies, where a series of animals is damaged and sacrificed at intervals, and that is that it is assumed that each slug is damaged equally and that each will regenerate at an equal rate. This is not necessarily so, as illustrated in Fig.46. Wounding by cautery, rather than by excision may cause more variable amounts of damage so that regressive stages may be apparent in the time sequence. Such difficulties can only be overcome by a more efficient mean of injuring the animal and numerous repetitive studies, which time did not allow in the current survey.

However, Fig.47 shows the more regular distribution of melanin within the blastema as well as the densely staining, closely-packed connective tissue. This last named tissue stains very strongly with

mercuric bromophenol blue and under the light microscope some areas appear paradoxically crimson. According to Mazia, Brewer and Alfert (1953) this is indicative of a very high protein content, and is not evident in the uninjured tissue. One would expect to find large amounts of protein in newly forming tissue, such as is found in the regenerate.

No cell division is detected in the blastema, either in the tissue examined by the light or electron microscopes, but it is possible that it may have been missed since only limited amounts of tissue at restricted time intervals were examined.

No metachromatic or alcianophilic material is evident in the Day 19 material, but between Days 22 and 29 one or two small mucous gland cells appear in the densely staining connective tissue of the regenerate. These gland cells display metachromasia and alcianophilia, and their contents are reticulate. They are occasionally almost spherical in shape, but mainly ovoid, there being no ducts visible between the epidermal cells, although this does not mean they are absent. The gland cells lie in the connective tissue below the epidermis, which appears columnar although not quite so ridged as in the normal tissue. The melanin is still not typically distributed in the regenerate, being in rather large aggregates in some places. The connective tissue of the regenerate is still very compact and stains strongly in mercuric bromophenol blue, indicating that more protein than normal is present.

Fig.48 shows Day 32 tissue with 'new' mucous gland cells, and the regenerate appears much the same as the Day 22 and 29 material, the melanin distribution and the densely staining connective tissue being the 'abnormal' features. The mucous gland cells are smaller and less numerous than in the normal tissue.

Fig.49 shows the Day 42 tissue. The mucous gland cells are more numerous and the more elongated ones have ducts passing between the epidermal cells. Those cells which apparently lack ducts are more ovoid in

outline. However, the epidermis covering the regenerate here is columnar at the edges but cuboidal towards the centre of the wound. This may be evidence that differentiation occurs from the tissue adjacent to the undamaged tissue inwards. Also, the mucous gland cells are more numerous at the edges of the wound.

A regenerated pigment/protein gland cell "shell" is also present in the Day 42 tissue, it is small, but elongated, and the only example of its type. It would seem that these cells develop after the mucous gland cells in damaged tissue.

Fig.49 further illustrates the point that regeneration does not occur equally in all animals since the epidermis was columnar in the Day 32 tissue (Fig.48). The melanin still appears abnormally in fairly large aggregates which lie deeper in the regenerate connective tissue than in normal (where it is mainly confined round the gland cells). The connective tissue of the regenerate continues to stain more deeply than that of the uninjured tissue and these two abnormalities are still apparent in the Day 46 tissue when the experiment ended.

R.LM.2 (ii) Conclusion

The results of this survey are rather disappointing in that the origin of the mucous gland cells is not clear, although the pigment cells appear to develop from fibroblasts derived from the connective tissue. Whether these fibroblasts can develop into other cell types is not shown by this survey.

A more extensive investigation, using fixation and embedding techniques devised for electron microscopy but examining 1µm sections (stained by toluidine blue) with the light microscope, might give rise to better results. Certainly the day 6 tissue examined in this way gives a fairly clear picture of fibroblasts containing melanin. It would also be necessary to have a series of slugs sacrificed between days 15 and 32 to

establish the origin of the mucous gland cells. The random sacrifices at 15, 19, 22, 27 and 29 days did not provide adequate coverage of this critical period.

R.EM.2. Ultrastructural Investigation of Regeneration

R.EM.2 (i) Introduction

A thorough investigation would have been an enormous task which time did not allow. Material from days 15, 19 and 22 was used covering the period during which, according to the light microscope survey above, mucous gland cells first appeared.

R.EM.2 (ii) Results

(a) Day 15

Plate 50 illustrates the epidermis over the blastema. The individual cells are almost cuboidal although still slightly elongated laterally, being about 8.5μm high and 13μm wide. This is in contrast to the normal columnar cells (Plate 39) which measured about 14.2μm high by 2.9μm wide. The flatness of the regenerate epidermis is very clear, as opposed to the ridging in the normal tissue. The flatness is probably the result of variable contractions of the underlying muscles, and since the musculature has not regenerated by Day 15 the epidermis is flat. However, the exposed surface of the regenerated epidermis is covered with microvilli which are about 1μm long, which is slightly greater than in the normal tissue examined earlier, but more measurements on more specimens would be necessary to substantiate this variation. The microvilli bear the "fuzzy coat" of radiating fibrils (Plate 51). The frequency of microvilli is almost identical to that on top of the ridges of the normal mantle.

The epidermal nuclei tend to lie towards the bases of the cells in the regenerate, as in the normal mantle, but are ovoid with slight

elongation laterally, although this is not consistent. They measure about 5.8μm long (the axis that is perpendicular to the cell surface) and 8μm wide as opposed to about 7.3μm long by 2.2μm wide normally. From these measurements of the nuclei, and those for the whole epidermal cell (p.194) it is possible to see that the nucleus of the regenerate occupies a much larger volume than its counterpart in the normal tissue. A single, large nucleolus can be distinguished in each cell, and the chromatin is present peripherally as a very thin band and as quite numerous isolated patches in the nucleoplasm. In the normal tissue these patches are fewer and larger. The nuclear membrane is about 300Å across (Plate 51) somewhat wider than in the ordinary material, but this measurement is only an average.

The cytoplasm of the regenerated epidermal cell is fairly granular and contains scattered mitochondria (Plate 51). Their outer double membrane measures 120Å approximately and they are usually ovoid in shape, but occasionally very elongated. The number of mitochondria in the apical cytoplasm appears less in the regenerate, as does the amount of apical (supranuclear) cytoplasm. There are two examples of Golgi complexes above the nucleus, the cisternae of which are quite long, in the region of 1.35μm and contain a substance of medium electron density, but the distended ones are electron-translucent. There appear to be five or six cisternae in each complex. There is possibly a little rough endoplasmic reticulum present, particularly in the region below the nucleus, but the print is not clear.

The cell junctions are not distinct because of oblique sectioning, but there appears to be a zonula adhaerens with an extensive septate desmosome below. However, there are also quite large gaps between the membranes below the septate desmosome. These gaps were noted in the normal mantle but are much larger in this tissue, so much so that at the bases the adjacent cells often do not communicate (Plate 50).

Although the epidermis of the regenerate is returning to normal by Day 15 the tissue below it is apparently quite disorganised. The area is filled with numerous elongated cells with long processes emanating from them and they are "suspended" in an electron-translucent medium, in which there is no evidence of collagen or muscle fibres or protein or mucous gland cells. These cells often have large nuclei (Plate 52), relatively little cytoplasm (of medium electron density) in a narrow band round the nucleus, and granules of melanin. They resemble closely the description of the fibroblasts of Australorbis glabratus, given by Pan (1958). It is not easy to give measurements for these cells since they are variable. The larger specimens measure about 12µm long and 2.3µm at their widest point, whilst the smaller ones are about 3.3µm long by 1.25µm wide. These measurements exclude any of the long processes. The nuclei are elongated and contain peripheral and isolated patches of chromatin. Plate 52 shows one cell with an enlarged patch of material in the nucleus, it may be a nucleolus. The nuclear membrane measures 500Å across.

Organelles are not visible in the cytoplasm at this magnification, but some cells contain spherical black granules of the same diameter as in the normal mantle. Occasionally, the cells contain spherical deposits of much greater diameter (Plate 52) .... whether the melanin granules condense from these larger patches is not known. The cell indicated in Plate 52 appears to contain a myelin figure and this could indicate that it is in an abnormal condition. On the other hand this cell could be a phagocyte. Plate 53 shows one of these "fibroblast-like" cells at higher magnification. The nucleus, with peripheral and central patches, is elongated in the cell at the left of the plate and fills a large portion of the cell. A small amount of rough endoplasmic reticulum is also visible and contains an almost electron-translucent substance. In the cell in the centre of the same plate the cytoplasm

contains rough ER together with some poorly defined Golgi complexes adjacent to which are numerous vesicles of medium electron density, which may instead be sections through the Golgi cisternae. These vesicles, if they are so, may fuse to give the large, less electron dense, spherical deposits (premelanin) seen in the cell at the right of Plate 53. They may condense to give the dense melanin granules.

Two points are to be noted in connection with the central cell in Plate 53. One is that the melanin tends to be deposited at each pole of the cell; and secondly there is a spherical body of medium electron density, bounded by a double membrane in the cell. It could be a pre-melanin mass or else a rather unusual mitochondrion, it is not possible to decide which. Lainé's (1971) description of melanocytes agrees quite well with the above except that she found no Golgi complexes. The sequence of development of melanin granules has been suggested by previous workers, for instance Wellings and Siegel (1963). However, the rod or plate-like formations described by them in the melanosomes (the stage before the formation of mature melanin) are absent in Arion hortensis nor were they seen by Lainé in Agriolimax. These cells containing granules of melanin account for the aggregates seen in the light microscope survey of the regenerating tissue at this time and later. They are possibly fibroblasts (from the connective tissue) in origin since they resemble those, bearing pigment, described by Pan (1958). Whether all the fibroblasts present develop into pigment cells is not known, for there are some smaller ones which lack deposits. The melanin is presumably developed early in the regenerate to provide some form of protection, possibly against light.

There is an alternative to the above explanation, that is that the cells are phagocytes which have taken up melanin debris, and this may be the case in one or two instances where myelin figures are present, as mentioned earlier (p.196). In general, however, the shape of the cells

does not seem to be consistent with their being phagocytes, and the presence of what are believed to be premelanin granules suggests that the cells are actually producing melanin.

Two more features are to be noted in connection with the Day 15 tissue. The first is the cell (X) indicated on Plate 52 which has a quite different shape from the fibroblasts, although it is elongated along one axis, measuring 15.5μm by 8.7μm. The nucleus is very large and ovoid, about 7.2μm by 6.2μm, and contains a prominent nucleolus. There are some peripheral patches of chromatin and small, isolated patches in the nucleoplasm. The nuclear membrane measures about 420Å. The cytoplasm is slightly less electron-dense than that of the pigment cells and at this magnification it is only possible to discern the presence of a fairly large amount of rough endoplasmic reticulum, containing a fairly pale substance. Also at one end of the cell is a rather distended vacuole, or vacuoles, whose nature is unknown, and at the other end there are fine aggregate granules which might be glycogen.

Plate 53 shows part of one of these cells at a higher magnification. The rough endoplasmic reticulum can be clearly seen, with its palely electron-dense contents. The shape of the cisternae varies, in some cases they are long and narrow (about 500Å wide), in others they are ovoid (1750Å wide) and in still others they are almost spherical (4750Å in diameter). At this magnification, elongated mitochondria can be seen although the details are not clear.

The nature of the above cell is not known, although it appears to be producing a protein at some point, because of the presence of ribosomes. It is seen in the Day 19 tissue and will be discussed later (R.EM. 2 (ii) b. p. 201-202).

The only possible evidence of nerve regenerating is seen in Plate 54, where the "circular profiles" indicated could be sections through axons. It resembles, quite closely, the nerves seen in Plate 43

for the normal mantle. On the other hand, it might be some debris resulting from the destruction of a nerve during wounding.

R.EM.2 (ii)

(b) Day 19

Plate 55 shows the regenerated epidermis 19 days after wounding. The epidermal cells are columnar, 11.6μm long by 5.6μm wide, but are still shorter and wider than the measurements taken for the normal mantle (p.170). However, there is likely to be variations in cell size between different animals. The nuclei are basal and ovoid, with the longer axis perpendicular to the cell surface (6μm by about 4μm), as in the mantle, although in the latter case they are more elongated. There is some chromatin round the membranes and a few isolated patches in the nucleoplasm. A single nucleolus is visible, and the nuclear membrane measures about 290Å across. The microvilli measure approximately 1μm in length and show the radiating fibres. By this time the epidermis begins to show some very slight ridging and this is possibly related to the presence of a few muscle fibres in the blastema.

A zonula adhaerens and a septate desmosome are found between adjacent cells but again large spaces are present below this. The epidermal cell cytoplasm appears as in the normal mantle, with numerous mitochondria, particularly in the supranuclear region. These mitochondria are elongated, up to about 1950Å, and in some instances show diagonal cristae. A "terminal web" is present below the microvilli. At the magnification on the print it is not possible to distinguish any other organelles.

Plates 56 and 57 show the condition of the subepidermal tissue after 19 days. Mature mucous gland cells are present at the edge of the wound and these appear as in the normal mantle.

Muscle and collagen fibres are present in the blastema (Plate

56) and these were absent in the Day 15 tissue (Plate 50). Plate 58 shows the periodicity of the collagen fibres. The muscle fibres appear as in the normal mantle but are less numerous. Plate 57 shows a cell which may be a myoblast. The nucleus is surrounded by muscle fibrils and measures about 3640Å by 2000Å, whilst the cell itself measures 5400Å by 2940Å. The chromatin is distributed peripherally and in two or three more central patches. Extensions from the nuclear membrane can be seen passing into the cytoplasm, where it presumably represents the endoplasmic reticulum (rough). However, the rest of the cytoplasm is filled with muscle fibrils, which are striated.

In general, the sub-epidermal tissue still appears much more loosely packed and disorganised in comparison with the normal mantle.

Very few of the fibroblast-like cells, noted in the Day 15 tissue, are present in the Day 19 material - only one can be seen in Plate 56. The nucleus is elongated, and in this section the chromatin is peripheral. The cytoplasm is confined to a narrow band round the nucleus and, in a projection from one pole, pigment granules can be seen. The bulk of the granules are present in membrane bound bodies as seen in the normal mantle (Plate 43).

In Plate 56, there is what appears to be a melanocyte showing the various stages of melanin production. The elongated nucleus adopts a basal position, and rough endoplasmic reticulum is present, together with numerous ovoid mitochondria in the supranuclear cytoplasm. There are one or two areas which could be Golgi complexes, but they might also be elongated mitochondria. Two large areas of secretory material are present one being palely electron-dense and homogeneous in appearance, the other being fairly electron-dense with whorls. It could be that this last is a condensing stage between the pale premelanin granule and the mature granules which are also present in the cell. These whorls may be equivalent to the rods and plates described by previous workers, for

instance Wellings and Siegel (1963) in mammalian tissue. They were not noted in the Day 15 tissue, but the quality of prints is better in the Day 19 tissue. The cytoplasm shows some long, thin projections from its surface, which may indicate its similarity with the fibroblast-like cells described in Day 15.

In the centre of Plate 56 there is another, almost stellate cell, which has long thin projections from its surface. This could mean that it is an immature melanocyte derived from the fibroblast-like cells seen in Plate 52, or else that this is a second cell type derived from the fibroblasts, possibly the cell (X) described in the Day 15 material. It is not possible to decide since only cisternae of the endoplasmic reticulum are visible. The other cell type, described as (X), is also present in the Day 19 material, but it is much more numerous being the more common type in the regenerate, whilst it was rare in the earlier tissue. Examples of it can be seen in Plates 56 and 57, and a higher power representation can be seen in Plate 58, where the cell organelles are clear. The nucleus is elongated and the chromatin is peripheral and in more central, isolated patches. A single nucleolus is also evident. The nuclear membrane measures about 200Å across and contains a substance of medium electron density, a similar substance being seen in the rough ER. The nuclear pore indicated on the Plate measures about 740Å in diameter. The ER cisternae are long and narrow (on average 450Å across), but they show dilatations in some places. Mitochondria are evident, and one appears exceedingly long (although this could be two overlying each other). Cristae are numerous and the outer double membrane measures about 180Å. A Golgi complex, with five cisternae, is visible at the edge of the print. They contain a substance of medium electron density, as do some secretion-containing vacuoles present on the mature face. Vesicles appear to be present at the tips of the Golgi cisternae but these could be sections through their ends. The nature of this secretion is unknown,

although it is possibly proteinaceous. Occasionally, single melanin granules are seen associated with these cells (Plate 56) but they could be intrusions from other cells (the appearance resulting from an unusual sectioning angle) since there is no indication of developmental stages. However, there is no definite evidence that these cells are, or are not, melanocytes. Alternatively, they could be very young mucous gland cells, although the preponderance of ER and the paler contents of the Golgi complexes is rather different from the situation appertaining in the mature mantle gland cells.

They might also be very young protein cells but the ER cisternae are more narrow. Dilation, due to the production of more protein, may occur as the cell ages and matures.

Lastly, they might be producing collagen - although there is no evidence of the inter-communicating vesicular system or the bordered pores described by Plummer (1966) in the collagen cells of the Achatinidae, but an extensive rough endoplasmic reticulum is present. Plummer quoted Jackson's (1964) view that collagen fibrils may be polymerising extracellularly, and it is possible that the substance produced by (X) could polymerise to produce collagen. However, it is not possible to reach any conclusions about this cell, except that it might be a young mucous gland cell, since many young cells have an extensive rough ER, on the other hand it could be any one of the other cell types indicated above. It might be significant that this cell is rare in the Day 15 regenerate and that collagen is absent, whilst both are present by Day 19.

R.EM.2 (iii) Conclusion

It would seem that the pigment cells derive from the fibroblast-like cells described in the Day 15 regenerate. If these are fibroblasts then the pigment cells at least have a connective tissue

origin. The unidentified cells (X) may have a similar origin. However, there is no conclusive evidence for the origin of the mucous gland cells, although the (X) type could give rise to them. Pan (1958) found that the fibroblasts of Australorbis gave rise to pigment cells and believed them to be involved in tissue repair also. At the edge of the wound, the mucous cells, at least, have reformed whilst there is no evidence of their formation in the centre. It would seem, therefore, that a much more extensive investigation of regeneration in the 19 to 22 day regenerates is required, in the hope of establishing the gland cell origin. They might be more likely to derive from the still relatively disorganised sub-epidermal tissue than from the well-formed epidermal cells. No evidence of cell division is seen in this investigation but Dyson (1965) also experienced difficulty in observing it, but stated that it reached a peak after seven days. It is possible that a more detailed study of the first week of development might reveal its presence.

Thus, although the results suggest that the pigment cells may have a connective tissue origin, there is no proper evidence for the mucous and protein gland cells.

R.EM.2 (iv) Comparison with other works

Dyson (1965) described the stages involved in the regeneration of the front edge of the mantle in Arion hortensis. She wounded the tissue by excision since it was believed that cautery caused damage beyond the apparent wound margin (for instance, by denaturing protein and destroying enzymes) and this is believed to be the reason why there is a time lag between the stages of regeneration in the two studies.

Within minutes Dyson noted that epithelial cells were released from the basal lamina (not evident in the current investigation) and began to extend and migrate over the wound. Because there was some

piling up of cells at the free edge of the migrating sheet, she suggested that individual cell movement might play a part in the migration. A similar "piling up" is seen in the Day 1 tissue in this investigation. At the same time, Dyson noted that blood cells agglutinated and muscular contraction restricted the size of the blood spaces, reducing further loss. Demolition and removal of dead tissue began within 24 hours, accompanied by a heightened level of acid phosphatase, two types of phagocytes were recorded, one being a blood cell and the other a tissue cell. Possible phagocytes are seen in the Day 1 tissue of this work, and similarly both works recorded the presence of glycogen in the wound region at this time, presumably acting as an energy supply for growth.

After three days Dyson recorded that the dorsal epidermal cells appeared squamous to cuboidal, but the ventral ones were still flattened. Such a situation is noted in the dorsal cells by Day 6 in the current work. They may have been in existence longer but no tissue was investigated between Days 1 and 6. Wound closure was complete by Day 5 in Dyson's tissue and by Day 6 in this work.

In both works an increased staining of the connective tissue of the wound region, at Day 1 by Dyson and Day 11 onwards here. Dyson believed that this might indicate a rise in the amount of hyaluronic acid present, which could act as a tensile matrix increasing wound strength.

Dyson recorded the appearance of blastema cells, derived from epithelial, blood, connective tissue and nerve sheath cells, two days after wounding. It is noted by Day 6 in this work, but it probably appeared earlier. Dyson found that myoblasts were local in origin formed by the de-differentiation of local muscle cells. Demolition and de-differentiation occurred simultaneously for some time, but after five days demolition was succeeded by repair and mitotic

division reached a peak after seven days.

After six days, Dyson stated that the number of phagocytes had dropped but that those that remained contained pigment, as noted in the Day 6 tissue here.

In both works, a division of the blastema into a demolition and fibroblast zone was noted, by Day 6 in this work.

Differentiation of the blastema into epithelial cells, fibroblasts, muscle fibres and some connective tissue was evident after 8 days. It began in the cells adjacent to the uninjured tissue and progressed outwards to the rest of the blastema. In this work, it was noted that the more developed wound cells appeared adjacent to the normal tissue.

By Day 14, Dyson recorded a rise in the amount of SS and SH groups in the wound, reaching a peak at 21 days. This was the stage at which myoblasts were differentiating, and Dyson believed that the increase reflected the amount of fibrous protein being produced. The other blastema cells also showed increased staining and this was believed to be the result of protein synthesis (necessary for growth) following cell division. A rise in protein staining is noted in the Day 19 tissue of this work.

Occasionally, dorsal mucous gland cells were noted by Dyson in the Day 14 tissue, resembling type III, and both protein and dorsal mucous cells were present by Day 21 and the epidermal cells appeared normal. The mucous gland cells appear to arise between days 15 and 22 in the current work, but protein cells were not seen until Day 42.

In both works, by Day 42, the regenerated tissue could only be distinguished by the heightened staining of the connective tissue and the unusual distribution of pigment granules.

Thus, there are considerable parallels between the two investigations, although there is a time lag in the cauterised tissue,

the main difference relating to the origin of the mucous gland cells, Dyson believing them to be epidermal.

Guardabassi and Piacenza (1958) studied the regeneration of the pallial epithelium of Helix pomatia, and whilst this work was principally concerned with the re-formation of the shell, they noted a strong PAS reactivity and intense metachromasia (in toluidine blue) in the damaged mantle, 24 days after wounding. This is in agreement with Dyson's findings and corresponded to those in the current work.

Arcadi (1965) excised a segment from the lateral portion of the foot pad of Lehmania poirieri and after three hours only a general disorganisation could be seen with an invasion of what appeared to be fibroblasts. These were not seen in the 24 hour tissue of the current work, being detected after 6 days. However, both Dyson and this worker (Lawrence) recorded the presence of phagocytes at this time. After two days, Arcadi noted that the epidermis adjacent to the wound had begun to migrate and completely covered the wound after four days. In this work the epidermis had covered the wound after six days but did not appear normal until Day 32 or later. Arcadi stated that the tissue appeared normal after 17 days but only accumulations of alcianophilic material, deep in the subcutaneous tissue, were evident. Arcadi believed this to mark the beginning of the regenerating mucous gland cells, and by days 21 to 22, they appeared normal. Thus, it would seem that they derive from fibroblasts or interstitial cells containing alcianophilic granules. In Arion mucous gland cells were seen as early as Day 14 by Dyson and by Day 22 in this work.

Binot (1965) studied the regeneration of the pedal sole of Oncidiella celtica, and found the demolition period to extend between days 1 and 5, whilst in Dyson's (1965) work on Arion it appeared to extend until the sixth day and in the current work until at least day 8. In Oncidiella a thin epidermis was evident between the fifth and

seventh days, whilst in both works on Arion it was present after only 24 hours. Between Days 36 to 42 the epidermis returned to normal, whilst round cells, rich in ER, appeared in the depths of the connective tissue. These were believed to be young mucous cells, and they could be equivalent to the (X) cells described in the current work on Arion, although these were evident between Days 15 and 19.

Thus, the evidence from Arcadi and Binot suggests a connective tissue origin for the mucous gland cells, whilst Dyson believed them to be epidermal. They are suspected to have a connective tissue origin in this work.

D DISCUSSION

This research has attempted to describe the structure of some of the gland cells associated with the skin of Arion hortensis, and to define the nature of their secretions.

The function of the slime produced by molluscs has already been considered (LM.3 pp 17-18) and in Arion that produced by the pedal gland and A/B gland cells in the sole (a chondroitin) is concerned with lubrication for locomotion. The function of the less numerous, granular C gland cells, also in the sole, is unknown but their secretion product may modify that of the other cells of the foot. This secretion is a mucoprotein, there being evidence of sulphated groups and tyrosine. The M/m cells of the dorsal surface also produce an acid mucopolysaccharide, but a low sulphate chondroitin, apparently combined with protein. It may keep the skin moistened and clear of debris, as well as allowing some cooling of the body by latent heat of evaporation. Pigment/protein cells are present in the dorsal integument, and the protein may have a repugnatorial effect and/or change the viscosity of the slime. The pigment appears to be a dietary waste product, since the newly hatched slug lacks the yellow colo ration, at least in the sole.

Interestingly, the counts of gland cells (Histograms I and II) revealed that more are present at the anterior end of the animal and this might be expected in an animal which proceeds forwards. More slime would be required at the front to protect the dorsal and ventral surfaces as they pass through the substrate.

In terms of the relative numbers of gland cells distributed over the body surface, the M/m and A/B mucous gland cells are most numerous followed by the pigment/protein and then the C gland cells.

The secretion produced by the mantle groove mucous gland cells is different again, being granular and apparently more sulphated, combined with protein and neutral mucopolysaccharide. Its function is

unknown, but the gland cells' position might suggest that the slime lubricates the groove and reduces the friction between the undersurface of the mantle and the sides of the body. Campion (1957, 1961) described granular PAS-positive cells in the mantle collar of Helix aspersa; and Barr (1927-1928) described granular cells in the mantle margin of Arion ater, as did Chétail & Binot (1968b) in Arion rufus, but their function was not considered. The finding of them in the shelled Helix is interesting, possibly indicating that they are retained from the stage in evolution when the "slugs" were shell-bearing.

Some A/B gland cells are aggregated in the tissue forming the ventral part of the peripodial groove, and their slime may reduce friction between the sides of the body and the top of the foot. However, the slime of the mantle groove mucous cells and that of the peripodial groove cells are quite different in character so it is not possible to say that they serve a similar function in reducing friction. The secretion of the peripodial groove A/B gland cells also laterally supplements the slime produced by the pedal gland, but it has not been possible to assess how extensively this last secretion spread over the foot. Nor is it known whether the pedal gland mucus is more fluid than the secretions released directly on to the sole, but it would be advantageous if this were the case since it has some distance to travel within the gland lumen, and, furthermore it could be more easily distributed in this condition. However, the numerous, long cilia in the lumen could also effectively move a viscous slime. The secretion, produced by the gland cells distributed over the sole, may increase the thickness of the "cushion" of slime on which the animal moves ... this would buffer the animal's foot against abrasion.

The histochemical investigation did not reveal the components of the acid mucopolysaccharide produced by the caudal gland, but its function is as a recognition mark or stimulant during courtship (Barr

1927-1928). This is where the'courtship ropes' of the other species, for instance, Arion ater, derive from, and the slime for that would have to be very viscous.

The broad categories of gland cell types are much as described by other workers on other molluscs, although it has been possible to characterise some of the secretions more extensively here using modern techniques. However, it was not possible to identify calcium or lipid gland cells, for instance, as described by Campion (1957, 1961), but Dyson (1965) noted calcium cells in the mantle of Arion hortensis . It is likely that this substance is associated with the pigment/protein cells in the current investigation, but the staining procedures adopted were not as comprehensive as those used by Dyson.

There is no obvious and consistent difference in the literature concerning the nature of slug and snail slime, but this is not to say there is none. The diversity of techniques used at different periods of time makes any exact comparison very difficult. It does seem likely that the granular cells, a, b and c, described by Laine (1971) in her light microscope survey of Agriolimax reticulatus , are equivalent to the pigment/protein cells of Arion.

Considering Dyson's (1965) light microscope investigation of the mantle of Arion hortensis, apart from the difference relating to the calcium cells (already mentioned), there is the question of the presence or absence of a basal lamina. Dyson found one to be present, but it could not be seen in the current investigation, even using the electron microscope. It is likely that what she was seeing is the band of collagen and muscle fibres seen lying below the epidermal cells (Plate 39), and around the bases of the gland cells (Plate 42), which under the light microscope might have looked like a basal lamina. The function of the

lamina is not known, but it may provide an anchoring mechanism for the epidermis.

The ultrastructure of the mucous cells of the mantle and pedal gland varies in the relative proportions of the endoplasmic reticulum and Golgi complexes, the former filling a large area of the pedal mucocyte and the latter being predominant in the mantle mucous gland cells. This may indicate that protein is a major component of the pedal gland secretion.

The most interesting structure revealed by the electron microscope was the tubular system within the endoplasmic reticulum, and whilst this has been reported before a new interpretation of its form is presented here. The tubules, believed to be precipitated protein, are thought to extend from one wall of the ER cisterna to the other, being shallowly S-shaped and with ribosomes at either end. Wondrak's interpretation of their organisation was rather different, in the form of radiating tubules, but this is not compatible with the plate-like cisternae seen in Arion hortensis.

In order to examine the nature of the small tubules in the endoplasmic reticulum of the pedal gland mucocytes, deamination and pepsin techniques were devised and used on tissue subsequently prepared for the electron microscope.

The pathway for the production of secretion in the pedal gland appears to be blebs from the ER, to vesicles, to the Golgi complex to secretion sacs. In the mantle gland cells, however, the amount of ER is greatly reduced and no blebbing or vesicles are evident. Whether this indicates that the pathway is different is not known, and without an autoradiographic investigation it is not possible to comment adequately on this point.

Two other features are apparent from the ultrastructural work. One is the presence of microvilli along the upper epidermis of the pedal gland lumen and on the mantle epidermis. Their function is presumably absorptive but what is being absorbed is not known. The second point is the paucity of nerve supply, particularly in the vicinity of the pedal gland. Even where nerves are evident in the mantle, their connection with gland cells could not be seen. This lack of innervation has not generally been commented on by previous workers, although Lainé (1971) found that techniques for staining nervous tissue did not give good results. It could be that this absence of nerves reflects the relatively primitive nature of the slugs.

The regular form of the gland cell necks indicates that discharge is merocrine, agreeing with Campion (1957, 1961) and Lainé (1971). The other workers considered in the historical background have not commented on the mechanism of discharge. The merocrine method would be advantageous since it presumably causes less disruption of the epidermis, and is less wasteful in terms of cell organelles. Since there is little evidence of developmental stages, except perhaps in the case of the pigment/protein cells, it seems likely that the gland cells function throughout the life of the animal.

Unfortunately, there is little information on the origin of the gland cells, although there is circumstantial evidence for a connective tissue origin.

One would expect a young gland cell to contain a fairly extensive rough endoplasmic reticulum composed of narrow cisternae (which have not filled with secretion components), numerous mitochondria, supplying energy, together with some Golgi complexes, and the nucleus would probably have a prominent nucleolus. This description agrees quite well with the (X) cells seen principally in the Day 19 regenerating mantle tissue, but the secretion present in the Golgi cisternae is unlike

that in the mature mantle gland cells. However, in the early stages the cells would contain little of their characteristic secretion and it is possible this may have gone undetected, although one might have hoped some detail would be evident. Thus, they could be young mucous cells and since they were present in the blastema below a well-formed epidermis, this suggests that the gland cells could have a connective tissue origin. One would expect young cells derived from epidermal cells to contain less endoplasmic reticulum and fewer Golgi complexes than those derived from connective tissue cells since these former do not normally have a secretory function.

Much more work needs to be conducted in order to ascertain the origin of the gland cells in Arion hortensis. A more detailed study on the regeneration of mantle tissue, particularly with respect to the ultrastructural aspect, might have provided some evidence of gland cell origin. In the text of that section it was said that a more thorough investigation of the Day 19 and 22 regenerates might establish the origin of the mucous gland cells at least. However, it would also be of value to examine the regenerate tissue prior to this time to see the 'arrival' of the "fibroblast-like" cells and (X) cells. Quite valuable results might be obtained if autoradiographic techniques could be combined with regeneration studies since they might allow for a more effective tracing of the origin of cells, by labelling their components. The regeneration results as they stand at present are inadequate and can only be regarded as a pilot survey. They need to be substantiated by much more work, but unfortunately this was not possible in the time allotted.

It has been possible to correlate the structures seen using both the light and electron microscopes but, in general, fixation for the electron microscope appears to be better since the cells are full and not shrunken and empty. This is partly because only small pieces of tissue are used and therefore fixation is more rapid, and partly because care is

taken with the molarity and pH of the fixatives for high resolution work and this is not the case for light microscopy. Unfortunately, the number of histochemical techniques available to the electron microscopist is limited, at present being mainly confined to the general identification of mucopolysaccharides. Also, the embedding of the tissue in resin tends to preclude the possibility of staining thick sections (1μm) with conventional histochemical stains. It might be useful to fix small pieces of tissue in glutaraldehyde and osmium but to clear in benzene and embed in wax, instead of propylene oxide and resin. However, it would still not be possible to cut sections thinner than about 5μm on an ordinary microtome. One manifestation of better fixation is seen in the mantle where large and small mucous gland cells were believed to be present after the light microscope examination. After fixation for the electron microscope the cells are found to be of a fairly uniform size and the small mucous gland cells are believed to be produced by the excessive contraction and discharge of some gland cells when the live animal is introduced into the light microscope fixative. Also the secretion in the mantle cells is seen to be coarsely reticulate after fixation in Susa whilst finely stranded material in sacs is evident in the ultrastructural investigation. Perhaps the most obvious damaging effect of chemical fixation, for the light microscope, is seen on the pigment/protein cells where often only a proteinaceous shell is visible.

A number of problems were encountered in the current investigation and these will be outlined below, together with some possible solutions. One is the identification of the secretions produced and another is determining the mechanism by which they arise.

The usual method of characterising the different secretions is to use stains, which have a varying specificity. In the past few years there has been a considerable proliferation and modification of techniques particularly with respect to the use of various salt concentrations

with the stain. Thus, for example, in the case of methyl green-pyronin (Scott 1967) competing salt ions prevent any substance but RNA staining with pyronin, whereas non-nucleotide polyanions stain as well in ordinary circumstances ... as has been demonstrated in this work. When staining acid mucopolysaccharides, the tests available simply indicate that such a substance is present, but do not characterise the type of group involved. The use of salts and pH extinction curves has, to some extent, made stains like Alcian blue more specific, but when using a range of tests on a complex substance such as slug slime, conflicting and unexpected results may be obtained simply because methods of detection are too crude. Many mucous secretions are composed of a combination of mucus and protein and the proteinaceous part may go undetected since its normal staining properties are masked by the acidic groups. Hence, protein was apparently only indicated by deamination in the mantle mucous gland cells. Yet it could not be established from histochemical tests whether the acidic groups were attached to the amine groups of protein or to the same groups in the suspected galactosamine unit of the mucous part of the molecule. Similarly, for this same secretion, the histochemical tests indicated that both carboxyl and sulphate radicals were present, but it could not be ascertained whether they were attached to the mucoid or protein moiety. Enzymes may help to characterise the mucous part of the secretion, and their action is usually held to be specific, but not all substances are susceptible to digestion - for example, some sialic acids are not affected by neuraminidase.

Thus, although histochemical methods can provide evidence for some of the types of units present within a secretion, they can give no indication of their arrangement. Biochemical and electrophoretic methods may be able to establish the nature of a given secretion, or at least identify large components within it, but problems of linkages may still remain. However, this work is beyond the scope of the present

investigation. If a better understanding is to be obtained about the nature of the secretions produced by Arion hortensis then it would be necessary to subject them to these methods, although the difficulty of obtaining adequate, pure samples would be considerable.

The second major problem to be faced is that of making dynamic interpretations from the static evidence provided by the slide or the micrograph. This is particularly acute when developmental sequences of gland cells are being established, for instance, the Y cells of the dorsal surface of Arion. From the histochemical results, and the images presented, it is only possible to say that they are probably the precursors of the pigment/protein cells. Only if intermediate stages are evident can the conclusion be less than speculation, and these are not common. Using the electron microscope, the situation is worsened since it is necessary to establish the direction of, for example, secretion processes where vesicles may be moving to or from an organelle. The interpretation of the cycle of events is crucial here since a completely false picture could be constructed. Autoradiographic techniques are of considerable importance in establishing pathways and developmental cycles, providing that suitable substances can be found to be labelled and administered with the diet or by injection. It would have been of some value to the current work if such methods had been used, since it was particularly difficult to establish developmental cycles. Labelling techniques would also have established a time sequence for the production of secretion, since such information could not be deduced from the present material.

Thus, the use of a biochemical assay and autoradiographic techniques would provide more information about the nature and production of the secretions in Arion, and the developmental sequence of the cells concerned.

However, a better way of establishing the origin of the gland cells, but even more lengthy, would be an investigation of the embryology

of Arion hortensis. Tissue was prepared for this but no time was available for its examination. A most valuable contribution might have been made if the embryo could have been examined using the electron microscope. However, a light survey would have to be undertaken first to establish the time of initiation of the gland cells, to conduct the whole investigation with an electron microscope would be an enormous task.

Lastly, although nerves have been implicated in the mechanism of discharge, as already mentioned their attachment to either gland cells or muscle fibres has not been established. This might be done by a 3D reconstruction of an area of tissue, using electron micrographs of serial sections. Micrographs would probably be necessary since the nerves are, in many instances, very small and would be best detected ultrastructurally. Although such a reconstruction may provide results for the mantle tissue, it may not be so successful for the pedal gland since nerves were never seen in the gland itself.

Thus, the results of the current investigation have provided additional problems, which might be solved by use of the techniques outlined above. However, it has been possible to characterise the secretions rather more extensively than before, to describe the ultrastructure of the pedal gland and that of regenerating slug tissue, albeit over a limited time interval, neither of which has been done before.

SUMMARY

1. Slug tissue was fixed principally in Susa or liquid nitrogen and exposed to 25 techniques for polysaccharides and mucopolysaccharides (including fluorescent methods); seven for protein; two for nucleic acids; two for lipid; two for calcium; one for connective tissue; one for elastic tissue and one for melanin.

2. Six types of mucous gland cells, two mucous glands and pigment/protein cells were described.

3. The reticulate M and m cells of the dorsal surface were believed to be one and the same type of cell, m being the discharged form. They were thought to contain a low sulphate chondroitin, possibly combined with protein.

4. The pigment/protein cells and what may be their young stages (Y) were also found in the dorsal surface, the proteinaceous part containing tyrosine and the pigment being either a carotenoid or a flavone (probably derived from the diet). They were usually severely damaged after chemical fixation. Discharge appeared to occur after 'severe attack' and the cells might have a repugnatorial effect.

5. The granular mantle groove mucous cells were found on the ventral surface of the mantle, and apparently contained a sulphated and carboxylated mucopolysaccharide with protein, possibly together with a neutral mucopolysaccharide.

6. The reticulate A/B cells were present in the sole, B being the less mature stage. They were believed to contain a chondroitin-like substance combined with protein.

7. These last cells resembled the mucocytes of the pedal gland. This gland's structure, with its complex epidermis, was described.

8. The granular C gland cells were present in the sole, they were less common than the others, and contained a mucoprotein. The mucoid part appeared to be sulphated and the protein stained for tyrosine. It was unusual in that it stained with Weigert's Elastin stain.

9. The caudal gland was described, but the nature of its mucoid secretion was not known.

10. Melanin was found over the dorsal surface, and a yellow pigment in the sole.

11. Deposits of calcium were present in the mantle, they were thought to represent the reduced shell.

12. Glycogen was detected in the connective tissue, presumably being a food reserve.

13. All gland cells were believed to discharge in a merocrine manner, possibly being effected by muscular contraction. Direct innervation of the gland cells could not be seen.

14. The gland cells were thought to have a connective tissue origin, although few developmental stages were seen. Because of this, it would seem that the slug is born with its full complement of gland cells.

15. (a) The ultrastructure of the pedal gland was described, with details of the gland duct epidermis and cell organelles. Fixation was accomplished by glutaraldehyde and osmium tetroxide, and staining with alcian blue and uranyl acetate in the block was attempted.

15. (b) Of particular note was the extensive tubule-filled endoplasmic reticulum. These tubules were thought to be precipitated protein. Deamination and pepsin techniques, applied to the blocks fixed for the EM, tended to substantiate this. Proposals were forwarded for their arrangement.

16. The ultrastructure of the normal mantle was examined, and the structure of the epidermis, M and pigment/protein cells considered. The m cells were not evident .... and were believed to represent excessively and unnaturally discharged M cells. In contrast to the pedal gland, the Golgi complexes were the most common organelles, and the endoplasmic reticulum lacked tubules.

17. Tissue from the same area of mantle was damaged, using a hot

wire, and examined using the light microscope. An epidermis composed of flattened cells covered the wound after 24 hours and mucous gland cells were evident between days 19 and 22. After 46 days the regenerate tissue could only be discerned by the abnormal distribution of melanin and a more strongly staining connective tissue.

18. Tissue was fixed for the EM 15 and 19 days after wounding. The ultrastructure of the regenerated epidermis was described, together with that of the blastema containing possible fibroblasts plus pigment and an unknown cell type (X). This last was most common at the Day 19 stage and could be a young mucous gland cell; however, this could not be established.

19. From the regeneration work it would appear that at least the gland cells of the mantle have a connective tissue origin, since the epidermis was formed before the blastema began to differentiate.

A1 APPENDIX I

Information Concerning the Eggs of Arion hortensis

The conditions in which these eggs were laid has already been described (C.M. pp4-6).

The eggs were found in groups, either partly or completely buried in the soil (to a depth of approximately 2.5 cms in the crystallising dishes). They were ellipsoidal, whitish and opaque, and measured about 3 mm on their long axis.

The eggs were transferred to filter paper (dampened by distilled water) in a petri dish, and placed in a 12°C-16°C (average 14°C) incubator with an 18 hour day.

They were checked for indications of fungus daily and contaminated specimens removed.

The following table gives details of the number of eggs found in each clutch and the number of days taken to hatch, where this information is available.

Table (i): Clutch size and Hatching times for A. hortensis

Clutch size	Hatching Time
11	45 days
17	44 days
20	44 days
6	)
	)
12	)
	)
14	) Time not
	)
16	) established
	)
20	)
	)
21	)

The average clutch size was 15.2 eggs. They were laid by slugs collected in October, just before the normal breeding season.

The newly hatched slugs were between 4 and 6.5 mm long (when fully extended) by about 1 mm in diameter and appeared pale grey on the dorsal surface, with a darker mantle. The sole was white.

A2 APPENDIX II

List of Suppliers of Dyes, Solutions and Enzymes, together with Colour Index Numbers and Batch Numbers

Stain	Supplier	Colour Index Number
Methyl Blue (for Mallory's Triple Stain)	G. T. Gurr	42755
Resorcin-Fuchsin (for Weigert's Elastin Stain)	G. T. Gurr	-
Toluidine Blue	G. T. Gurr	52040
Azure A (MacNeal)	G. T. Gurr	-
Alcian Blue	G. T. Gurr	74240
Revector Methyl Green	Hopkin & Williams	42585
Revector Pyronin G	Hopkin & Williams	-

Solution	Supplier	-
Thionyl chloride	Hopkin & Williams	-

Enzymes	Supplier	Batch Number
β-Glucuronidase: Dry powder 0.1gm., 0.05 EU/mg. or 900,000 Fishman Units/gm.	B.D.H. Biochemical	-
Hyaluronidase: 250mgm., ex-ovine testes, salt-free, lyoph. Activity 300 IU/mg.	Koch Light Labs.	45356
Neuraminidase: ex Vibrio cholerae. 0.05M Sodium acetate buffer with 1% NaCl and 1% $CaCl_2$ added. Activity 500 units/ml.	Koch Light Labs.	54342
Ribonuclease: ex-bovine pancreas.	Koch Light Labs.	49504

B BIBLIOGRAPHY

Andersen H., Møllgård K., & von Bülow F. A. 1970 Histochemie 22 362-375.

'On the Specificity of Staining by Alcian blue in the Study of Human Foetal Adenohypophyses.'

André J., & Marinozzi V. 1965 J. Microsc. 4 615-626.

'Présence dans les Mitochondries de Particules resemblant aux Ribosomes.'

Arcadi J. A. 1963 Ann. N.Y. Acad. Sci. 106 (2) 451-457.

'Some mucus-producing cells of the Garden Slug (Lehmania poirieri).'

Arcadi J. A. 1965 Ann. N.Y. Acad. Sci. 118 987-996.

'Histochemical observations on the Regeneration of some mucus-producing cells in the integument of the Garden Slug, Lehmania poirieri Mabille.'

Arcadi J. A. 1967 Trans. Amer. microsc. Soc. 86 (4) 506-509.

'Two Types of Mucous gland Cells in the Integument of the Slug, Lehmania poirieri (Mabille): A Study in Metachromasy.'

Barr R. A. 1926 Quart. J. micr. Sci. 70 647-667.

'Some Observations on the Pedal Gland of Milax.'

Barr R. A. 1927-1928 Quart. J. micr. Sci. 71 503-525.

'Some notes on the Mucous and Skin Glands of Arion ater.'

Beams H. W. & Kessel R. G. 1968 Int. Rev. Cytol. 23 209-276.
'The Golgi Apparatus.'

Behnke O. & Zelander T. 1970 J. Ultrastruct. Res. 31 (5-6) 424-438.
'Preservation of Intercellular Substances by the Cationic Dye Alcian Blue in Preparative Procedures for Electron Microscopy.'

Bennett G. 1970 J. Cell Biol. 45 (3) 668-673.
'Migration of glycoprotein from Golgi Apparatus to cell coat in the columnar cells of the duodenal epithelium.'

Bernardi G. 1957 Biochem. Biophys. Acta. 26 47-52.
'The molecular size, shape and weight of mucoprotein from cartilage.'

Binot D. 1965 Cah. Biol. Mar. VI (3) 325- 346.
'Histologie, histochimie, cytologie de quelques formations glandulaires d'Oncidiella celtica (Cuv.) (Gastéropode Pulmoné).'

*Chétail M.& Binot D. 1968 (a) Malac. Rev. 1 91-102.
'Histogénèse des mucocytes de la glande et de la sole pédieuse d'Arion rufus (Stylommatophora:Arionidae).'

Binot D. & Chétail M. 1968(b) Ann. Histochem. 13 73-82.
'Les mucocytes du manteau d'Arion rufus (L.) et leur developpement:Histologie et Histochimie.'

Blundstone E.R. 1885 Proc. roy. Soc. XXXVIII 442-445.
'On the occurrence of glycogen as a constituent of the vesicular cells of the connective tissue of Molluscs.'

Bolognani-Fantin A. M. 1965 Boll. Soc. ital. Biol. sper. 41 (5) 257-259.

'Le Cellule 'A Fenoli' nella Cute del Piede di Helix pomatia.'

Bolognani-Fantin A. M. & Bolognani L. 1964 Instituto Lombardo (Rend.Sc.) B. 98 (2) 343-354.

'Dati Biochimici ed Istochimici sulla Mucinogenesi in Helix pomatia.'

Bonneville M. A. & Weinstock M. 1969 Anat. Rec. 163 157 (Abst. only).

'The formation of the apical free surface of intestinal absorptive cells.'

Boycott A. E. 1934 J. Ecol. XXII (1) 1-38.

'The Habitats of Land Mollusca in Britain.'

Campion M. 1957 PhD Thesis. University of London.

'The Structure and Function of Some of the Cutaneous Glands in Some Molluscs.'

Campion M. 1961 Quart. J. Micr. Sci. 102 (2) 195-216.

'The Structure and Function of the Cutaneous Glands in Helix aspersa.'

Cardot H. 1924 J. Physiol. Path. gen. 22 575-586.

'Observations physiologiques sur les embryons des Gastéropodes Pulmonés.'

Carrick R. 1938 Trans. roy. Soc. Edinburgh. 59 563-597.

'Life history and development of Agriolimax agrestis L., the

grey field slug.'

Chandra A. 1968 Lab. Invest. 18 (4) 442-428.

'Undulating Tubules Associated with Endoplasmic Reticulum in Pathologic Tissue.'

Chétail M. & Binot D. 1967 Malacologia 5 (2) 269-284.

'Particularités histochimiques de la glande et de la sole pédieuses d'Arion rufus L. (Stylommatophora:Arionidae).'

*

Conklin J. L. 1963 Stain Tech. 38 (1) 56-59.

'Mucopolysaccharides: staining reactions after formalin containing fixatives.'

Curran R. C. 1964 Int. Rev. Cytol. 17 149-212.

'The Histochemistry of the Mucopolysaccharides.'

Dainton H. H. 1954 J. exp. Biol. 31 165-187.

'Activity of Slugs. I. The Induction of activity by changing temperatures.'

Ibid. 188-197.

'Activity of Slugs. II. The effect of light and air currents.'

Daniels E. W. 1964 Z. Zellforsch. 64 38-51.

'Origin of the Golgi System in Amoeba.'

De Martino C., Accini L., & Andres. 1969 Z. Zellforsch. 97 (4) 502-511.

'Tubular Structures Associated with the Endothelial Endoplasmic Reticulum in Glomerular Capillaries of Rhesus Monkey and Nephritic Man.'

Dorfman A. 1963 J. Histochem. Cytochem. 11 (1) 2-13.
'Polysaccharides of Connective Tissue.'

Drury R. A. B. & Wallingford E. A. 1967 4th Ed.
'Carleton's Histological Technique.' Pub. Oxford University Press.

Dyson M. 1965 PhD Thesis. University of London.
'An Experimental Study of Wound healing in Arion.'

Elves M. W. 1961 Proc. malac. Soc. London. 34 346-355.
'The histology of the foot of Discus rotundatus, and the locomotion of Gastropod Mollusca.'

Essner E. & Novikoff A. B. 1962 J. Cell Biol. 15 289-312.
'Cytological studies on two functional hepatomas: inter-relations of endoplasmic reticulum, Golgi apparatus and lysosomes.'

Ewer D. W. & Hanson J. 1945 J. R. micr. Soc. 65 40-43.
'Some Staining Reactions of Invertebrate Mucoproteins.'

Favard P. 1969 In: 'Handbook of Molecular Cytology.'
Ed. Lima-de-Faria A. Vol.15 in 'Frontiers of Biology'.
Series Eds. Neuberger A. and Tatum E. L.
Pub. North Holland Publishing Co. Amsterdam & London.
Chap.41 1131-1155. 'The Golgi Apparatus.'

Fawcett D. W. 1964 In: 'Modern Developments in Electron Microscopy.'
Ed. Siegel B. M. 257-333.
Pub. Academic Press. New York and London.

Fawcett D. W. 1966 'The Cell.'

Pub. W. B. Saunders & Co. Philadelphia and London.

Férussac 1819 Hist. Nat. Moll. 2 65.

Flickinger C. J. 1969 J. Cell Biol. 43 250-262.

'The development of Golgi Complexes and their Dependence upon the Nucleus in Amoebae.'

Fox D. L. 1953 'Animal Biochromes.'

Pub. Cambridge University Press.

Fox D. L. 1966 In: 'Physiology of Mollusca.' Vol.II.

Eds. Wilbur K. M. and Yonge C. M.

Pub. Academic Press Bew York and London.

Chap. 8. 249-274. 'Pigmentation of Molluscs.'

Freeman J. A. 1966 Anat. Rec. 154 121-147.

'Goblet Cell Fine Structure.'

Fullmer H. M. 1960 J. Histochem. Cytochem. 8 113-121.

'Effect of Peracetic acid on the enzymatic digestion of various mucopolysaccharides: Reversal of the PAS staining reaction of mucin.'

Geuze J. J. 1971 Z. Zellforsch. 117 (1) 87-102.

'Light and Electron Microscopic Observations on the Gastric Mucosa of the Frog (Rana esculenta). I. Normal Structure.'

Ghose K. C. 1963 Current Sci. 32 (4) 167-168.

'Morphogenesis of the pedal gland in the giant snail Achatina fulica (Bowdish).'

Goldblatt P. J. 1969 In: 'Handbook of Molecular Cytology.'
Ed. Lima-de-Faria A. Vol.15 in 'Frontiers of Biology.'
Series Eds. Neuberger A. and Tatum E. L.
Pub. North Holland Publishing Co. Amsterdam & London.
Chap.40 1101-1129. 'The Rough Surfaced Endoplasmic Reticulum.'

Graham A. 1957 Proc. malac. Soc. London. 32 (4) 135-143.
'The Molluscan Skin with Special Reference to Prosobranchs.'

Guardabassi A. & Piacenza M. L. 1958 Archs. Anat. microsc. Morph. exp. 47 (1) 25-46.
'Le manteau de l'escargot, Helix pomatia. Etude cytologique et histochimique.'

Gurr E. 1962 'Animal Tissues Practical and Theoretical.'
Pub. Leonard Hill. London.

Hogben L. & Kirk R. L. 1944 Proc. roy. Soc. B. 132 239-252.
'Studies on temperature regulation. I. The Pulmonata and Oligochaeta.'

Hollande E. 1966 C. R. Acad. Sci. Paris. 262 D No.16 1788-1791.
'Présence de mucoploysaccharides neutres et acides coincidant avec l'appareil de Golgi, dans les glandes multifides d'Helix pomatia.'

Hollmann K. H. 1963 Ann. N.Y. Acad. Sci. 106 (2) 545-554.
'The Fine Structure of the Goblet Cells in the Rat Intestine.'

Horn R. G. & Spicer S. S. 1964 Lab. Invest. 13 (1) 1-15.
'Sulfated Mucopolysaccharide and Basic Protein in Certain

Granules of Rabbit Leucocytes.'

Hubendick B. 1958 Ark. Zool. A.S. 11 (3) 31-36.
'On the Molluscan adhesive epithelium.'

Humason G. L. 1962. 2nd. Ed. 'Animal Tissue Techniques.'
Pub. W. H. Freeman & Co. San Franscisco and London.

Hunt S. & Jevons F. R. 1965 Biochem. Biophys. Acta. 101 (2) 214-216.
'The polysaccharide sulphate-peptide complex in the hypobranchial mucin of Buccinum undatum L.'

Hunter W. R. 1964 In: "Physiology of the Mollusca.' Vol.I
Eds. Wilbur K. M. and Yonge C. M.
Pub. Academic Press. New York and London.
Chap.3. 83-126. 'Physiological Aspects of Ecology in Nonmarine Molluscs.'

Hyman L. H. 1967 'The Invertebrates VI: Mollusca I.'
Pub. McGraw-Hill Book Co. New York and London.

Jackson S. F. 1964 In: 'The Cell' VI
Eds. Brachet J. and Mirsky A. C.
Pub. Academic Press. New York and London.
387-509 'Connective Tissue Cells.'

Jennings M. A. & Florey H. W. 1956 Quart. J. exp. Physiol. 41 (2) 131-153.
'Autoradiographic observations on the Mucous cells of the Stomach and Intestine.'

Kasten F. H. 1960 Int. Rev. Cytol. 10 1-100.

'The Chemistry of Schiff's Reagent.'

Kessel R. G. 1971 J. Ultrastruct. Res. 34 (3-4) 260-275.

'Origin of the Golgi Apparatus in Embryonic Cells of the Grasshopper.'

Kurosumi K. 1961 Int. Rev. Cytol. 11 1-124.

'Electron Microscopic Analysis of the Secretion Mechanism.'

Lainé H. A. 1971 MSc. Thesis. University of Keele.

'Some observations on the Structure of the Skin of Agriolimax reticulatus.'

Lane N., Caro L., Otero-Vilardebo L. R. & Godman G. C. 1964 J. Cell Biol. 21 339-351.

'On the site of sulfation in colonic goblet cells.'

Leppi T. J. & Stoward P. J. 1965 J. Histochem. Cytochem. 13 (5) 406-407.

'On the Use of Testicular Hyaluronidase for Identifying Acid Mucins in Tissue Sections.'

Lev R. & Gerard A. 1967 J. R. micr. Soc. 87 361-373.

'The histochemical demonstration of protein in epithelial mucins.'

Lev R. & Spicer S. S. 1964 J. Histochem. Cytochem. 12 309 (Letters to Ed.).

'Specific Staining of Sulphate groups with Alcian blue at low pH.'

Lewis R. D. 1969 Malacologia 7 (2-3) 295-306.

'Locomotor activity of Arion ater L: I. Humidity, Temperature and Light Reactions.'

Ibid. 307-312

'Locomotor activity of Arion ater L: II. Locomotor Activity Rhythms.'

Lockwood A. P. M. 1963 'Animal Body Fluids and their Regulation.'

Pub. Heinemann. London.

Mathews M. B. & Lozaityte I. 1958 Arch. Biochem. Biophys. 74 158-174.

'Sodium chondroitin sulphate protein complexes of cartilage. I. Molecular Weight and shape.'

Mazia D., Brewer P. A. and Alfert M. 1953 Biol. Bull. 104 57-67.

'The cytochemical staining and measurement of protein with mercuric bromphenol blue.'

Millonig R. W. & Marinozzi V. 1968 In: 'Optical and Electron Microscopy.' Vol.2

Eds. Barer R. and Cosslett V. E.

Pub. Academic Press. New York and London.

251-341. 'Fixation and Embedding in Electron Microscopy.'

Mowry R. W. 1963 Ann. N.Y. Acad. Sci. 106 (2) 402-423.

'The Special value of methods that colour both acidic and vicinal hydrocyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with PAS.'

Muir H. 1969 Amer. J. Med. 47 (5) 673-690.

'The Structure and Metabolism of Mucopolysaccharides (Glycosaminoglycans) and the Problem of the Mucopolysaccharidoses.'

Neutra M. & Leblond C. P. 1966 (a) J. Cell Biol. 30 119-136.

'Synthesis of the Carbohydrate of mucus in the Golgi Complex as shown by Electron Microscope Radioautography of Goblet Cells from Rats injected with Glucose -H^3.'

Neutra M. & Leblond C. P. 1966 (b) J. Cell Biol. 30 137-150.

'Radioautographic Comparison of the Uptake of Galactosamine -H^3 and Glucose -H^3 in the Golgi Region of various cells secreting Glycoproteins.'

Novikoff A. B. & Shin W-Y 1964 J. Microsc. 3 187-206.

'The Endoplasmic Reticulum in the Golgi zone and its relations to microbodies, Golgi Apparatus and Autophagic vacuoles in Rat Liver Cells.'

Ovtracht L. 1967 J. Microsc. 6 773-790.

'Ultrastructure des cellules sécrétrices de la Glande Multifide de l'Escargot.'

Ovtracht L., Morré D. J. & Merlin L. M. 1969 J. Microsc. 8 989-1002.

'Isolement de l'Appareil de Golgi d'une Glande Sécrétrice de Mucopolysaccharides chez l'Escargot, (Helix pomatia).'

Palay S. L. & Karlin L. J. 1959 J. biophys. biochem. Cytol. 5 363. (Unseen)

Pan C-T. 1958 Bull. Mus. Comp. Zool. Harvard. 119 235-299.

'The General Histology and Topographic Microanatomy of Australorbis glabratus.'

Pasquali-Ronchetti I., Greenawalt J. W. & Carafoli E. 1969 J. Cell Biol. 40 (2) 565-568.

'On the Nature of the Dense Matrix Granules of Normal Mitochondria.'

Pearse A. G. E. 1954. 2nd Ed. 'Histochemistry: Theoretical and Applied.'

Pub. J. & A. Churchill Ltd. London.

Pearse A. G. E. 1968. 3rd Ed. 'Histochemistry: Theoretical and Applied.' Vol.I.

Pub. J. & A. Churchill Ltd. London.

Plummer J. M. 1966 Proc. malac. Soc. London. 37 189-198.

'Collagen formation in Achatinidae associated with a Specific cell type.'

Prenant M. 1924 Bull. biol. 58 331-380.

'Contributions à l'étude cytologique du calcaire: I. Quelques formations calcaires du conjonctif chez les gastéropodes.'

Puchtler H. & Sweat F. 1960 Stain Tech. 35 347-348.

'Commercial resorcin-fuchsin as a stain for elastic fibres.'

Quick H. E. 1940 The Linnaean Society of London Synopses of the British Fauna. London. No.8. 'Slugs (Mollusca: Testacellidae, Arionidae,

Limacidae).'

Quintarelli G. & Dellovo M. C. 1965 Histochemie 5 196-209.
'The Chemical and Histochemical Properties of Alcian Blue. IV. Further Studies on the methods for the identification of Acid Glycosaminoglycans.'

Quintarelli G., Scott J. E. & Dellovo M. C. 1964 Histochemie. 4 99-112.
'The Chemical and Histochemical Properties of Alcian Blue. III. Chemical Blocking and Unblocking.'

Rambourg A., Hernandez W. & Leblond C. P. 1969 J. Cell Biol. 40 (2) 395-414.
'Detection of Complex Carbohydrates in the Golgi Apparatus of Rat Cells.'

Reynolds E. S. 1963 J. Cell Biol. 17 208-212.
'The use of lead citrate at high pH as an electron opaque stain in electron microscopy.'

Roth H. 1929 Z. wiss. Zool. 135 357-427.
'Zur Kenntnis Hautdrüsen von Helix pomatia.'

Ruthmann A. 1966. Revised 1970. 'Methods in Cell Research.'
Pub. G. Bell & Sons Ltd. London.

Saint-Simon A. de 1852 J. Conch. Paris III 278-282.
'Observations sur la glande caudale de l'Arion rufus.'

Sams W. M. Jr., Smith J. G. Jr. & Davidson E. A. 1962 J. Histochem.

Cytochem. 10 710-718.

'The Connective Tissue Histochemistry of Normal and Some Pathological Skin.'

Schmekel L. 1971 Z. Morph. Tiere. 69 115-183.

'Histologie und Feinstruktur der Genitalorgane von Nudibranchiern (Gastropoda, Euthyneura).'

Schneider K. C. 1902. 'Lehrbuch der Vergleichende Histologie der Tiere.' Pub. G. Fischer. Jena. (Unseen)

Scott J. E. 1967 Histochemie 9 30-47.

'On the Mechanism of the Methyl Green-Pyronin Stain for Nucleic Acid.'

Scott J. E. & Dorling J. 1965 Histochemie 5 (3) 221-233.

'Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions.'

Shatton J. & Schubert M. 1954 J. biol. Chem. 211 565-573.

'Isolation of a mucoprotein from cartilage.'

Sjöstrand F. S. 1967 'Electron Microscopy of Cells and Tissues.' Vol.I. Pub. Academic Press. New York and London.

Smith F. G. W. 1935 Phil. Trans. R. Soc. London B. 95 225.

'The development of Patella vulgata.' (Unseen)

Spicer S. S., Horn R. G. & Leppi T. J. 1967. In: 'The Connective Tissue.'

Eds. Wagner B. M. and Smith D. E.

Pub. Williams & Wilkins Co. Baltimore.

Chap.17. 251-329: 'Histochemistry of Connective Tissue Mucopolysaccharides.'

Spicer S. S. & Lillie R. D. 1961 Stain Tech. 36 365-370.

'Histochemical Identification of Basic Proteins with Biebrich Scarlet at alkaline pH.'

Spitznas M., Luciano L. & Reale E. 1971 Z. Zellforsch. 118 (3) 439-448.

'The fine structure of the Rabbit Sclera with Special Reference to a peculiar structure in the Fibroblast Rough Surfaced E.R.'

Stephenson J. W. 1962-1963 Proc. malac. Soc. London. 35 43-45.

'A culture method for Slugs.'

Stoward P. J. 1967 J. R. micr. Soc. 87 (1) 77-103.

'The histochemical properties of some periodate-reactive muco-substances of the pregnant Syrian hamster before and after methylation with methanolic thionyl chloride.'

Sumner A. T. 1966 J. R. micr. Soc. 85 (2) 181-192.

'The fine structure of digestive gland cells of Helix, Succinea and Testacella.'

Taylor J. W. 'Monograph of the Land and Freshwater Mollusca of the British Isles. (1). Structural and General.' 1894-1900.

Ibid. (2) 'Testacellidae, Limacidae and Arionidae.' 1900-1907.

Pub. Taylor Bros. Leeds.

Taylor R. E. 1963 Proc. XVIth Int. Congr. Zool. Washington. 1 86.
'Mucus of the Giant Slug, Ariolimax columbianus (Gould).'

Thiéry J. P. 1969 J. Microsc. 8 689-708.
'Rôle de l'Appareil de Golgi dans la synthèse des mucopolysaccharides. Etude cytochemique. I. Mise en Evidence de Mucopolysaccharides dans les vésicules de Transition entre l'Ergastoplasme et l'Appareil de Golgi.'

Toner P. G. & Carr K. E. 1968 'Cell Structure.'
Pub. E. and S. Livingstone Ltd. Edinburgh and London.

Watson M. L. 1966 J. biophys. biochem. Cytol. 1 257-270.
'The nuclear envelope. Its structure and relation to cytoplasmic membranes.'

Wellings S. R. & Siegel B. V. 1963 Ann. N.Y. Acad. Sci. 100 548-568.
'Electron microscopic studies on the subcellular origin and ultrastructure of melanin granules in mammalian melanomas.'

Whaley W. G. 1966 In: 'Organisation der Zelle. III.' Ed. Sitte P.
Pub. Springer-Verlag. New York.
340-371 'Proposals Concerning Replication of the Golgi Apparatus.'

Wondrak G. 1967 Z. Zellforsch. 76 287-294.
'Die Exoepithelialen Schleimdrüsenzellen von Arion empiricorum (Fér.).'

Wondrak G. 1968 Protoplasma. 66 151-171.

'Elektronenoptische Untersuchungen der Körperdecke von Arion rufus L. (Pulmonata).'

Wondrak G. 1969 Z. mikr.-anat. Forschung. 80 17-40.

'Elektronenoptische Untersuchungen der Drüsen - und Pigment - zellen aus der Körperdecke von Arion rufus L. (Pulmonata).'

Valeri V., Goncalves R. P., Cruz A. R. & Laicine E. M. 1971 J. Ultrastruct. Res. 35 (1-2) 197-200.

'Tubular Structure within the Granular Endoplasmic Reticulum of the Pars Intermedia of Toad Hypophysis.'

Zeigel R. F. & Dalton A. J. 1962 J. Cell Biol. 15 (1) 45-54.

'Speculations based on the Morphology of the Golgi systems in several types of protein-secreting cells.'