Functional aspects of gastropod mucus

Rajshree Shirbate

FUNCTIONAL ASPECTS OF GASTROPOD MUCUS

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TEXT

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by
Rajshree Shirbhate, M.Sc. Nagpur, India.
Department of Zoology, University of Durham.

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The work was carried out while the candidate was under supervision by Dr. A. Cook, whose name also appears on these papers as co-author.

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ABSTRACT

The gross anatomy and histochemical properties of the mucus-producing glands of three prosobranchs from different habitats (Littorina littorea, Bithynia tentaculata and Pomatias elegans), and a pulmonate slug (Limax pseudoflavus) were investigated.

A supra-pedal gland was present in Pomatias and Limax that secreted mucoprotein and acid mucopolysaccharide in the former and neutral mucopolysaccharide in the latter. All species examined except Pomatias possessed glandular areas at the leading edge of the foot secreting a mucoprotein, and widely distributed glands on the ventral surface of the foot secreting a variety of mucosubstances. The ventral secretory cells of Pomatias, which contained protein and both neutral and acid mucopolysaccharides, were confined to a band either side of a deep medial pedal furrow. The secretory cells on the exposed dorsal surface of all four species contained a variety of mucosubstances and protein. However, a neutral mucopolysaccharide component was found to be absent in the dorsal body mucus of Limax. In the prosobranchs examined, specialized cells of the opercular groove and disc produced mucopolysaccharide and at least two differently staining proteins.

In Pomatias and Bithynia the dorsal mantle epithelium possessed no secretory cells whereas that of Littorina possessed cells secreting mucopolysaccharide. At the mantle edge of Pomatias and Littorina two types of glands produced a variety of mucosubstances but at that of Bithynia one cell type produced mainly protein.

The hypobranchial gland of Pomatias contained no specialised cell types but that of Bithynia and Littorina possessed cells secreting mainly protein and acid mucopolysaccharide respectively.

The mode and mechanism of discharge of the mucus-producing gland cells of these gastropods and functional aspects of the distribution of cell types and their mucus are discussed. It is suggested that the pedal mucus of Limax is especially suitable for the gastropod creeping system. Many areas of the body surface of Limax and the foot of Pomatias, Bithynia and Littorina are capable of producing both fluid (neutral or weakly acid) and viscous (acid) mucus. It is postulated that such an arrangement allows for both adhesion and lubrication at different times.

CHAPTER I

INTRODUCTION

The gastropod epidermis interacts with its external environment through a layer of mucus. Since different areas of epidermis are involved in different functions, it is to be expected that the nature of the superficial secretion will vary with these functions. The prosobranchs occupy freshwater, marine and terrestrial habitats and thus provide the opportunity for studies of animals of similar design but living within these varied habitats. Although the general histology of the epidermis of gastropods has attracted a great deal of attention because of the variety of gland cells that are present in or below the epidermal surface (Herfs, 1922a and 1922b; Houssay, 1884; Delahaye, 1974; Simkiss and Wilbur, 1977), very little detailed histochemical work has been applied in those studies carried out on prosobranchs. Although the mucus-producing glands of several terrestrial pulmonates have been examined histochemically (Chetail and Binot, 1967; Campion, 1961; Lawrence, 1972; Dalal and Pandya, 1976), no extensive study since that of Lawrence has been aimed at elucidating their functions. Various lines of research have implicated the epidermis of gastropods in general functions such as respiration (Zaaijer and Wolverkamp, 1958; Jones, 1961) and osmoregulation (van Aardt, 1968; Greenaway, 1970) but very little attention has been paid to their structural basis.

Most studies of gastropod organs like the foot or hypobranchial gland almost completely ignore the nature of the secretions which allow the organs to operate as they do. Although it is now generally accepted that the shell is secreted by the mantle edge most of the studies have been made on the histology and the glandular nature of the margin of the mantle. There are very few histochemical studies of the mantle edge (Kapur and Gibson, 1968; Timmermans, 1969; Walsh, 1981 and Umadevi, Hanumantha Rao and Shyamasundari, 1984) even though the use of histochemical methods would have been useful to get information about the manner in which the mantle is involved in the process of shell formation. Similarly in view of the large number of studies on the histochemical and chemical composition of gastropod opercula (Vovelle, 1967 and 1969a,b; Degens et al., 1967; Hunt, 1970a and 1970b) it is perhaps surprising that no attempt has been made to discover the distribution or the detailed histochemical nature of the opercular secretory glands in the gastropods.

The general structure of the foot, the mantle and the hypobranchial gland in prosobranchs is well known (Hyman, 1967; Fretter and Graham, 1962; Houssay, 1884; Herfs 1922a, 1922b) but the detailed histochemistry of mucus-producing cells is known only for a very restricted range of species. Previous studies of the epidermis and its associated glands in the gastropods have shown structural differences throughout the class. There are differences in the cell types

present and the nature of their secretions dependent on the species, habitat, and the role of mucus in the particular gastropod.

Apart from locomotion the function of epidermal secretions in prosobranchs would clearly show some differences from those in slugs, the main difference being due to the secretion of shell and operculum in the prosobranchs. Nevertheless, the slugs are more dependent on their mucus than prosobranchs because they have no shell into which to withdraw and they are more dependent on behaviours such as huddling (Cook, 1981) and homing (Cook, 1979) which have as their basis the pheromonal qualities of their mucus. Opisthobranchs are not included in the present study, mainly because very little previous information was available, the mucus secretion in opisthobranchs is relatively unknown (Eales, (1921); Thompson and Slinn (1959); Schmekel and Wechsler, (1967); Trench, (1973) and they are anatomically diverse and very different from the other groups of gastropods. They are also entirely marine.

It was decided, therefore, to examine the glands which contribute to the mucus covering of foot, operculum, mantle and hypobranchial gland of prosobranchs from different habitats Pomatias elegans (Muller) (terrestrial), Bithynia tentaculata (Linnaeus) (fresh water), Littorina littorea (L) (marine) and the gland cell types contributing to the surface coating of mucus of the limacid slug Limax pseudoflavus Evans, with particular reference to the

histochemical nature of the glands; using the same techniques in an attempt to relate different glandular cell types to the varying physiological, behavioural and environmental requirements of each species.

CHAPTER II

IDENTIFICATION

The different species of gastropod molluscs from various habitats used in this study were identified from A field guide to the Land Snails of Britain and North West Europe by M.P. Kerney and R.A.D. Cameron (1979), British Prosobranchs by Graham (1971) and British Land Snails by R.A.D. Cameron and Margaret Redfern (1976).

Pomatias elegans (Round mounted snail or Land snail).

It used to be known as Cyclostoma ('round hole') a name which reflected its outstanding character, an almost perfectly circular opening to the shell. The name Pomatias comes from the Greek word for 'door' and refers to the operculum, a thick calcareous plate, which closely fits the mouth when the animal is retracted.

This is a prosobranch found on land. It always lives on highly calcareous soils and spends most of its time burrowing in the loose friable soil. The snail will not usually be found in those parts of the substrate where the soil is basically clay with flints.

The shell is conical with 4 -5 strongly rounded whorls. It is thick, solid and has spiral ridges and transverse striations. Its colour is pale greyish-violet to yellowish, with a variable patterning of darker spots or interrupted spiral bands. The animal has a long flexible proboscis and

the eyes are present at the base of the two cylindrical tentacles. The front end of the foot is forked and the sole is divided longitudinally into halves which move independently in crawling, which shows a stepping form of locomotion, as can be observed when the animal moves over a glass plate.

Bithynia tentaculata

This has been variously spelt Bithynia, Bythinia and Bithinia though the first is currently standard and derived both from the Greek bythos meaning the depth of the sea and from the name of the province Bithynia on the south western shore of the Black Sea. Tentaculata (Lat.) means "with tentacles".

This is a calciphile species common in all kinds of bodies of fresh water. In Britain, absent or rare in Devon and Cornwall, N. Wales and much of highland Scotland. The shell is rather glossy externally and very glossy internally. There is a thin periostracum. The operculum is concentrically ringed, dark and partly calcified. The colour is of various shades of horn, often more brown apically. There is a dark line round the peristome marking a periostracal thickening. The head has a rather long snout cleft in the middle anteriorly. The mouth, ventral to the snout is a vertical slit when shut. The tentacles are long and slender, thicker basally where they bear eyes which are not on separate stalks. The mantle edge is simple and rather thin. The foot is broad anteriorly and extended laterally

short. In a normal unirritated animal the foot and body mucus are colourless but the dorsal mucus of agitated animals is yellowish in colour whilst the dorsal mucus produced in response to mechanical and/or chemical stimulation is very watery.

Limax pseudoflavus - Confusion over classification

The identity of Limax pseudoflavus has been a source of confusion. This animal has been assigned to L.flavus (as a colour variant, Chatfield 1971), subsequently being classified by Chatfield (1976) as Limax grossui Lupu (originally described by Lupu, 1970). The main external difference is a dorsal yellow stripe on L. flavus, contrasting with the irregular green mottling of the new species. Since then it has been classified as L.pseudoflavus (Evans 1978a,b) and Limax maculatus (Wiktor and Norris 1982).

Evans' (1978a,b) reclassification to L.pseudoflavus was based on biochemical work and the different relative proportions and morphology of the terminal genitalia in L.pseudoflavus compared with L.flavus and L.grossui. The differences were corroborated by Dr. Lupu.

The classification by Wiktor and Norris (1982) depends largely on external characters and they dismissed the taxonomic characters used by Evans attributing them to the level of sexual maturity of the slug and the manner of its preservation. However, I shall continue to use the classification put forward by Evans.

CHAPTER III

COLLECTION AND MAINTENANCE

Pomatias elegans

Pomatias elegans were collected from the South downs in Sussex (near Slindon) and from Swansea. They were kept in a plastic lunch box. The box had a lid with holes which allowed the air to circulate. The snails were kept on a few damp tissue papers. They were supplied with a variety of food which included mostly lettuce and cabbage. The fresh food was supplied at least once a week when the tissue papers were replaced by clean, damp ones, to avoid excessive contamination by the faeces and slime. In these conditions Pomatias survived very well but were generally inactive and contracted into the shell. The inactivity was probably due to lack of loose, friable calcareous soil in which they spend most of their time burrowing.

Bithynia tentaculata

These were purchased from Gerrards Biological centre, Rustington. All Bithynia, about 15-20 at a time, were kept in a small fish tank of 300cm in length, 240cm in width and 190cm deep in the indoor departmental aquarium. The tank was furnished with some healthy growing weeds. The water was aerated and the tank was covered with a loosely fitting lid to keep the snails in and dust out and to prevent the evaporation of water. The water was changed every 1 to 2

weeks. In these conditions Bithynia survived very well.

Littorina littorea

Littorina littorea were collected from Portstewart, N. Ireland and also from the Whitburn Beach, Sunderland. Most of the conditions were the same as those for Bithynia tentaculata with only a few differences. Littorina being a marine species the tank was filled with sea water and was furnished with gravel. Mostly Littorina was to be found outside the water, either on the gravel or clinging to the sides of the tank and for this reason the tank needed to be covered with a tightly fitting plastic lid with holes for aeration.

Limax pseudoflavus

Adult and juvenile Limax pseudoflavus were collected from Londonderry and Coleraine (Northern Ireland) and immediately fixed.

Patella vulgata (L)

Patella vulgata were collected from Portstewart, N. Ireland and all the conditions for Patella were the same as for Littorina littorea.

Lymnaea stagnalis (L)

These were collected from the pond in the campus of the University of Ulster, Coleraine, Northern Ireland.

CHAPTER IV

LIGHT MICROSCOPY

1. Introduction

The main aim of this study was to investigate the glandular cells of the foot, operculum, mantle and the hypobranchial glands of prosobranchs from different habitats and the mucus producing glands of the pulmonate slug Limax pseudoflavus. In order to examine the gland cell types and the histochemical nature of their contents it was necessary to fix, embed and section the animals. The general distribution of materials making up cell structure visible by light microscopy should be preserved by adequate fixation and here the choice of fixative can be critical. The fixative must be able to diffuse rapidly, before autolysis begins in the depth 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. Finally, the cell structures should still be susceptible to absorbing contrast-producing agents such as stains, metals and coloured products of histochemical reactions.

In the living cells protein, carbohydrate, lipid, and complexes of several or all of these, are loosely and weakly

associated by hydrogen bonds and salt linkages. These weak associations should be replaced during ideal fixation by stable linkages that will not break down in dehydration, when water is lost from the system.

According to Ruthmann (1970) the best available fixatives are those that lead to the denaturation of the cellular proteins without recognizable precipitation and thus provide an 'equivalent image' of cell structure at any given level of microscopic resolution. However, it is necessary and useful to realize that the universal fixative is not yet available. Only that best suiting the substance to be studied should be selected (for instance, 95% alcohol for calcium or Baker's formaldehyde calcium for Lipids), and even then some changes will have occurred.

2. Method

From the wide variety of literature available on the subject, fixatives were chosen particularly from the works of Pantin, (1946); Gurr, (1962); McManus and Mowry, (1964); Preece, (1972); and Pearse (1980). The fixatives selected were alcoholic Bouin, Susa, Zenker, Buffered neutral formalin and formalin with 0.5% cetylpyridinium chloride which were particularly recommended for animal tissues in general.

The present investigations started with the study on the slug Limax pseudoflavus and it was noticed that the fixatives containing formaldehyde result in the production of an extremely thick mucous coating. In alcoholic Bouin

the animals contracted into a 'U shape' which rendered sectioning rather difficult.

The tissues fixed in Susa sectioned well, but the final choice of Zenker's fixative was made, since it provokes the minimum of mucus secretion and when placed in Zenker the slug seemed not to contract excessively. Zenker's is not the ideal fixative for all the histochemical tests used. It gives, however, a good general fixation of gastropod tissues and allows the comparison of serial sections stained with different techniques. Using one fixative avoids the problem of identifying the same cell type when fixed differently. Furthermore, according to Preece (1972) "Zenker fixed tissues stain brilliantly. Zenker fixation is compatible with most stains, in fact many stains require this fixative as a mordant to ensure a specific staining reaction". Exceptions to the use of Zenker's fixative had to be made for material used to locate calcium (fixed in 95% alcohol, Dahl 1952) and lipid (10% formaldehyde and Baker's formaldehyde calcium, Pantin, 1946; and Pearse, 1980). For all the animals studied the same fixatives were used and the same histochemical procedures were applied to maintain comparability throughout the work.

Bithynia tentaculata, Pomatias elegans, Lymnaea stagnalis and Patella vulgata were anaesthetized prior to fixation in 0.08% Nembutal (in distilled water for Bithynia, Pomatias and Lymnaea and in sea water for Patella vulgata) to reduce the contraction of the tissues (Table, Page 13a), (Runham et al., 1965).

TABLE I

Showing the time required by the animals for the relaxation

Animal	Time	Chemical used
Pomatias elegans	24 to 72 hrs	0.08% Nembutal in distilled water
Bithynia tentaculata	20 to 36 hrs	0.08% Nembutal in distilled water
Lymnaea stagnalis	8 to 12 hrs	0.08% Nembutal in distilled water
Patella vulgata	1½ to 3 hrs	0.08% Nembutal in Sea water

No histological or histochemical differences were found due to anaesthetization.

Depending upon the fixative employed the animals were washed, dehydrated and cleared in methyl benzoate which was used as an intermediate clearing agent. The specimens were then transferred to benzene and later, when the use of benzene in the laboratory was prohibited, to toluene before embedding in paraffin wax. The tissues were embedded finally using 'L' brass blocks. (See Appendix 1 for the routine instituted for each fixative used).

Serial sections of 7μ thickness were cut on a "Cambridge" Rocker microtome or on a Spencer rotary microtome. Sections were then transferred to a cleaned slide smeared lightly with glycerine/albumen. Generally one consecutive section was mounted on each slide for each method used to safeguard the staining properties of the same cell type when the various staining procedures were applied. The slides were then transferred to a hot plate where the sections extended and flattened. When dry they were ready for staining.

To build up a picture of the structure, contents and distribution of gland cells throughout the animals the sections were treated in a prearranged order.

A number of animals were examined in the form of frozen sections. This was necessary to allow the staining of lipids which are normally lost after chemical fixation. The cryostat used was the Bright freezing microtome.

The animals were fixed in Baker's formaldehyde calcium for 2-3 days. After fixation the tissue needed from the animal was transferred to a small metal cup containing isopentane. The cup was fixed in a wire basket and was held in liquid nitrogen vapours for a short time. Tissues from the cup were transferred immediately to the cryostat chamber and fixed on a cold microtome block holder using a Bright Cryo-M-Bed paramount. The block was left in the cryostat chamber and was cut after an hour or two.

Sections were cut at 10μm and transferred to the cleaned slides. Slides were left at room temperature for some time before staining. This was found to be very useful as otherwise if the sections were directly taken from the cryostat and stained they had a tendency to float in the solution used and thus there was a greater risk of losing them while staining.

Specimens were prepared for the scanning electron microscope by fixation for 1 hour in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) before being wiped to remove superficial mucus. They were then dehydrated in acetone, critical point dried and coated with gold.

CHAPTER V

HISTOCHEMISTRY

1. Introduction

The morphologist investigating animal tissues can obtain additional information about the structure and function of the cells by histochemistry. Proteins, carbohydrates and lipids are the basic constituents of animal tissues. The histologist will describe their location, the histochemist will categorise some of their chemical structure and the biochemist may be able to determine their complete chemical identity (Drury and Wallington, 1980).

The growing number of modern histochemical reactions for proteins, carbohydrates, lipids and their constituents allows a fairly good identification of many types. It was hoped that a wish to recognise microchemically at least some of the substances in the tissues could be achieved by histochemistry.

2. Methods

A survey was devised using a selection of widely applied and also specific modern histochemical techniques for the demonstration of proteins, mucopolysaccharides, calcium and lipids as all these have previously been reported in other gastropod skin tissue.

2.1 Histochemical identification of proteins:

Proteins are the most widely distributed and are important for histochemical purposes. Proteins can be divided into simple proteins and conjugated proteins. All simple proteins can be broken down by hydrolysis into amino acids only, while the conjugated proteins are composed of amino-acids combined with carbohydrates (glycoproteins), lipid (lipoproteins), or nucleic acids (nucleoproteins). The precise histochemical reactions of proteins are due to their reactive groups and these are not peculiar to one amino acid or one type of protein. Histochemical methods for the reactive end-groups of amino acids gives some identification of the structure of protein compound.

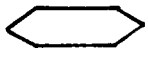
The classical methods which have been employed are:-

2.1.1. Diazotization coupling reaction.

2.1.2. Mercuric bromophenol blue.

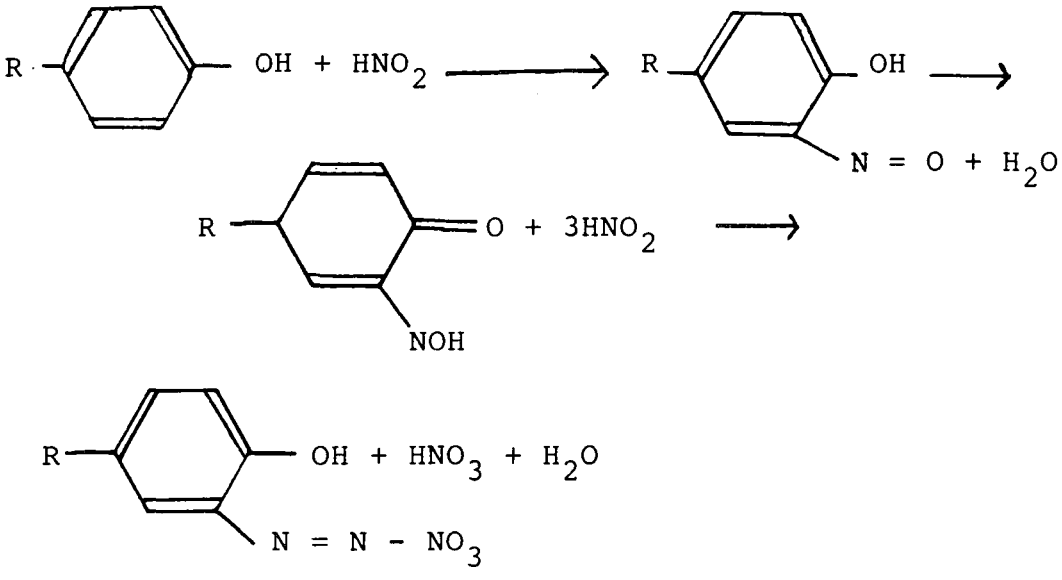
These reactions determine only a fraction of the constituent amino acids of any particular protein, nevertheless a positive reaction has been taken as an indication of the presence of protein since free amino acids do not occur in tissue preparations from animal sources (Pearse 1980).

2.1.1. Diazotization coupling reaction:

In this histochemical method for amino acid the phenyl  OH is the reactive end group and tyrosine can be demonstrated.

The diazotization coupling reaction was not entirely reliable until Glenner and Lillie (1959) converted the method into one of an absolute reliability and specificity.

The prolonged treatment of sections with cold HNO_2 resulted in the C-nitrosation of the tyrosine groups of the proteins. The C-nitroso groups were then progressively converted into the quinone-oxime tautomer and thence, by further reaction with HNO_2 into diazonium nitrates.



These diazonium nitrates can be coupled with amines in alkaline solution to give coloured reaction products. Lillie used 1-amino-8-napthol-5 sulphonic acid (S-acid) for this purpose.

2.1.2. Mercuric Bromophenol Blue:

This is a widely applied histochemical method for the general identification of proteins. This stain was devised by Durrum (1950). The use of mercuric bromophenol blue (Hg-BPB) for the histochemical detection of protein was

introduced by Mazia, Brewer and Alfert (1953) who found that tissue section staining conforms to the Beer-Lambert law and that dye binding of nonprotein substances is negligible. It has been widely used in filter paper electrophoretic studies of protein spots. The method was also applied by Bonhag (1955) for investigating the composition of the ovary of the milkweed bug Oncopeltus fascinatus.

2.2 Histochemical identification of carbohydrate mucosubstances:

In animal tissues a large number of compounds containing carbohydrates are present. They can be divided into polysaccharides and mucosubstances.

The mucosubstances are a larger group of different compounds which are often found as mixtures. Mucosubstances include the mucins and are mucoid carbohydrates which can be divided into three main types (Stacey and Barker 1962).

1. Mucopolysaccharides: Polysaccharide-protein complexes that are predominantly carbohydrate.

Mucopolysaccharides may be neutral or acid.

2. Mucoproteins: Polysaccharide-protein complexes that are predominantly protein.

3. Mucolipids: Polysaccharide-fat compounds.

A considerable number of different types of reactions are involved in the methods used for the demonstration of mucosubstances. The two which are the most important are the Periodic Acid Schiff reaction (PAS) and the basic dye methods or the combination of both. The most widely used

basic dye is Alcian Blue.

The PAS reaction and Alcian Blue staining either separately or as the combined Alcian Blue - PAS reaction, give a considerable amount of information. Only glycogen, neutral mucopolysaccharides and glycolipids will be PAS-positive and Alcian Blue (pH 2.5) negative. Glycogen is digested by diastase and glycolipids will be stained by Sudan black leaving the neutral mucopolysaccharides which can be identified by using the Periodic Acid Paradiamine technique (Spicer, 1966) for neutral mucopolysaccharides.

Acid mucosubstances can be demonstrated by a number of metachromatic dyes. Carboxylated and sulphated compounds are metachromatic whilst the neutral compounds are orthochromatic. In the present study the Toluidine Blue method for the demonstration of acid mucosubstances (standard method - Pearse, (1980)) was employed.

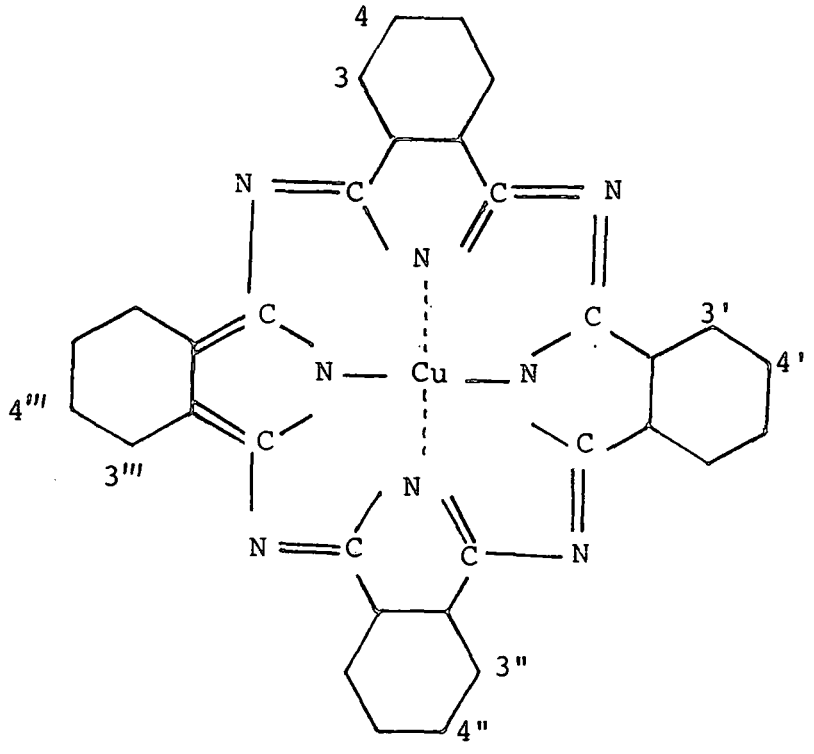
2.2.1 Toluidine Blue method:

Toluidine blue is the most commonly used metachromatic dye, which means that the tissue structure may be stained a colour different from the colour of the dilute dye solution. Different terms are used depending on the degree of metachromasia; β metachromasia describes a moderate effect (purple colour), γ metachromasia for a strong effect (red colour) (Michaelis, (1947)). The exact mechanism of the metachromatic staining reaction is not known. It is supposed that the change in colour is caused by polymerization of the dye molecules. The presence of

oriented negative surface charges in the tissue is essential. The result depends on the fixative used, temperature, pH, the concentration of the dye and the salt concentration of the staining solution.

Alcian Blue 8GX:

Alcian Blue is a water soluble phthalocyanin dye possessing a blue colour because of its copper nucleus. The copper phthalocyanine (CuPC) has a basic structure of the type shown below.



The use of dilute solutions of Alcian Blue 8GS as a specific stain for mucins was introduced by Steedman (1950). In about 1957 the original Alcian Blue 8GS was replaced by a new Alcian Blue 8GX. Mowry (1960) showed that the results given by Alcian Blue 8GX were superior to those of the

original techniques.

The usual method is with a staining solution at pH2.5.

2.2.2 Alcian Blue pH2.5-PAS (Mowry, 1963):

The Alcian Blue-PAS combination has been described as one of the most routine tests. This can be used for the demonstration of all mucosubstances and it distinguishes neutral and acid compounds. The section is first stained with Alcian Blue and Alcian Blue positive mucosubstances that are also PAS positive will not then react with PAS. Only neutral compounds will be PAS positive. When mixtures are present there will be a combination of the blue and red colours.

The next problem is the separation of Alcian Blue positive mucosubstances. Lev and Spicer (1964) showed that if used at pH0.5 Alcian Blue will distinguish between carboxylated and sulphated mucopolysaccharides as carboxyl groups will not stain at this pH.

2.2.3 In the present study the Alcian Blue pH0.5/Eosin method was used for the demonstration of sulphated mucopolysaccharides and mucoproteins. In this method eosin was used as a counterstain.

Acidic compounds can also be differentiated into sulphated and carboxylated mucopolysaccharides by a combined Alcian Blue/Alcian Yellow technique.

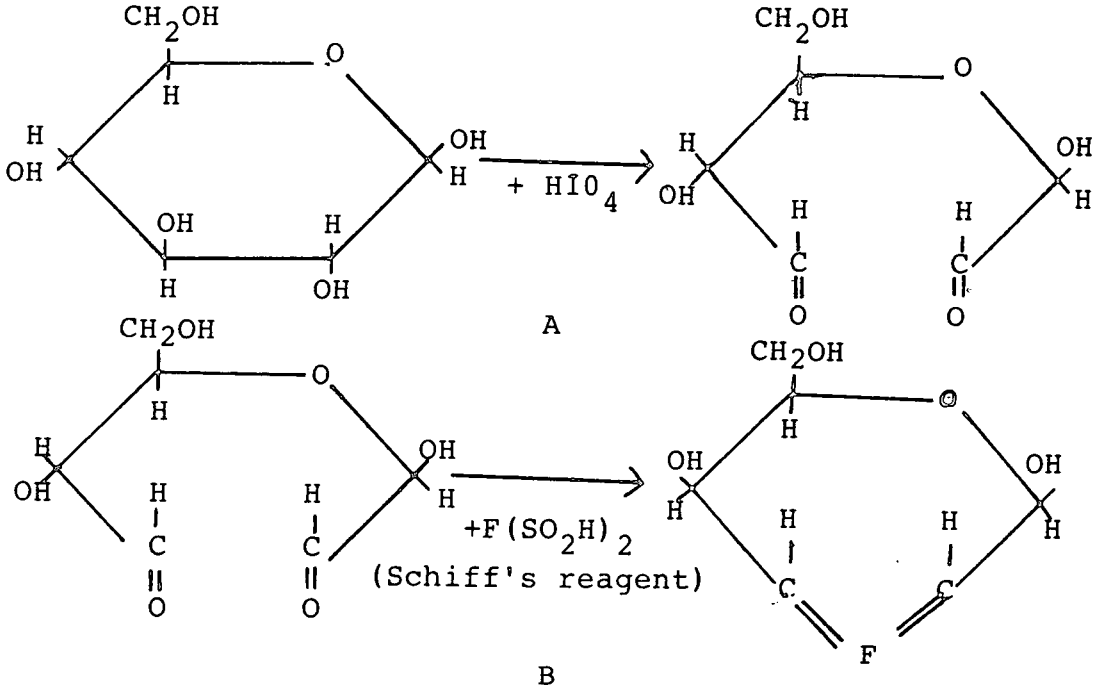
2.2.4 Alcian Blue pH0.5/Alcian Yellow pH2.5 technique (Ravetto 1964):

Alcian Blue at low pH(0.5) will stain sulphated

compounds but the carboxyl groups are not ionized and will not stain at this pH but they will stain with Alcian Yellow at pH2.5 and when mixtures are present there will be a combination of the blue and yellow colours.

2.2.5 The Periodic Acid Schiff technique (PAS) (McManus, 1946):

This is a true histochemical method. McManus (1946) described the use of the periodic acid in the PAS reaction for the staining of mucins. Periodic acid oxidises the 1-2 glycol groups (CHOH-CHOH) in tissues to produce aldehydes that are coloured by Schiff's reagent.



The chemical changes in the periodic acid-Schiff reaction. (A) Periodic acid oxidation. (B) Recoloration of Schiff's reagent by aldehyde groups.

The PAS reaction is histochemically specific but many different components of tissues, which include

polysaccharides (glycogen), neutral mucopolysaccharides, mucoproteins, glycoproteins and glycolipids, give a positive reaction. Therefore to improve the value of the PAS reaction for identification of a single tissue component or group of substances, it is used in conjunction with chemical controls, blocking techniques or with additional staining methods (e.g. Alcian Blue pH2.5/PAS). However, lipids have been dissolved for the greater part during dehydration and embedding in paraffin, small molecules such as amino acids are lost during fixation and in protein the reactive groups of the individual amino acids are mostly bound in the peptide chain and in that case are not oxidised by periodic acid.

2.2.6 Periodic Acid Diamine method (PAD) (Spicer 1966):

Spicer and Jarrels (1961) observed that in aqueous solutions N, N-dimethyl-p-phenylenediamine rapidly formed orange brown condensation products with periodate engendered aldehydes. With acid groups of mucopolysaccharides on the other hand, it gave rise to black complexes after prolonged reaction time. The effect of periodate oxidation on the diamine reaction cannot be interpreted in chemical terms (Munch and Ernst, 1964). There is no apparent reason why some mucosubstances retain their basophilia while others lose it. Spicer (1965) presumes that condensation of the diamine with periodic engendered aldehyde groups may bring the positive charges of the disubstituted amine into such proximity to the negatively charged residues of the mucosubstances as to neutralize their attraction for

positively charged dye molecules. Expressed in another way, PAD sensitive materials should be PAS positive. In the present study this method was particularly useful for the identification of the neutral mucopolysaccharides.

2.3 Histochemical identification of Calcium :

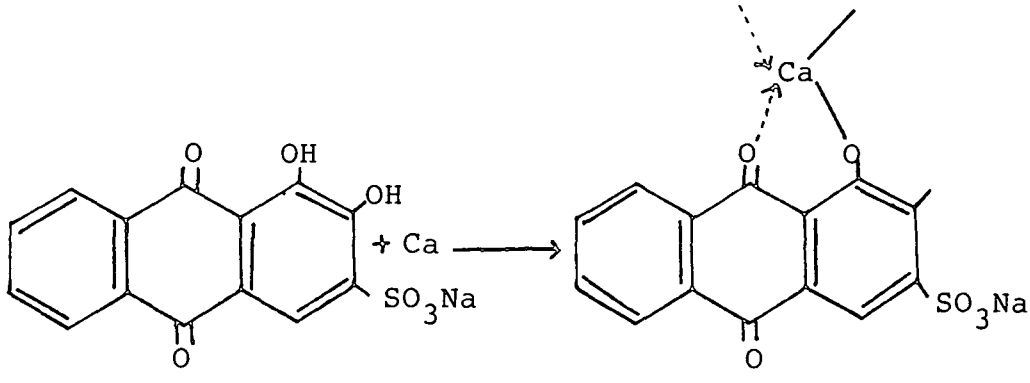
Calcium is contained in tissues in crystals and in complexes. There are only a few worthwhile methods for its demonstration (McManus and Mowry 1964). The ones which are most widely used were employed in this study. These techniques fall under two main headings, metal substitution techniques and dye lake reactions:

2.3.1 Alizarin Red 'S' Method for Calcium is a dye lake reaction.

2.3.2 von Kossa is a metal substitution technique.

Alizarin Red 'S' method (Sodium alizarin sulphonate method):

Sodium alizarin sulphonate is an anthraquinone dye. It forms a birefringent reddish orange coloured precipitate with calcium in which the metal is held by strong covalent linkages.



None of the anthraquinone dyes is strictly specific for calcium (Pearse, 1980) and magnesium, manganese, barium and iron are the most common interfering substances. According to Barka and Anderson (1963) the reactions of these elements are easily distinguished from that due to calcium.

For the demonstration of calcium a sensitive technique using alizarin red 'S' was evolved by Dahl (1952). He stressed the importance of pH in the performance of the test. The sensitivity of his alizarin method was stated by Dahl to be greater when used histochemically than in a spot test.

von Kossa method for calcium:

This is much used to demonstrate calcium while it is actually a method for phosphate and some carbonates. In animal tissues, however, the insoluble phosphates and carbonates are nearly always those of calcium and the test is usually regarded as sufficiently specific for calcium (Pearse 1980).

The original principle of the method was the conversion of either salt into its silver equivalent and subsequent reduction of this by means of sunlight or ultra violet light or by means of a strong reducing agent to block metallic silver. Pizzolato and McCrory (1962) however, showed that neither light nor reducing agent was necessary to bring about the second stage of the reaction.

2.4 Histochemical identification of lipids:

In the present study Sudan black B was used to

investigate whether lipids are present in the tissue. Neutral fats and phospholipids can be demonstrated with this method.

Neutral fats are esters of unsaturated or saturated fatty acids with glycerol. Phospholipids or phosphatides are characterized by their phosphoric acid contents.

2.4.1 Sudan Black B Method:

Natural fats and phospholipids are stained blue black, particularly the latter compounds (Pearse 1980). Staining is due to the fact that the dye dissolves in the lipids (Cain 1950). Protein bound phospholipids are practically unable to dissolve the Sudan stain and therefore remain unstained.

Controls were used for Periodic Acid Schiff's (absence of periodic acid); Alizarin Red 'S' (Extraction in HCl) and Sudan Black B (chloroform/methanol extract or Lipase at 37°C) method.

All the technical details for these procedures will be given in the Appendix.

Preparation of figures and plates:

A camera lucida was used for the preparation of drawings.

Photographs of stained sections were obtained using a Nikon photomicroscope loaded with Kodacolor Gold 100 film.

While measuring the sizes of the gland cell types, in all instances the 'length' of the cell is taken as being the axis perpendicular to the epidermis and 'width' as the area

in between the two lateral cell walls with an axis parallel to the epidermis.

CHAPTER VI

HISTORICAL RESUME

Histological data on the mucus-secreting areas of the gastropods are plentiful, but mostly as part of a general anatomical description. In only a few species is there a histochemical study available related to the chemical nature of the mucus secretion.

The relevant areas of gastropods can be considered as:

1. The foot.
2. The mantle.
3. The hypobranchial gland.

At this juncture only brief mention will be made of the literature since it can most usefully be examined in detail in comparison with the present study, and will therefore be discussed in Chapter VIII.

1. The foot

1.1 Prosobranchs

The histology of the prosobranch foot has been the subject of repeated investigations. The literature of the pedal glands of the prosobranch has been summarized by Touraine (1952) Fretter and Graham (1962) and Hyman (1967).

Carriere (1882) was the first to notice the diversity of the pedal glands among the prosobranch gastropods. Houssay (1884), Garnault (1887) and later Herfs (1922a) also gave general descriptions of these glands in gastropods.

However, recently a number of comprehensive studies

have been carried out on the pedal glands.

Gainey (1976) studied locomotion in the gastropods and also described briefly the functional morphology of the foot in Neritina reclivata and Thais rustica.

In examining the foot of Neritina he observed three types of mucocytes. The anterior mucocytes were found to be arranged in longitudinal bundles, whilst the subepidermal mucocytes were found unevenly distributed throughout the ventral portion of the foot. The third type of mucocytes were the epithelial mucocytes.

Gainey described the foot of Thais rustica as blunt in the front and tapered in the rear with two clefts, longitudinal and transverse, on the sole. As in Neritina, here also three types of mucocytes were found, although the anterior mucocytes were smaller and found to be less organized than those in Neritina. The subepidermal mucocytes were present along the entire sole except for the region above the longitudinal cleft and anterior to the transverse cleft. The epithelial mucocytes were also present.

Pomatias elegans has received much attention and two papers will be described in relation to Pomatias elegans.

Delahaye (1974a,b) studied the pedal glands of Pomatias and described basically two glands in the foot. The anterior pedal gland was composed of an outer part, where three types of cells could be observed; and an inner part which was found to be made of only one type of secretory

cell. The other gland was the tubulous gland which Delahaye believed might be involved in osmoregulation.

Recently Bensalem and Chetail (1982) studied the hydrocalcic metabolism and pedal glands in Pomatias elegans. Their work was more interesting in that they proposed an interesting role for the pedal glands in hydrocalcic equilibrium, in addition to secreting the lubricant of the pedal surface. They described three kinds of pedal glands: the anterior pedal gland, the sole gland and the tubulous gland. Bensalem and Chetail (1982) observed two types of cells in the outer part of the anterior pedal gland whilst the inner part consisted of one type of cell.

In examining the sole, Bensalem and Chetail described the sole gland with its developing stages of mucocytes. The third type of gland was the tubulous gland.

Grenon and Walker (1978) have made a comprehensive study of the pedal glandular system of two limpets, Patella vulgata L. and Acmaea tessulata Muller. Their work gave a more detailed account of the histochemical nature of the various secretions produced by the pedal glands. Some attempts at functional interpretations of the distribution of mucus-producing cells in these species have been made by these authors.

In the pedal glandular system of Patella vulgata, Grenon and Walker described nine different gland types, six of which poured their secretions to the sole. Proteins secreted by the marginal gland into the marginal groove were

believed to be used for locomotion, especially when the limpet moves off following a period of adhesion. Another protein-secreting, subepithelial gland cell type was found to be sparsely distributed throughout the foot and its secretion was supposedly used in locomotion. A third gland type was present in the epithelium of the sole. The other three sole glands secreted highly viscous mucus and these authors thought that their secretion apparently had an adhesive function. Three glands were also found to be contained within the side wall. Of these, two were believed to secrete mucus to protect the animal against desiccation during emersion. The third gland was found only in young Patella vulgata and its secretion was thought to be used in consolidating the debris before it was cleared from the pallial cavity.

In examining the foot of Acmaea tessulata, Grenon and Walker described six different gland types which corresponded in position, composition and function to the six glands of the sole of Patella vulgata. In this species no glands were found within the side wall.

1.2 Pulmonates

The pulmonate epidermis and associated glands have been the subject of numerous investigations and some species of pulmonate gastropods have received detailed histological and histochemical study.

Some papers on the pedal glands of shelled pulmonates will be examined in the literature review that follows

together with those on naked varieties but in addition to these terrestrial genera, work on the pedal glands of aquatic pulmonates will also be examined, since these are exposed to approximately similar conditions as the terrestrial and aquatic prosobranch species studied in the present work and one would expect comparable structures to be evolved.

1.2.1. Terrestrial pulmonates

Roth (1929) was the first to describe the one type of mucus gland cell in the sole of Helix pomatia Linne.

More work on Helix pomatia was published by Bolognani-Fantin and Bolognani in 1964. Their's was a biochemical and histochemical investigation into the nature of the secretion produced by the cells of the foot in active and hibernating animals. Besides mucus cells they described calcareous, eosinophilic and phenolic cells. The last gland cell type was the subject of a further paper (Bolognani-Fantin, 1965). 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.

Campion (1961) described mucus-secreting areas of the mantle and general body surface of Helix aspersa and found eight types of gland cells: mucocytes A,B,C & D; protein;

calcium; lipid and pigment. The details of the mucus gland cells found in the mantle will be discussed in Chapter 2 on the mantle.

However, in examining the foot structure, she stated that gland cells type 'C' were numerous in the sole and often found in clusters. Their secretion appeared to be granular in the cell; but in ducts and particularly near the surface the secretion had a somewhat reticular texture. She also found occasionally gland cells of type 'D' to be scattered among the sole glands. They appeared to be very similar to gland cell type 'C', but differed histochemically. She thought that these two cell types had a common origin.

The next work to be considered was by Elves (1961) who described the structure of the foot of Discus rotundatus (Muller) in which he found one type of mucus gland cell to be scattered throughout the sole and also aggregated in foot fringes, together with amoebocytes (equivalent to Barr's (1926-28) calcic cells), both types being embedded in the connective tissue.

He also described a pedal gland which was composed of two cell types, one type being in a discharged state and the other a developing or discharging state. Furthermore, a caudal gland was present and was composed of mucous gland cells and amoebocytes.

The comparative paucity of gland cell types in slugs was born out by Barr (1926) who described the pedal gland of

Milax sowerbii (Ferussac) and found it to be composed of a mass of secretory cells traversed by a duct, whose 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.

Barr (1928) also described the glandular system of Arion ater var. Castagnea. Similar mucus gland cells were described in the tissue of the mantle margin, foot sole and foot fringe, the aggregation of such mucus 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 ater the pedal gland was attached to the tissues of the foot and discharged into intercellular spaces or directly into the gland duct. In many respects the gland resembled that of Milax sowerbii but the duct in the case of A.ater was never secondarily branched, did not project beyond the secretory cells at the posterior end as occurred in M.sowerbii and did not show any evidence of an excretory function.

Two forms of pigment cells were found in A. ater var. Castagnea, in the skin and subepidermal tissues. One contained 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 excretory product deposited in mature mucous gland cells and in the intercellular spaces.

Arcadi (1963) described the gland cells of Lehmania poirieri (Mabille) a limacid 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.

Chetail and Binot produced a series of papers (1967 and 1968) on the glandular structures of Arion rufus Linnaeus. The description of the pedal gland (1967) was much like that given by Barr (1928) in Arion ater Linne but they divided the gland into three regions, anterior lateral, anterior median and posterior. They also described three histochemically distinct cell types a1, a2 and b. a1 cells were taken to be the less mature form of a2, since the secretions were alike but stained more intensely in the a2 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 a1 and a2 but produced a

different type of secretion. They also found four gland cell types in the sole of the foot: I, II, III and IV.

Type I were most numerous in the sides and middle of the sole. Type II were like Type I but showed less intense staining and were the most numerous cell types in the sole, being found throughout the foot. Type III had granular contents and 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 but they were the least numerous being found only in the middle of the sole.

A paper produced by Wondrak in 1969 described the mucous gland cells of Arion rufus and 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 Sohlendruse) as it was distributed all over the sole, and the second type he designated the "lateral sole gland cell" (Lateral Sohlendruse) which was found in the lateral areas of the sole below the peripodial groove.

More recently Lawrence (1972) did a comprehensive study of the skin glands in Arion hortensis Ferussac and in the sole region reticulate type A/B cells were found to be present, B being the less mature stage. She thought that these gland cells resembled the mucocytes of the pedal gland. The granular type C gland cells were also found in the sole but they were less common than the others and contained a mucoprotein type of secretion.

Dalal and Pandya (1976) studied briefly the structure and histochemistry of the suprapedal gland of the garden slug Laevicaulis alte Ferussac. They observed that the suprapedal gland was loosely attached to the anterior part of the body and opened just below the mouth. This gland was found to be composed of exclusively granular cells and no muscle fibres were present. The granular cells were tall columnar and epithelial and discharged into the lumen of the gland.

However, the most recent work is by A. Cook (1987) on the mucus-producing glands of the systellommatophoran slug, Veronicella floridana Leidy.

In examining the foot, Cook divided it into three parts - the suprapedal gland, the foot ridges and the foot margin.

In Veronicella the suprapedal gland was found to be extremely short and contained two types of secretory cells.

In Veronicella the foot possessed transverse ridges and the four types of gland cells were associated with each ridge (V3, V4, V5 and V6). Cook suggested the possibility that type V4 cells might be the inflated ducts of the type V3 cells.

The foot margin of Veronicella consisted of two halves, one on the foot side and the other on the body side. A small group of secretory cells was present on the side of the foot. Cook thought that these were histochemically undistinguishable from type V6 cells and the type V7 cells which were present on the head, mantle etc. At the edge of

the body near the foot there were type V8 cells containing refractile granules.

The next studies to be considered are the investigations on aquatic pulmonates.

1.2.2 Aquatic pulmonates

Pan (1958) studied Biomphalaria glabrata Say (Australorbis glabratus) and Bolognani-Fantin and Virgo (1967a,b) studied Lymnaea stagnalis L and Planorbis corneus. However, the work to be considered in particular detail is the more recent study by Zylstra (1972) on fresh water pulmonates, who carried out a comprehensive investigation of the histochemistry and ultrastructure of the epidermal and subepidermal gland cells in Lymnaea stagnalis L and Biomphalaria pfeifferi Say. This was a study of the whole animals. He described the 13 different subepidermal gland cell types in addition to the epidermal goblet cells. Three of the gland cell types were found to be distributed ubiquitously whilst four cell types were peculiar to the foot, two types to the lips and five types to the mantle.

At this point only the gland cell types with a general distribution and the cell types peculiar to the foot will be considered, whilst the gland cell types found in the mantle will be discussed in Chapter 2 on the mantle.

The gland cells with a general distribution were intraepithelial goblet cells and subepidermal muciparous and nonmuciparous gland cells. Muciparous gland cells were the most common of the ubiquitous subepidermal gland cell types.

The foot was found to possess an abundance of gland cells both in number as well as in type, and Zylstra described four subepidermal gland cell types peculiar to the foot region. In the anterior part of the foot two gland cell types were found which could be distinguished due to their location, internal structure and staining features.

The third type was the sole gland cells which were located in the ventral region of the foot and the fourth gland type was referred to as the lateral foot edge gland cells.

However, in his investigations on Biomphalaria pfeifferi Zylstra found striking differences in comparison with Lymnaea stagnalis L both in the overall distribution and the number of subepidermal gland cell types. In this species he could distinguish only seven types of subepidermal gland cells, of which two were the lip gland cell types and the other five types were present in the foot. Three gland cell types specific to the foot were quite similar in staining reactions as well as in location to the foot sole gland cells and two pedal gland cells in Lymnaea stagnalis .

He believed that lubrication was the primary role for the foot sole and the anterior and posterior pedal gland cell types. The same role was thought to be played by lateral foot edge gland cells but he suggested that they also might have an additional function.

2. The Mantle

The edge of the mantle is an important area of growth and secretion and apart from its shell-secreting function, it also has important glandular and sensory activities.

Over the past years a wealth of literature has accumulated on the histology and glandular nature of the margin of the mantle. The primary aim of most of these studies has been to determine the various structures responsible for secretion of the various layers of the shell.

2.1 Prosobranchs

In regard to the structure of the mantle edge glands, the following investigations are typical. Fisher (1904) studied the anatomy of Lottia gigantica and remarked on the presence of a dorsal, median and ventral group of glands in the margin of the mantle. The ventral gland was composed of large pouch like cells which pierce the epithelium. Fisher mentioned that these glands were probably concerned with the secretion of the shell.

In Helcioniscus ardosiaeus, Thiem (1917) described one marginal gland consisting of flask shaped cells opening to the surface of the mantle edge. However, he offered no suggestion concerning their function.

In two species of Paludina, Neumann (1928) described the submarginal glands in detail at the optical level. He believed that the periostracum in these species was not

derived from the glands but from the mantle groove.

Gland clusters opening at the dorsal side of the mantle edge are very common in the prosobranchs. Tullberg (1882) and Dakin (1912) found them in Buccinum, Bregenzer (1916) in Bythinella and Starmuhlner (1952) in Valvata.

More recently, Bevelander and Nakahara (1970) studied the formation and structure of the periostracum of Littorina littorea at an electron microscopic level. They described the three distinct groups of glands at the mantle edge in between the dorsal and ventral epithelium. The dorsal and ventral glands were found to be composed of cells which were similar in size and appearance. The third gland consisted of an elongated array of mucous cells. Bevelander and Nakahara believed that the secretory granules of the dorsal and ventral gland cells had an important role in the formation of the periostracum. However, they offered no suggestion or evidence concerning the function of the third type of gland.

Further studies were carried out on the mantle edge by Walsh and an abstract of his work was published in 1981. His studies included the histology and cytochemistry of the mantle edge of Busycon carica and Busycon canaliculatum. Homologous structures were found in the mantle edge of both species. The subepithelial gland cells were found to be present on the outer shell side epithelium. The inner mantle epithelium was composed of pigmented, ciliated, columnar epithelial cells and goblet cells. Walsh believed

that the secretion of the supramarginal and distal supramarginal glands had an important role in the formation of the periostracum. Both of these glands poured their secretion into the shallow, ciliated supramarginal depression.

The only difference found in these two species was due to the presence of inner subepithelial gland cells of unknown function in B.canaliculatum.

The most recent work on the histology and histochemistry of the mantle edge was by Umadevi, K. Hanumantha Rao and Shayamasundari (1984). They studied the mantle edge in Pila virens and described the supramarginal groove and the mantle edge gland. The mantle edge gland was composed of highly modified cells comprising tall, glandular, flask shaped cells arranged in bundles. They assumed that the secretion from the mantle edge gland was responsible for the formation of the ostracum and the protein secretion from the supramarginal groove for the formation of the periostracum.

2.2 Pulmonates

2.2.1 Terrestrial pulmonates

A series of papers have been published on the mantle of Helix aspersa Muller.

Villepoix, (1892) described the structure of the marginal gland in Helix aspersa and referred to cytoplasmic granules in the gland cells. He suggested that these granules might be involved in the formation of the ostracum and organic substances. Prenant, (1924) using

haemalum as his only stain described two types of mucus glands. Roth, (1929) also distinguished, but not very clearly, two kinds of mucus glands. However, the most comprehensive study on the mantle of Helix aspersa has been done by Campion (1961).

She divided the mantle into two regions, the shell gland or the ventral surface and the median surface. Campion described two types of mucus glands involved. Mucus gland cells type A exhibited reticular or bubbly contents, whilst the gland cell type 'B' was distinguishable because of the evenly granular nature of its secretion. Protein glands were abundant in the collar and appeared somewhat ribbon-like in section. These three types of glands were common in the mantle collar. However, she also described two other kinds of gland which were found occasionally. These were pigment glands which had a characteristic deep yellow-coloured secretion, and a gland with a globular secretion which was rarely found in frozen dried material.

More recently Saleuddin (1976) carried out ultrastructural studies on the formation of the periostracum in Helix aspersa and as the periostracum is secreted by the mantle, he described briefly the structure of the mantle edge.

In examining the mantle in Helix aspersa he detected three different zones: (1) ventral glandular epithelium, (2) periostracal groove, and (3) the dorsal epithelium. He

observed many mucus and calcium cells opening externally through the ventral epithelium. However, he did not describe them as his study was mainly concerned with the formation of the periostracum. The periostracal gland was extended along the entire mantle edge and was deeply embedded in the connective tissue. He believed that the outer epithelium had an important role in the formation of the calcareous part of the shell.

Numerous papers have been published in relation to the mantle in Helix pomatia and in most of them (Villepoix, 1892; Prenant, 1924; Roth, 1929 and Baecker, 1932) three zones have been detected: (a) an inner glandular zone, (b) the periostracal groove and (c) the general outer epithelium.

Abolins-Krogis (1973) gave an excellent account of histological and histochemical changes that occur in the mantle during shell regeneration in Helix pomatia in the region of injury. However, the work to be considered in detail is by Saleuddin (1970) who studied the structure and histochemistry of the normal mantle of Helix pomatia and the effect of shell injury on the general outer epithelium using light and electron microscopy. Like the earlier authors Saleuddin (1970) also divided the mantle into three distinct zones: glandular epithelium, periostracal gland and the outer epithelium.

Mucus cells were found to be present beneath the glandular epithelium. The most characteristic of all the

cell types found within the connective tissue were the calcium cells which appeared among the Leydig cells. Leydig cells contained an appreciable amount of RNA. Lipid cells were also present in between the cells of the glandular epithelium. Saleuddin also mentioned the cells of an unknown type in the centre of the mantle which were frequent but not abundant. The epithelial cells of the periostracal gland were pyramidal with the secretory surface less broad than the basal regions. The outer epithelium was composed of tall columnar cells with light brown inclusions near the secretory surface. The cells of the outer mantle epithelium which he believed had an important role in the shell formation and shell regeneration showed different histological structures from those of the periostracal groove and the glandular epithelium. The most characteristic feature of the outer epithelium was the presence of lamellar organelles.

Saleuddin suggested that the secretory droplets in the cells of the periostracal groove indicated their secretory nature and he believed that it might have been involved in the secretion of certain components of the shell.

Chetail and Binot, (1968a) studied the gland cells of the mantle of Arion rufus. They found two types of cells and a third type of gland cell was found in the groove between the mantle and foot tissues.

Lawrence (1972) described the three types of mucus cells and the pigment/protein cell in the mantle region of

the slug Arion hortensis. However, she believed that the reticulate 'M' and 'm' cells were the same type of cell, m being the discharged form. The pigment/protein cell and the 'Y' cell which was thought to be the young stage of the pigment protein cell were found on the dorsal surface. On the ventral surface of the mantle, granular mucus cells were found.

In Veronicella (Cook, 1987) the mantle was found to be dominated by type V9 cells. Type V11 cells in this region showed the same staining as type V9 and Cook suggested that these two might be the same cell type in a different secretory phase. The type V10 cells were large and contained protein possibly combined with neutral mucopolysaccharide.

2.2.2 Aquatic pulmonates

The mantle of the fresh water pulmonate, Lymnaea stagnalis has been repeatedly studied: by Timmerman, (1969, 1973); Kniprath, (1970, 1971 and 1972); Zylstra, (1972), and Zylstra, Boer and Sminia, (1978). Most of these studies concerned the structure and formation of the periostracum. However, the work to be considered in detail is by Zylstra (1972) and Zylstra et al. (1978) as these are the most recent papers published on the histology and histochemistry of the mantle in Lymnaea stagnalis.

In 1972, Zylstra described the gland cells associated with the inner mantle epithelium in Lymnaea stagnalis and Biomphalaria pfeifferi.

In Lymnaea six types of subepidermal gland cells were found to be present in this region of which four types were specific to the mantle whilst the other two were ubiquitous cell types which have been described already with the pedal glands.

Of the four gland cell types specific to the mantle, two were muciparous and the other two were nonmuciparous. The mantle muciparous gland cell types 'A' and 'B' were found to differ in their form and internal structure.

The two nonmuciparous subepidermal gland cell types specific to the mantle produced a granular secretion.

In examining the inner mantle epithelium of Biomphalaria pfeifferi, Zylstra found two types of gland cells in this region. She thought that type 'A' were rather analogous to the mantle muciparous gland cell type 'A' in Lymnaea stagnalis whilst the type 'B' were analogous to the ubiquitous nonmuciparous gland cells in Lymnaea stagnalis in structure and staining responses.

Zylstra et al. (1978) studied the histology, histochemistry and the ultrastructure of the mantle edge of Lymnaea stagnalis and Biomphalaria pfeifferi. They found that the morphological features of the mantle edge of Lymnaea stagnalis and Biomphalaria pfeifferi were very similar. The mantle edge gland divided the mantle epidermis into two regions: the inner mantle epithelium and the outer mantle epithelium. The mantle edge gland structure was found to consist of the periostracal groove and the belt

region posterior to the groove.

On the basis of histological and histochemical studies Timmermans (1969) divided the mantle edge of Lymnaea stagnalis into five zones and this division was also adopted by Zylstra et al. (1978). In the periostracal groove of Lymnaea stagnalis, seven distinguishable subepithelial gland cell types were found to be present. Four of these gland cell types were located below the periostracal groove with their necks opening into the basal region of the groove, whilst the cell bodies of the other three were found to be scattered throughout the connective tissue surrounding the groove. They believed that the gland cell types 1 and 2 were the same respectively as the mantle muciparous gland cell type 'B' and the mantle nonmuciparous gland cell type 'B' (Zylstra, 1972). These two gland cell types were not distinguished by Kniprath (1970).

Only a few histochemical differences were found between cell types 3, 4, 5 and 6. Although they showed only few histochemical differences they differed in both cell organelles and in secretion granules (Zylstra et al; 1978, Kniprath 1972).

The periostracal groove of Biomphalaria pfeifferi was found to be very similar to the periostracal groove of Lymnaea stagnalis with regard to the presence of gland cells in the base of the groove. However, only three gland cell types, differing in their structure and secretion granules, were found. In this respect, Zylstra et al. (1978) thought

that Biomphalaria pfeifferi showed similarities with the freshwater pulmonate Helisoma duryi duryi in which also fewer cell types were found to be present in the groove region compared to Lymnaea stagnalis.

Saleuddin with his associates produced a series of papers (1975, 1979) on the mantle in Helisoma duryi. However all of his studies were electron microscopic and concerned with the formation of shell and periostracum. The paper produced in 1979 appeared to be interesting as in it Saleuddin described the cells at the periostracal groove and called them periostracal cells (Chan and Saleuddin 1974). These cells were found to be lying beneath the epithelium at the base of the groove and opened into the lumen of the groove by long apical processes. He believed that the periostracum was secreted by these gland cells and the secretion of the mantle edge gland cells was responsible for the thickening of the maturing periostracum when it was moving away from the periostracal groove. Mucous cells were also present at the base of the groove, and Saleuddin suggested that the mucus secretion might be helping in the movement of the developing periostracum along the groove.

In the same paper (1979) Saleuddin also described the mantle edge of Physa. The basic plan for the mantle edge resembled that in Helisoma. In this species 3 cell types were found to line the dorsal face of the periostracal groove and he believed that two of them were involved in the formation of the periostracum.

3. The hypobranchial gland

The presence of a hypobranchial gland located in the mantle of some gastropod molluscs has been well known since ancient times owing to the purple coloured pigment which was used by Phoenicians. A number of the morphological studies have been carried out on the middle part of the hypobranchial gland in Muricaceans where the formation of the purple secretion takes place. There has also been histological and histochemical research concerning mainly the purple producing cells in the central portion (Grynfeltt, (1911 and 1913); Lison, (1933); Vialli, (1934 and 1965); Gabe, (1959) and Fainmaurel, (1967)). However, few comprehensive studies of the hypobranchial gland have been carried out in non-purpurigen species of prosobranch. Bernard (1890) did a comparative study of the hypobranchial gland in a number of prosobranchs and found that the epithelium was generally composed of three types of cells ciliated cells, mucocytes and neurosecretory cells. Dakin (1912) supported Bernard's view as he also found in Buccinum a hypobranchial epithelium consisting of ciliated cells, mucocytes and neurosecretory cells. In general terms this description applies to most of the hypobranchial glands which have been studied although other authors have found more than one type of gland cell and detailed descriptions may differ quite appreciably.

Tarao (1935) investigated the hypobranchial gland of

Haliotis and found four types of gland cells: rod-shaped mucocytes, small granule-containing eosinophil cells, large granule-containing eosinophil cells and brown granule-containing cells.

In examining the hypobranchial gland of Busycon canaliculatum Ronkin (1952) found it to be composed of ciliated cells which were conical and three types of mucous cells, two of which he thought were probably different developmental stages of the same cell type.

Starmuhlner (1952) studied the hypobranchial gland of the fresh water species Viviparus and found it was composed entirely of granular cylindrical cells.

More recently, Hunt (1967, 1973) produced a series of papers on the hypobranchial gland of Buccinum undatum L. Hunt (1967) described the two different cell types in the hypobranchial gland of Buccinum. Type 'A' cells were large and contained a frothy granular secretion, whilst the type 'B' cells were much narrower and less bulbous than type 'A'. Sandwiched between these two types of mucus cells were numerous ciliated cells with spindle-shaped nuclei.

In 1973, Hunt studied the fine structure of the hypobranchial gland of Buccinum undatum and also described the protein-producing cells which he named 'goblet cells'. These cells appeared to be smaller in size, with denser contents which responded strongly for protein reactions.

Ottaviani (1978) studied the hypobranchial gland in the mantle of Paludina vivipara Draparnaud, a fresh water

gastropod. This work was more interesting in that it gave a detailed account of the histochemical nature of the various secretions produced by the hypobranchial gland. In examining the hypobranchial gland Ottaviani divided it into three areas: two side areas (branchial and rectal) and a central area. The protein-producing cells appeared to be very much alike in all these 3 areas. However, the mucocytes which were found to be numerous in the two side areas compared to the central area reacted differently to the histochemical procedures used. Two types of cells were found to be present in the central area.

The most recent work is by Bolognani-Fantin and Ottaviani (1981), who studied the hypobranchial gland in a number of prosobranchs living in different habitats.

In the central area of the hypobranchial gland in Murex brandaris (Marine species), they found in addition to the purple producing cells, six other epithelial cell types. These were (1) ciliated cells, (2) empty cells, (3) cells with big granules, (4) cells with fine granules, (5) cells with homogeneous contents and (6) Mucocytes.

Bolognani-Fantin and Ottaviani found three cell types in the two lateral areas of the hypobranchial gland in Murex. They were supporting cells, mucocytes and cells with different sized granules. The authors named these granular cells picrophilic cells because of their strong affinity for picric acid.

The hypobranchial type glandular system was found to be

much simpler in the mantle of Viviparus viviparus L (a freshwater species) than in that of Murex.

In the hypobranchial gland of Viviparus the two lateral areas were very thin and the only specialized cells in these areas were the mucocytes. However, in the main region two cell types were found.

In Pomatias elegans (a terrestrial species), the hypobranchial type glandular system was extremely simplified and no separate areas could be distinguished. The glandular epithelium of the hypobranchial gland was composed of very tall cylindrical supporting cells and one type of secreting cells.

CHAPTER VII

RESULTS

SECTION A

Prosobranchs

Pomatias elegans, Bithynia tentaculata and Littorina littorea were chosen for the present study since they all belong to the order Mesogastropoda of the same subclass Prosobranchia although they live in different habitats. As far as mucus is concerned, there are four areas of the skin which play a particularly important part in the life of a gastropod mollusc. These are the skin over the foot, the operculum, at the mantle edge and the hypobranchial gland.

The general distribution of cells in the foot, the operculum and at the mantle edge is shown in Figure 1. Pomatias elegans, Figure 4, Bithynia tentaculata and Figure 8, Littorina littorea. Staining procedures were diverse and are summarized in Table 1.

1. The Foot

The glandular areas of the foot of these prosobranchs can generally be divided into the suprapedal gland and three areas on the surface of the foot. The anterior pedal gland, the sole of the foot, and the dorsal and lateral epithelium.

1.1 Pomatias elegans

The foot of Pomatias is longitudinally divided into two parts by a deep median furrow. The entire ventral foot epithelium is a layer one cell thick that consists of closely packed columnar cells devoid of cilia. There are no

secretory cells generally distributed on the ventral surface of the foot and there is no specialized glandular area at the anterior margin of the foot, but there are some epidermal and subepidermal gland cells in the lateral pedal epithelium, and specialized glandular areas on either side of the median furrow (Figures 1A and 2A, B and C).

In Pomatias the foot therefore can be divided into:

1.1.1 The suprapedal gland

1.1.2 The sole

1.1.3 The dorsal and lateral epithelium.

1.1.1 The suprapedal gland: This gland lies on the foot musculature in the anterior third of the foot. It consists of an outer part in which three cell types are apparent and an inner part consisting of just one cell type. These two parts are separated by the suprapedal duct which opens into the anterior part of the median pedal furrow (Figure 1A and Plate 1A and B).

(a) The outer part : Three kinds of gland cells are located in the outer part of the suprapedal gland. When lining the outer part, the suprapedal duct consists of an epithelium that is made of cylindrical cells of about 15-20μm in size, and in between these cells the ducts of the gland cell types P1, P2 and P3 can be seen (Plate 2A).

Cell type P1:

These cells are very few in number and generally lie near the epidermis of the duct. They are flask shaped, with an ovoid cell body about 15-20μm in diameter and a short

neck opening between the epithelial cells. The nucleus is not visible at all in these cells (Figure 3A and Plate 2A). The contents of these cells are generally granular but may appear reticulate. However, the granular contents stain more deeply than the reticulate material.

The histochemical results as shown in Table 2 indicate that detectable amounts of protein are present in the cytoplasm of these cells. The contents of these cells are negative to all the stains tried for mucopolysaccharides, calcium and lipid and therefore their presence can apparently be discounted. However, the protein reactions appear to be strong since a bright colouration is obtained with the mercuric bromophenol blue and diazo coupling reactions (Plate 3A and B).

Thus the secretion of P1 cells is proteinaceous, containing tyrosine (Table 3).

Cell type P2:

These are numerous cells which are closely packed together. P2 cells have an irregular shaped, distinct cell body which is 10 to 12μm in size and an extremely long thin duct leading to the epidermis between the P3 type cells. The contents of these cells are finely granular (Figure 3A and Plate 2B).

The histochemical results from Table 2 indicate that a mixture of substances is present in these gland cells. The positive reactions for the mercuric bromophenol blue and diazo coupling tests show that a protein containing tyrosine

is present (Plate 3A and B).

The contents of these cells stained blue with Mallory's triple stain suggesting that possibly some type of mucosubstance, either acid or neutral, could be causing this staining, though no other methods for acidic mucopolysaccharide such as toluidine blue, Alcian Blue pH0.5/Eosin, and Alcian Blue pH0.5/Alcian Yellow pH2.5 gave positive results. Nevertheless, the reaction with Periodic Acid Schiffs (Plate 2A) and the brown colour shown with Periodic Acid Paradiamine (Plate 6B) indicate that neutral mucopolysaccharide is present.

The contents of these cells also reacted positively for calcium tests and stained reddish orange with alizarin red (Plate 4A) and black with the von Kossa method. This staining is found in alcohol fixed sections as well as in fresh frozen sections; however, negative reactions are obtained in Zenker fixed sections. This suggests that the calcium deposits are soluble in acid which is present in Zenker's fixative and this in turn suggests that the calcium is deposited in the form of calcium carbonate.

How a neutral mucopolysaccharide would fit into the molecule can only be guessed. Possibly neutral mucosubstances are attached to the protein units which may form link units and calcium may be involved in the ionic linkage. However, with the tests applied it is not possible to be sure about linking and binding of the components, since biochemical methods were not employed.

Cell type P3:

These cells are usually club shaped with a somewhat ovoid cell body but they may adopt unusual shapes (Figure 3A and Plate 2A and B). Their length ranges between 40 to 80μm and their width is 20-25μm. Some of these size variations 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 cells) and any section taken off centre will give a reading smaller than the actual measurements. However, a number of measurements of the gland cells with various sizes have been taken assuming that the typical gland cells will have a size included in that range.

When visible, the contents of these cells are reticulate in texture but they appeared empty with most staining procedures used. In many cases this may result from the discharge of secretion either before or at the time of fixation.

The results from the histochemical tests applied (Table 2) show that cell type P3 contains mainly acidic mucopolysaccharides, particularly when considering the results from the combined Alcian Blue pH2.5/PAS and Alcian Blue pH0.5/eosin techniques (Plate 5A and B).

The Alcian Blue pH2.5/PAS sequence shows that acidic groups possibly containing hyaluronic acid are present in the mucosubstances as indicated by the bright blue colour;

and the staining at low pH in Alcian Blue pH0.5/eosin indicates that sulphate groups are also present. With the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique the contents of cell type P3 stained blue/green and if sulphates as well as hyaluronic acid are present, as would be suspected, then one would normally expect green or yellow/green staining with this technique but this did not happen. This is supposed to indicate that there is no carboxyl group present or alternatively very weak carboxyl groups are present.

However, the sulphate groups present are stacked in such a way as to give β metachromasia in toluidine blue (Plate 5C). There is no evidence of lipid or calcium in the secretion and none of the protein tests was positive (Table 2). Therefore, the secretion appears to contain an acidic mucopolysaccharide, mainly containing sulphate groups.

(b) The inner part: This part of the suprapedal gland is thicker than the outer part. The duct epithelium lining the inner part becomes flat, discontinuous and then disappears completely. This means that exactly in the middle the duct epithelium is lacking and the cells directly face the lumen of the duct. Only one type of cell (P4) is observed in this part of the suprapedal gland (Figure 2A, and Plates 5 and 6).

Cell type P4:

These cells constitute the inner part of the gland. They are tightly packed, irregular cells of diameter about 20-22μm and possess reticulate contents. No ducts were

observed associated with these cells and no sort of secretory activity was seen. Therefore it must be concluded that they are not exocrine in function. However, the histochemical results (Table 2) seem to indicate that a mixture of substances is present in the inner part of the suprapedal gland.

A bluish - purple staining with the Alcian Blue/PAS technique (Plate 5A) shows that periodate-reactive and alcinophilic components are present. A periodate-positive substance could possibly be present only in the form of a neutral mucosubstance as indicated by the brown colour shown with periodic acid paradiamine method (Plate 6B) and none of the tests for protein was positive.

The contents of cell type P4 stained blue with the Alcian Blue pH0.5/Eosin which is supposed only to show sulphates at this pH (Plate 5B). The results obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique shows that the contents are stained yellow-green which indicates the presence of sulphate and carboxyl groups (Plate 4B). However, these end groups do not apparently confer metachromasia in toluidine blue (Plate 5C), possibly this could be because these groups are either not 'stacked' in such a way to confer such metachromasia or else the neutral mucopolysaccharide present in the secretion is suppressing the metachromasia. Substantial amounts of calcium also seem to be associated with the neutral and acidic mucosubstances as indicated by the von Kossa and alizarin red methods

(Plate 4A), but lipid was not evident (Table 2).

Bensalem and Chetail (1982) thought that the main function of the inner part of the gland is the storage of calcium and water in its cells. However, the present study reveals that in addition to calcium ions acidic and neutral mucopolysaccharides are also present.

Tubulous gland

The tubulous gland is located in the foot musculature just behind the supra-pedal gland. It consists of two tubes which join before opening into the median pedal furrow. Their common duct opens posteriorly to that of the duct of the suprapedal gland (Figure 1A and Plate 1B). Each tube originates independently in the midst of the pedal musculature from some irregular clusters of small, oval cells that arrange themselves in groups to form the tubules and ultimately these tubules join into two tubes. The wall of the tube is made of cylindrical ciliated cells which are always visible with a big oval nucleus (Figure 2D and Plate 7A). They stained with PAS and the positive reactions with alizarin red, von Kossa and Sudan black B methods show that calcium and lipids are present (Plate 7). However, they contain neither mucopolysaccharide nor protein and are included here only for completeness.

This gland is thought to be concerned with the uptake of salts from the soil water (Delahaye 1974) but recently Bensalem and Chetail (1982) suggested that the tubulous gland acts as a "salt gland" that excretes calcium ions to

obtain the necessary water.

1.1.2 The sole

The epithelium of the sole and the median furrow is made of tall, closely packed columnar cells devoid of cilia. The height of the sole epithelial cells is about 20 to 25µm but the height of the epidermis tends to decrease towards the bottom of the furrow and there the epidermal cells are about 15-20µm in height. However, at the base of the furrow two types of subepidermal secretory gland cells are present (cell types P5 and P6) and here the epithelial cells are tall and are about 25µm in height. This area of the foot is illustrated in Figure 2 and Plate 8. The free apical surface of these epidermal cells is lined by a continuous mucous film, produced by the secretory cells of the suprapedal gland and at the median pedal furrow (Plate 8A and B).

Cell type P5:

These are flask shaped cells with a somewhat ovoid cell body of about 10-12µm in size and their long necks open at the median furrow. They are about 45-60µm in length and their contents are reticular in appearance but sometimes may have a more granular texture. The nuclei can occasionally be visible occupying a basal position. The P5 gland cells tend to occupy a slightly more superficial position than P6 type cells, that is, they lie nearer the epidermis (Plate 8).

From the histochemical results (Table 2) it seems that

periodate-positive substance is present in P5 cells as indicated by the strong reaction obtained with Schiff's reagent in the general Periodic Acid Schiff's technique and the Alcian Blue pH2.5/PAS sequence (Plate 8A and B). A periodate-positive substance is present possibly in the form of neutral mucosubstance, together with what could be some protein. On the basis of the tests employed it is possible to suggest that the protein as well as neutral mucopolysaccharides are present in these cells, as evidenced by the positive reactions obtained with mercuric bromophenol blue (Plate 9A) and diazo coupling methods for protein and the PAD technique for neutral mucopolysaccharide (Plate 9C). Moreover, the alizarin red and von Kossa techniques show that calcium is also present in these gland cells (Table 2). However, there is no evidence of any acidic mucopolysaccharide components or lipid in the secretion. Thus, the contents of P5 cells appear to be a neutral mucoprotein associated with some calcium (Table 3).

Cell type P6:

These cells group together to form a bulky mass nearer the mid line than type P5 cells and lie deeper in the foot musculature. Sometimes they may extend up to a depth of 100 µmfrom the surface. These gland cells have fibrous contents and occasionally the large nucleus can be visible occupying the basal position (Figure 2C).

The secretion produced by these cells is quite interesting as its responses to most of the histochemical

techniques applied are the same as type P3 cells indicating an acidic mucopolysaccharide secretion (Table 2 and Plate 8).

The difference between P3 and P6 cell types lies principally in their reactions to the alizarin red and von Kossa methods which indicate the presence of calcium; and in both cases positive results were obtained for P6 type cells. The similarity between the secretion of these two cell types could be related to the fact that P6 type cells need to produce more mucus to keep the foot sole lubricated. This is likely since the mucus trail produced by the suprapedal gland (cell type 3) and these cells is essential for locomotion and also to trap the calcium ions in their secretion as suggested by Bensalem and Chetail (1982).

The pedal mucus of Pomatias therefore consists of a complex secretion from the suprapedal gland and acid and neutral mucopolysaccharides together with protein from the sole itself. All these secretions are delivered to the longitudinal median furrow.

1.1.3 The dorsal and lateral epithelium:

The dorsal and lateral epithelium is composed of columnar cells about 20-25μm high at the dorsal surface and about 12-25μm at the lateral surfaces. Interspersed with these epithelial cells are four types of goblet cells and one type of subepidermal secretory cell (Figure 2B).

The goblet cell types can be differentiated according to their texture and the histochemical nature of their

contents (Figure 3B and Table 3). These goblet cells are generally distributed but occur sparsely on the ventral surface of the foot. However, they are particularly common on the dorsal and lateral body surfaces. Their height ranges from 12-25μm depending on the area in which they are present.

Cell type P9:

These goblet cells are cuboidal in appearance and their contents are homogeneous with a smooth texture. A basal nucleus can occasionally be visible (Figure 3B and Plate 10A). The contents of these cells did not show any positive reactions to the methods used for protein, acidic mucopolysaccharides and lipid (Table 2). However, the PAS reaction appears to be stronger since a bright red colour is obtained with the Periodic Acid Schiffs (Plate 11A) and the Alcian Blue pH2.5/PAS technique . This periodate-positive group could possibly be neutral mucopolysaccharide as evidenced by the brown staining obtained with the periodic acid paradiamine method (Plates 10A and 11C). The presence of calcium in the secretion is doubtful as only a slight reaction is obtained with the alizarin red method and the contents did not show any response to the von Kossa method (Table 2). Therefore, the contents of these goblet cells appear to be neutral mucopolysaccharide (Table 3).

Cell type P10:

These cells have the same distribution and appearance as cell type P9 (Table 3). The nature of their contents

differs however, since most of the results indicate the presence of an acid mucosubstance as indicated by the bright blue colour obtained with the Alcian Blue pH2.5/PAS technique.

Staining at low pH in Alcian Blue pH0.5/eosin indicates that sulphate groups are indeed present and these groups display metachromasia in toluidine blue (Plate 11B). However, there is no evidence of any periodate-positive material in the secretion as shown by the PAS and Alcian Blue pH2.5/PAS techniques which in turn indicate that neutral mucopolysaccharides are not present. A negative response to the mercuric bromophenol blue and diazo coupling reaction for protein and the black colour obtained with the periodic acid paradiamine method for neutral mucopolysaccharides further confirm that these components are not present in the secretion (Table 2 and Plate 11C). Calcium and lipids are also not evident. Therefore, the contents of these goblet cells are acidic mucopolysaccharides.

Cell type P11:

These cells are sparsely distributed all over the body and their contents are finely granular (Figure 3B). There is no evidence of any acidic mucopolysaccharides as indicated by the toluidine blue, Alcian Blue pH 2.5/PAS, Alcian Blue/eosin and Alcian Blue pH0.5/Alcian Yellow pH2.5 techniques and of lipid as indicated by the Sudan Black B method (Table 2).

However, periodic-reactive substances appear to be

present as indicated by the red staining obtained with the PAS and Alcian Blue pH2.5/PAS techniques. The positive reactions with mercuric bromophenol blue and diazo coupling methods (Table 2) and the brown colour obtained with the periodic acid paradiamine technique (Plate 10A) show that protein as well as neutral mucopolysaccharides are present in the secretion. Nevertheless, as in the case of cell type P9 the presence of calcium in these cells is doubtful. However, it seems that most of the results indicate the presence of a mucoprotein type of secretion containing neutral mucopolysaccharide (Table 3).

Cell type P12:

Cell type P12 is the predominent goblet cell with large globular contents. The globules appears spherical and are about 2 to 4 µm in diameter (Figure 3B and Plates 10B and 11C). These cells are also localized in the opercular region and are numerous in the hypobranchial gland.

The results of the staining techniques show that a mixture of substances is present in the secretion of these cells (Table 3). The presence of sulphate groups is indicated by the positive staining at low pH in Alcian Blue pH0.5/eosin (Plate 10C). The bright colouration obtained with mercuric bromophenol blue and diazo coupling reactions show that protein containing tyrosine is present (Table 2 and Plate 10B). Red staining obtained with the PAS (Plate 11A) and Alcian Blue/PAS methods indicates the presence of periodate-reactive groups, and these groups appear to be

neutral mucopolysaccharide on the evidence of the response to the periodic acid paradiamine method (Plate 11C).

The sulphate groups which are present in the secretion did not show metachromasia in toluidine blue. However, this could be due to the protein and neutral mucoplysaccharides which suppress the metachromasia, this is again supported by the Alcian Blue pH2.5/PAS technique as only red staining is obtained with this method (Table 2).

Cell type P13:

These subepidermal cells occur mainly on the lateral sides of the foot and on the tentacles. They are flask-shaped and are about 70-80 µm in length (Figure 2B). Their ovoid or somewhat elongated cell body is 10-12µm in size and is embedded in the connective tissue and musculature of the foot and tentacles. The secretion of these cells is discharged to the exterior through a single long straight duct (Plate 12A). A basal nucleus can sometimes be visible. The histochemical results from Table 2 show that the contents of these cells did not respond to the stains used for acidic mucopolysaccharides, proteins, calcium and lipid. Therefore the presence of these components can be dismissed. However, strong reactions are shown with Schiff's reagent in the general PAS (Plate 12A) and Alcian Blue/PAS sequence. This indicates the presence of periodate-positive substances in the secretion which probably could be neutral mucopolysaccharide. The presence of neutral mucopolysaccharide is further confirmed by the

brown colour obtained with periodic acid paradiamine method (Plate 12B).

These histochemical results indicate that the contents of cell type P13 are composed of neutral mucopolysaccharide (Table 3).

1.2 Bithynia tentaculata

The sole of the foot of Bithynia is ciliated all over the surface. The foot shows considerable muscular movements during locomotion but these are comparatively local and do not take the form of locomotory waves. Ciliary movements are clearly visible and the ciliary locomotion may therefore be modified by these local contractions. Sometimes an attempt to relax the foot was not completely successful and longitudinal sections therefore are transversely folded to various extents at a point which corresponds to the insertion of the columellar muscle from above (Plate 13).

The ventral foot epidermis of Bithynia is a one cell thick layer consisting of general epidermal cells, cilia cells and goblet cells. Interspersed between these cells can be found the necks of subepidermal gland cells. When the snail is fully extended the epidermis is stretched and appears quite delicate in view of its integumentory function. The general epidermal cells vary from cuboidal to columnar in form with a height ranging between 10 to 25µm depending on the region of the foot. The oval shaped nucleus with the nucleolus lies in the basal half of the cell.

In Bithynia all the pedal glands are single-celled, each having its own duct or neck extending between the epidermal cells to the exterior surface, and the suprapedal gland as found in Pomatias is not present.

The anterior pedal gland is present and there are secretory cells distributed generally over the sole but there is a concentration of specialized cells in the transverse fold of the contracted foot. The general distribution of these cells is illustrated in Figure 4A and Plate 13.

Therefore, in Bithynia the glandular areas on the surface of the foot can be divided into:-

1.2.1 The anterior pedal gland

1.2.2 The sole

1.2.3 The dorsal and lateral epithelium

1.2.1 Anterior pedal gland:

Cell type B1:

The anterior pedal gland occupies an arc across the leading edge of the foot, and the majority of epidermal cells in this area are ciliated. This is the only region of the foot where the densely ciliated surface extends beyond or above the foot edge (Figure 5 and Plate 14A). Its cells are bunched together such that their necks form bundles that open in between the ciliated epidermal cells. The cell body has a reticulate appearance with the nucleus located centrally but occasionally the contents of these cells may appear granular. The gland cells have a cell body

approximately 12-18μm in width and can extend up to 580μm from the surface.

The staining responses to the histochemical methods used show that the secretion of this gland is complex and contains a number of substances (Table 4). A bluish/purple staining obtained with Alcian Blue pH2.5/PAS suggests that both alcinophilic components and periodate-reactive groups are present (Plate 14B). The Alcian Blue pH0.5/Alcian Yellow pH2.5 method indicates that acidic mucosubstances are composed of both sulphate and carboxyl groups as evidenced by the yellow-green staining obtained with this technique (Plate 14C). Furthermore, staining with Alcian Blue at low pH in Alcian Blue pH0.5/eosin shows that some sulphate groups are indeed present in the secretion (Table 4).

However, the reactions for proteins appear to be stronger and the bright colouration obtained with mercuric bromophenol blue and the diazo coupling method confirms that protein containing tyrosine is present (Plate 15A and B). The light brown colour shown with the periodic acid paradiamine method indicates that some neutral mucopolysaccharide is also present (Plate 15E).

From these results it seems that the secretion is a mucoprotein. The contents of B1 cells stained strongly for protein but only slightly for carboxylated and neutral mucopolysaccharides indicating that they secrete a mucoprotein containing weakly acidic and neutral carbohydrates (Table 5 and Plate 15). Calcium and lipid

were not evident in the secretion (Table 4).

1.2.2 The sole

The epidermis of the sole is specialized for ciliary locomotion. In view of that function practically the entire ventral surface of the foot is composed of ciliated, columnar cells with a height of 20-25 μm. Goblet cells are occasionally present on the ventral surface of the foot and in addition there are two types of subepidermal secretory cells which are peculiar to the sole (types B2 and B3). They are distinguished by location, internal structure and staining responses (Figure 4, Plate 13 and Table 4).

Cell type B2:

These gland cells are generally distributed over the sole of the foot (Plate 13). They are pyriform in shape with a large cell body embedded in the connective tissue and musculature of the foot and their necks opening between the epithelial cells (Figure 6A). They are about 80-110 μm in length and 25-45 μm in width. The contents of these cells have a reticular appearance and can be seen in the process of being discharged (Plate 14B). Most of the histochemical results indicate that the content of these cells are sulphated mucopolysaccharides as evidenced by the blue colour obtained with Alcian Blue pH0.5/eosin and Alcian Blue pH0.5/Alcian Yellow pH2.5 techniques (Plates 16B and 17B). These sulphate groups are stacked in such a way as to confer β metachromasia in toluidine blue (Plate 16A). No periodate-reactive substance was evident in the secretion,

as indicated by the black colour shown with the periodic acid paradiamine method, which in turn confirms that neutral mucopolysaccharides are not present (Plate 16C). The contents of these cells did not respond to the tests used for protein, calcium and lipid (Table 4). From these results it seems that the B2 cells are composed of sulphated mucopolysaccharides (Table 5).

Cell type B3:

These cells are confined to a transverse band in the middle region of the foot, on either side of the fold that forms when the animal contracts into its shell (Figure 4 and Plate 13) Frequently the outline of the individual cell is not clear. This is mainly because their fibrous contents stain very strongly and rather diffusely, thus obscuring the cell boundries(Plate 17A and B). Ducts filled with secretion are visible passing between the epidermal cells (Plate 17A). A nucleus is not visible at all in these cells (Figure 6B). The secretion of cell type B3 contains sulphated mucopolysaccharides as shown by the Alcian Blue pH0.5/eosin (Plate 16B). However, B3 cells exhibit only slight β metachromasia in toluidine blue (Plate 16A) and this could be due to the presence of neutral mucopolysaccharides in their secretion. The difference between the secretion of cell types B2 and B3 is mainly due to the presence of some carboxylated groups as indicated by the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique and neutral

mucopolysaccharides as evidenced by the periodic acid paradiamine method in the latter (Table 4 and Plates 16C and 17B).

However as in B2 cells, the contents of B3 cells also did not respond to the tests used for protein, calcium and lipid (Table 4). The function of the secretion of B3 cells may not be for lubrication as such during locomotion, since the mucus produced by anterior pedal gland and B2 mucus gland cells serves this function, but it may alter the viscosity of the pedal mucus.

The mucus on the sole of the foot therefore, contains a mucoprotein from the anterior pedal gland which becomes mixed with a sulphated mucopolysaccharide generally and with a more weakly acidic and neutral mucopolysaccharide from type B3 cells in the mid region of the foot.

1.2.3 Dorsal and lateral epithelium

The dorsal and lateral epithelium is composed of cuboidal cells with a height of 10-15 µm. Interspersed with these cells are three types of secretory cells, two of which are epidermal and the third subepidermal. All are generally distributed over this area (Figure 4).

Cell type B7:

This is a goblet cell type. The secretory material is reticular in appearance and can often be seen in the process of being discharged (Plate 18). The nuclei can occasionally be identified as slightly paler patches within the cells and they tend to be central and quite large (Figure 7B). These

goblet cells are spherical and are about 10-15μm in diameter, however variations in size do occur according to the height of the epidermis and the area of the snail body in which they are present.

Histochemical results from Table 4 show that the contents of these cells are composed of acidic mucopolysaccharides. The staining with the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique suggests that both sulphate and carboxyl groups could be present and the blue colour obtained with Alcian Blue pH2.5/PAS sequence shows that hyaluronic acid and/or a weakly sulphated mucosubstance is present (Plate 18A). However, the staining with Alcian Blue pH0.5/eosin confirms that sulphate groups are indeed present; but whichever end-groups are present they are stacked in such a way as to give strong β metachromasia in toluidine blue (Plate 18B). No calcium, lipid, protein or periodate-reactive substance such as neutral mucopolysaccharide was found in the secretion (Table 4). These results show that B7 type cells secrete acidic mucopolysaccharides (Table 5).

Cell type B8:

These goblet cells are few in number but are concentrated in the epithelium joining the head and foot and on the dorsal epidermis of the foot (Figure 4 and Plate 18A). They have a somewhat ovoid cell body and a basal nucleus, which generally lies against the cell wall (Figure 7B). However, the nucleus is often not visible in these

cells. Their contents are reticular and they are about the same size as B7 cells but again variations in size may occur. The difference between goblet cell types B7 and B8 derives principally from their responses to most of the histochemical techniques applied. Results from Table 4 show that the contents of B7 cells are acidic mucopolysaccharides. However, B8 cells did not show positive reactions to the stains used for acidic mucopolysaccharides but the strong reactions for protein tests and the brown colour obtained with the periodic acid paradiamine method (Plate 18C) show that protein and neutral mucopolysaccharides are present in the secretion of these cells. Calcium and lipid are not evident in them. Thus, the contents of cell type B8 are neutral mucoprotein (Table 5).

Cell type B9:

These subepidermal cells are present on the dorsal and ventral surfaces of the foot and also at the base of the tentacles. The cell body is ovoid or slightly elongated and is about 10-12μm in size. The duct is much narrower than the cell body (Figure 7A). These cells usually lie near the epidermis amongst the B2 type cells but occasionally the cell body can be seen deeply embedded in the connective tissue and the musculature of the foot (Plate 19) and tentacles. They have granular contents and a centrally located nucleus. The granular secretion of these cells reacted negatively to most of the histochemical tests applied (Table 4). However, the bright blue staining with

mercuric bromophenol blue, and purple with the diazo coupling method (Plate 19), indicate the presence of protein containing tyrosine. Thus, from these results it seems that the secretion of B9 cells is proteinaceous (Table 5). It is suggested that the function of the protein secreted by these gland cells may be to alter the viscosity of the secretion or it may serve to lower the surface tension of the secretion.

1.3 Littorina littorea

In Littorina the foot is wedge shaped, being broadly truncated at the front and tapering to a rounded point behind. Two types of anterior pedal gland cells are present and as in Bithynia tentaculata there is no suprapedal gland. The other three types of cells present are distributed over the sole. The general distribution of cells is illustrated in Figure 8A and Plate 20.

In Littorina littorea the glandular areas over the surface of the foot can be divided into:

1.3.1 Anterior pedal gland

1.3.2 The sole

1.3.3 The dorsal and lateral epithelium.

1.3.1 Anterior pedal gland:

At the anterior part of the foot, the epidermal cells are columnar in shape with a height of about 25-40µm, and as in the foot sole the majority of these epidermal cells are ciliated. The cells of the anterior pedal gland (L1 and L2) are situated in an arc across the leading edge of the foot.

They are distinguished by location, internal structure and the nature of their contents as shown in Figure 9 and Plates 21 and 22).

Cell type L1:

This is the main cell type in an anterior pedal gland that occupies the major part of the leading edge of the foot. All the gland cells are grouped together to form a number of lobes. They are reticular in appearance with a centrally located nucleus (Figure 9).

Results with the PAS (Plate 21B) and Alcian Blue/PAS sequence suggest that some periodate-reactive material is present in the secretion of these cells which possibly could be a neutral mucopolysaccharide. Red staining with the Alcian Blue/eosin method (Table 6) would seem to indicate that protein could be present. The reactions for protein appear to be strong and the bright colouration obtained with mercuric bromophenol blue (Plate 21A) and the diazo coupling reaction (Plate 22) further confirms that the secretion of this gland is proteinaceous. The question whether any sort of mucosubstance is present in the secretion or not is difficult to answer as the colour obtained with the periodic acid paradiamine method (Plate 23A), which is used particularly for the detection of neutral mucopolysaccharides, is evanescent and rather pale. It is particularly interesting that slight β metachromasia occurs in toluidine blue (Plate 23B). This suggests that there is some molecule present in the cytoplasm stacked in

such a way as to give metachromasia. The nature of the molecule is uncertain: it could be an acid mucopolysaccharide, though no other methods for acid mucopolysaccharides gave positive results (Table 6). However, other possible explanations for these histochemical results could be that only a small amount of mucosubstances are present in the secretion, or else the protein which is the main component of the secretion is suppressing the reactions for mucopolysaccharides.

Calcium and lipid were not found using the alizarin red, von Kossa and Sudan black B methods (Table 6).

Thus most of the results indicate that the secretion of L1 is proteinaceous, containing tyrosine, and also contains some acidic and neutral mucopolysaccharides (Table 7).

Cell type L2:

These cells tend to occupy a more superficial position in the connective tissue than L1 cells, that is they lie nearer the epidermis (Figure 9 and Plate 21A). The L2 cells may extend up to 150 μm from the surface. These are smaller than type L1 with a cell body which is about 10-15 μm in size and the duct is much narrower than the cell body. Their contents are granular and the ducts filled with secretion are visible passing between the ciliated epidermal cells (Plate 21A). However, the nucleus is not visible at all in these cells. Most responses of L2 gland cells to the histochemical tests applied are the same as L1 indicating the presence of protein (mercuric bromophenol blue) in the

secretion (Table 6). However, the secretion of L2 differs from L1 in that the contents of these cells did not respond to the diazo coupling reaction (Plate 22) which indicates that tyrosine is not present; and L2 gland cells do not show metachromasia in toluidine blue (Plate 23B). Furthermore, the brown colour obtained with the periodic acid paradiamine method confirms the presence of neutral mucopolysaccharide in the secretion. As in L1 cells calcium and lipid were not evident in these cells (Table 6). Therefore, the secretion of L2 cells is mucoprotein containing neutral mucopolysaccharide (Table 7).

It seems unlikely that Littorina would possess two types of cells at the leading edge of the foot both producing the secretion necessary for lubrication. The secretion produced by L1 cells would seem to serve this function as the large size and the large nucleus in these cells suggest that they are very active. Therefore, it is suggested that the function of the secretion produced by L2 cells would be to change the viscosity of the mucus, this is further supported by the fact that neutral mucopolysaccharide is present in the secretion of the L2 cells.

1.3.2 The sole

The ventral epidermis of the sole is ciliated and composed of columnar cells with a height which ranges between 25-40μm. Three types of secretory cells are present (Plate 20) and these cells are found throughout the length and breadth of the foot sole, except at the very front edge

of the foot, which is occupied by the anterior pedal gland cell types. The largest number of these cells are found in the first third of the foot sole, which might be expected in an animal that progresses on the mucus trail and whose ciliated foot surface spreads the mucus across the whole foot. The appearance of these mucus cells is shown in Figure 10.

Cell type L3:

These are pyriform cells with a centrally located small nucleus. However, the nucleus is often not visible. They are 150-225μm in length and about 10-12μm in diameter and the cell body lies in the dermis with its duct passing between the epidermal cells. The contents of these cells may appear reticular or fibrous and can often be seen in the process of being discharged (Plate 24A). Probably therefore only a small part of the contents of these cells is visible and shows the slight positive reactions.

From the histochemical results (Table 6) it seems that the secretion of these cells consists of acidic mucopolysaccharides as indicated by the Alcian Blue pH2.5/PAS sequence and toluidine blue method (Plate 24A and B). Blue staining obtained at low pH with the Alcian Blue pH0.5/eosin method further confirms that sulphate groups are indeed present (Plate 25A). The positive reaction with alizarin red indicates the presence of calcium (Plate 24C). Black colour is obtained with the periodic acid paradiamine method, confirming that neutral mucopolysaccharide is not

present (Plate 25B). The contents of these cells also did not respond to the tests used for the detection of protein, and lipid (Table 6). Thus, the secretion of L3 cells appear to be acidic mucopolysaccharides with some calcium (Table 7).

Cell type L4:

The L4 cells tend to occupy a slightly deeper position in the connective tissue than L3 gland cells being often found deeply embedded in the dermis of the sole and lying about 200-275μm from the surface (Figure 10 and Plate 25B). These cells are generally distributed over the sole but are found in clusters, particularly at the front end of the foot (Plate 21B). They have an oval cell body of 10-12μm in size and a long duct leading to the epidermis. Their contents are reticular to slightly granular in appearance and their ducts filled with secretion are visible passing between the epidermal cells.

A yellow/green colour obtained with the Alcian Blue pH0.5/Alcian Yellow pH 2.5 method indicates that sulphate and carboxyl groups are present in the secretion (Table 6) and the staining with the Alcian Blue pH0.5/eosin method further confirms the presence of sulphate groups (Plate 25A); and these groups show strong metachromasia in toluidine blue (Plate 24B).

However, there are two unusual results; one is that the strong reaction with PAS (Plate 21B) shows that a periodate -reactive substance is present which could be mucoprotein and/or neutral mucopolysaccharide; but the black

colour obtained with the periodic acid paradiamine method indicates that no periodate-reactive substance is present and this in turn confirms that neutral mucopolysaccharide is not present in the secretion. The contents also reacted negatively to the tests used for protein (Table 6). The difficulty involved in interpreting histochemical results is well illustrated here, for they are not entirely specific and can only indicate the type of substance and groupings present, not the actual secretion as a whole. Nor can they show the ordering of linkages within the substance.

However, the positive reactions with alizarin red (Plate 24C) and the von Kossa method confirm that calcium is present in the secretion of these cells.

Cell type L5:

These cells are few in number and are often found deeply embedded in the dermis of the sole; sometimes just below the main glandular layer or among the cell types L3 and L4 (Figure 10 and Plate 26A). They are about 250-375μm in length and the cell body is ovoid or elongated with a duct that is much narrower than the cell body and tapers even more towards the epidermis. Their contents are granular (Table 7).

The histochemical results (Table 6) show that the granular contents of these cells did not respond to the tests used for acidic mucopolysaccharides, calcium and lipid and therefore the presence of these substances can be

discounted. However, the reaction with Schiff's reagent appears to be strong and the red staining obtained with the PAS method (Plate 26B) and the Alcian Blue/PAS sequence (Plate 24A) indicates that periodate-reactive substance is present, which possibly could be neutral mucopolysaccharides associated with some protein. The positive reactions and the bright colouration obtained with mercuric bromophenol blue and the diazo coupling method (Plate 26A and C) confirm that protein is indeed present; and the light brown colour obtained with the periodic acid paradiamine method may indicate the presence of some neutral mucopolysaccharide (Plate 25B). Thus the granules of L5 cells are composed of mucoprotein.

In Littorina therefore the pedal mucus consists of a complex mucoprotein from two types of cells in the anterior pedal gland which becomes mixed on the sole with sulphated and carboxylated mucopolysaccharides from cell types L3, L4 and a small quantity of protein and neutral mucopolysaccharide from cell type L5.

1.3.3 Dorsal and lateral epithelium

The dorsal and lateral epithelium of Littorina is composed of columnar cells with a height ranging between 25-30μm and interspersed between these cells are the pigment cells and two types of secretory goblet cells (Figures 8 and 9 and Plate 21B). These goblet cells are generally distributed over this area but are more frequent in the region of the operculum and are also present at the mantle edge (Figure 11) and in the hypobranchial gland.

Cell type L9:

These are epidermal goblet cells (Figure 11 and Plate 27). Their secretory material is homogeneous in appearance but sometimes it may appear reticular. They are about the same size as the epidermal cells (25-30 μm) and their nucleus is not visible.

Reactions with Toluidine blue and the Alcian Blue/PAS methods show that acidic mucopolysaccharides are present (Table 6 and Plate 27A). However, the blue colour obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique suggests that only sulphate groups are present and the presence of sulphate groups in the secretion is further confirmed by the blue colour obtained with the Alcian Blue pH0.5/eosin method (Plate 27B). Negative results obtained with the tests used for the detection of protein, neutral mucopolysaccharides, calcium and lipid indicate that these components are not present in the secretion (Table 6).

Therefore, the contents of these cells appear to be sulphated mucopolysaccharides (Table 7).

Cell type L10:

These goblet cells have a similar distribution to cell type L9. They are cuboidal cells with a height ranging between 25 to 30μm. The nucleus can occasionally be seen, lying pressed against the basal cell membrane (Figure 11). Their contents however differ from those of cell type L9 since they are granular and have shown negative responses to all the methods for acidic mucopolysaccharides (Table 6).

Protein reactions however, appear to be stronger as indicated by the mercuric bromophenol blue and diazo coupling reaction (Plate 27C) and therefore protein containing tyrosine is present. The brown colour shown with the periodic acid paradiamine method confirms the presence of neutral mucopolysaccharide in the secretion of these cells.

Thus, these results indicate that the secretion of these goblet cells is neutral mucoprotein (Table 7).

2. Opercular glands

2.1 Pomatias elegans

Six types of cell can be identified from the opercular region of Pomatias. Four of these are ubiquitous goblet cells (P9, P10, P11 and P12) which are more common on the dorsal and lateral body surfaces. There are also two subepidermal cell types which are confined to the opercular region (P7 and P8). All these cell types are arranged systematically in this region (Figure 12). In Pomatias the operculum is free from the foot epithelium for about half of its length at the posterior of the foot and in this area the opercular gland cells are distributed along the under-surface of the opercular disc. The middle of the operculum is attached to the opercular disc by a simple cuboidal epithelium. The opercular secretory cells are present at both the anterior opercular groove and the opercular cleft (Figure 12 and Plates 28 and 29).

Cell type P7:

These subepidermal cells occur only in the opercular region. They are flask-shaped and about 40-45μm in length, and their cell body is ovoid or slightly elongated along the axis perpendicular to the surface. The duct is much narrower than the cell body. The contents, which occupy the bulk of the cell body and duct, have a fibrous appearance (Figure 13 and Plate 30).

The contents of these cells have given negative responses to all the methods used for the detection of acidic mucopolysaccharides, calcium and lipids, therefore the presence of these can apparently be discounted (Table 2).

However, a periodate-positive substance appears to be present in the cytoplasm of these cells as indicated by the general PAS and the Alcian Blue pH2.5/PAS sequence (Plate 30A). Possibly these periodate-reactive groups could be represented by components such as neutral mucopolysaccharide or mucoprotein but this last can be dismissed as the tests applied for protein gave negative results (Table 2). Nevertheless, the strong positive reaction obtained with the periodic acid paradiamine method confirms that neutral mucopolysaccharides are the periodate-reactive material (Plate 30B). Therefore, it seems that the secretion of P7 cells is composed of neutral mucopolysaccharide (Table 3).

Cell type P8:

These cells are the same as type P7 in distribution and appearance but the histochemical nature of their secretion is different from that of P7 cells (Table 3). Ducts of these cells, filled with secretion, are visible passing between the epidermal cells; but the nuclei cannot be identified in these cells mainly because of the fibrous nature of their contents (Plate 30). The histochemical results indicate that a number of substances are present in the secretion of these cells. The Alcian Blue pH2.5/PAS sequence shows that acidic groups and periodate-reactive substances are present as indicated by the purple colour (Plate 30A). The yellow/green staining obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique indicates that acidic groups are composed of both sulphate and carboxyl groups. The presence of the sulphate group is again evidenced by the blue staining at low pH when the Alcian Blue pH0.5/eosin technique is applied. These sulphate and carboxyl groups display metachromasia in toluidine blue (Table 2).

The secretions of the cell types P7 and P8 are similar as far as the periodate-reactive substance is concerned as in both cases the periodate reactive group is represented by neutral mucopolysaccharide (Plate 30B). However, the periodic acid paradiamine and the Periodic Acid Schiff's techniques reveal a stronger colouration for type P7 cells than for type P8 cells. It seems that more neutral mucosubstance is present in P7 cells assuming that stronger

colouration can be interpreted to mean a quantitative variation (Table 2).

No protein, calcium or lipid was found in the secretion. Thus, the contents of P8 type cells are acidic and neutral mucopolysaccharides (Table 3).

2.2 Bithynia tentaculata

In Bithynia the opercular glands are present on the under-surface of the opercular disc as well as in the opercular groove (Figure 14 and Plate 31). In these regions three types of epidermal granular secretory cell are present in addition to those of the general epithelium.

Cell type B4:

These epidermal gland cells are prominent and contain large globules which appear spherical and are about 2 to 6μm in diameter. They are the dominant cell type in the cleft but can also be found sparingly on the dorsal epidermis of the foot. These cells are ovoid or somewhat elongated in shape and are about 25-40μm in height, which is approximately the same size as the epidermis of the subopercular cleft (Plates 31B and 32B).

From the histochemical results it seems that these cells contain mucopolysaccharides as well as protein (Table 4 and Plates 33 and 34). However, the slight responses of their contents to the Alcian Blue pH0.5/Alcian Yellow pH2.5 and Alcian Blue pH0.5/eosin (Plate 33A) techniques suggest that only weakly acidic mucopolysaccharides are present in the secretion. Nevertheless, the strong reaction with PAS

(Plate 32B) indicates the presence of periodate-reactive groups which possibly could belong to neutral mucopolysaccharides or mucoproteins. The bright colouration shown with the mercuric bromophenol blue and diazo coupling reaction confirms that protein is indeed present (Plate 34) and the result obtained with the periodic acid paradiamine method shows that neutral mucopolysaccharide is also present (Plate 31B).

From these results, the secretion of B4 cells appears to be a mucoprotein containing weakly acidic and neutral mucopolysaccharides (Table 5).

Cell type B5

These long, narrow cells have densely packed fine granular contents with the granules less than 1μm in diameter. These cells are few in number compared to cell type B4. The granular secretion of these cells did not show a positive reaction for the techniques used for acidic mucopolysaccharides, calcium and lipid so these can be dismissed from consideration (Table 4). However, the Periodic Acid Schiff's method and the Alcian Blue/PAS sequence suggest that periodate-positive substances are present in the secretion (Plate 32B) and the bright brown colour obtained with the periodic acid paradiamine method (Plate 31B) confirms that neutral mucopolysaccharides are displaying the periodate-reactive groups. The strong positive staining in mercuric bromophenol blue and by the diazo coupling reaction (Plate 34) indicate that protein is present.

Thus, the granules of B5 cells are composed of a neutral mucoprotein (Table 5).

Cell type B6

These cells are very few in number and are found amongst the other cell types in the subopercular cleft. Their granular contents are highly refractile with a faint yellow colour, but did not stain with any of the procedures used (Table 4 and Figure 14B). The presence of mucopolysaccharides, protein, calcium and lipid can apparently be discounted and their contents are therefore unknown.

2.3 Littorina littorea

In Littorina the operculum grows asymmetrically from its anterior edge where it is embedded in the foot. In fixed animals the foot is retracted and the operculum folds at its anterior edge distorting its insertion and giving the impression of a subopercular cleft. Three types of subepidermal cells are present lining the anterior opercular groove and the posterior subopercular cleft of Littorina. The frequency of such cells is always greater in the anterior opercular groove (Figure 15 and Plate 35).

Cell type L6:

These cells have an oval cell body which is about 25-30 μm in length and 8-10μm in width. Their reticular contents are frequently accumulated in one corner. Consequently they often have a rather empty appearance (Plates 36 and 37). It can be seen that the nucleus is not visible and it was

impossible to trace the ducts of these cells to the epidermis. This could be because the sections were not cut through the right plane. The contents of these cells reacted negatively to all the tests tried for the detection of protein, calcium and lipid, so these can be dismissed from consideration. Furthermore, the reactions with PAS and the periodic acid paradiamine method indicate that no periodate-reactive substance is present and in turn this confirms that neutral mucopolysaccharide is not present in the secretion (Table 6). However, the blue colour obtained with Alcian Blue pH2.5/PAS and Alcian Blue pH0.5/eosin shows that acidic mucopolysaccharides are present (Plates 36 and 37), and these groups are responsible for the β metachromasia observed in toluidine blue.

Cell type L7:

These cells have a cell body which is about 25-30μm in length and 10-12μm in width containing large goblules which appear spherical and are about 1μm in diameter (Figure 15 and Plate 38). Again it was impossible to trace the ducts of these cells but occasionally the ducts filled with the secretory product of these cells are visible passing between the epidermal cells (Plate 39A). Histochemical results from Table 6 indicate that acidic mucopolysaccharide, calcium and lipid are not present in the secretion as none of the tests used for these components gave positive results. However, the presence of protein with some neutral mucopolysaccharide is indicated by the positive reactions obtained with

mercuric bromophenol blue (Plate 38A), diazo coupling (Plate 38B), and periodic acidparadiamine method (Plate 39B). Thus the globules of these gland cells appear to contain neutral mucoprotein (Table 7).

Cell type L8:

These cells are scarce. Their contents are homogeneous in appearance (Plate 38A), and most histochemical results obtained for these cells are similar to those for type L7 cells. The difference between these two cell types is mainly that the contents of type L8 cells reacted only slightly to PAS stain (Plate 39A) and the result with the periodic acid paradiamine method indicates that neutral mucopolysaccharide is not present (Table 6). It is suggested that these cells could be a precursor of cell type L7 since they are histochemically almost similar.

3. Mantle

The skin of the snail is very glandular and secretes a copious supply of mucus. The secretory gland cells responsible are present either in or below the epithelium of most external surfaces of Mollusca.

However, the mantle is an important area of growth and secretion on the surface of the prosobranch body and this secretory activity broadly constitutes that responsible for shell formation. In view of this and other specialized functions it is to be expected that the mantle will display specific histological features which differ considerably from those of the epidermis of other parts of the body.

The epithelium of the mantle region is peculiar in that a dorsal and ventral epithelium can be discerned. The dorsal epithelium lies underneath and adjacent to the shell while the ventral epithelium lies opposite the head and is exposed to the external environment. The mantle edge separates these two epithelia.

Therefore, the mantle can be divided into:-

1. The dorsal mantle epithelium.
2. The mantle edge.
3. The ventral mantle epithelium.

This section will include a description of the structure of the free margin of the mantle in Pomatias elegans, Bithynia tentaculata and Littorina littorea with special reference to the glands which occur at this site.

For this purpose a detailed description of the gland cells and the histochemical nature of their contents is given.

3.1 Pomatias elegans

3.1.1 The dorsal mantle epithelium:

The dorsal epithelium is made up of a single layer of small, flattened, cuboidal epidermal cells. These cells are about 8-10µm in height. In Pomatias the dorsal epithelium is devoid of any type of secretory gland cells (Figure 16 and Plate 40).

3.1.2 The mantle edge:

At the mantle edge the epidermis changes from being a simple cuboidal to a tall columnar epithelium of about 25µm in height. As in most gastropod molluscs, Pomatias also has two types of glands opening at the dorsal aspect of the mantle edge (Figure 16 and Plate 40A and B). However, these gland clusters are very small and are not present throughout the width of the mantle edge.

Mantle edge gland type A (P14):

This gland is composed of a cluster of cells with an oval cell body about 10-12µm in size and a centrally placed nucleus. They have an extremely long, thin duct leading to the epidermis, such that the cells of this gland can extend up to 90µm from the surface (Figure 16). Their contents are reticular in appearance and stained bluish-purple with the Alcian Blue pH2.5/PAS (Plate 40A) and purple with toluidine blue which indicate the presence of acid mucosubstance in the secretion. The yellow-green staining obtained with

Alcian Blue pH0.5/Alcian Yellow pH2.5 technique shows that the acid mucosubstances are composed of both sulphated and carboxylated groups. The presence of sulphate groups in the secretion is further confirmed by the blue staining at low pH in Alcian Blue pH0.5/eosin technique (Table 8). However, only slight reactions are obtained with the PAS and the periodic acid paradiamine method (Plate 40B). Neither calcium nor lipid were found. Therefore, most of these results indicate that the secretion of this gland consists of acidic mucopolysaccharides probably associated with some neutral groups.

Mantle edge gland type B (P15):

This gland is very slender and composed of only a few gland cells. Therefore in any 7µm section of mantle edge only 3-4 cells are visible. These gland cells have a distinct oval cell body about 8-10µm in size and a long neck which opens in between the columnar epidermal cells. A nucleus is not visible in these cells, because their contents stain very strongly.

The contents of these cells reacted negatively to all the tests for the acidic mucopolysaccharides, calcium and lipid, therefore the presence of these can apparently be discounted. However, periodic-positive substances appear to be present in the secretion as indicated by the red staining obtained with the PAS and the Alcian Blue/PAS techniques (Plate 40A). This substance could possibly be in the form of neutral mucopolysaccharide or mucoprotein. The positive

reactions with the mercuric bromophenol blue and diazo coupling for protein (Table 8) and the bright brown colour obtained with the periodic acid paradiamine method (Plate 40B) confirm that protein and neutral mucopolysaccharides are present. Thus, the secretion of the mantle edge gland (P15) is a neutral mucoprotein (Table 9).

3.1.3 The ventral mantle epithelium:

In Pomatias, on the ventral surface the epidermis is made up of tall, columnar cells which are about 25-30μm in height. An oval nucleus lies in the basal half of the cell. Interspersed between these cells are four types of ubiquitous goblet cells which show the same staining properties as similar cells in the other regions of the body (Figure 16 and Plate 40C and D).

3.2 Bithynia tentaculata

3.2.1 Dorsal mantle epithelium:

The dorsal mantle epithelium in Bithynia is a typically low epithelium being made up of small cuboidal cells of 8-10 μm height. The epidermal pigment cells lie a little further forward from the edge and contain a basally placed nucleus and yellowish-brown pigment granules located distally in the supranuclear area. However, as in Pomatias, the entire dorsal epidermis is devoid of ciliated cells and any type of secretory cells (Figure 17 and Plate 41A).

3.2.2 The mantle edge:

At the mantle edge the epidermis changes its form from being cuboidal to become composed of tall columnar cells

about 25μm in height. In Bithynia the dorsal mantle gland cluster opens at the dorsal side of the mantle edge.

Mantle edge gland (B10)

The cells of the mantle edge gland are bunched together such that their necks form bundles that open to the outside between the columnar epidermal cells (Plate 41 A and B). These gland cells have an oval or somewhat elongated cell body about 5-7μm in size, and within this lies the large, spherical centrally placed nucleus, which shows patches of chromatin and a distinct nucleolus. These gland cells may extend up to 100μm from the surface and their contents have a reticulate appearance (Figure 17 and Plate 41C).

The cytoplasm of these gland cells stained red with the Alcian Blue/eosin technique which probably suggests that the contents are proteinaceous. The strong reactions for protein and the bright colouration obtained with mercuric bromophenol blue and the diazo coupling method confirms that protein including tyrosine is indeed present (Plate 41A and B). Whether neutral mucopolysaccharide is present in the secretion or not is difficult to determine as the slight reaction obtained with PAS suggests the possibility of its presence in the secretion but with the periodic acid paradiamine method, which is used for the detection of neutral mucopolysaccharides, the colour is evanescent and rather pale (Table 10). Again the possible explanation for these results could be that protein being the main component of the secretion, is suppressing the reaction for neutral

mucopolysaccharides.

The contents of these gland cells did not respond to the tests used for acidic mucopolysaccharides, calcium and lipid, therefore the presence of these can apparently be discounted.

Thus, most of the histochemical results indicate that the secretion of this gland is proteinaceous, containing tyrosine and possibly some neutral mucopolysaccharide (Table 11).

3.2.3 Ventral mantle epithelium:

The ventral epidermis is composed of columnar epidermal cells with a height ranging between 18 to 20 µm. Amongst these epidermal cells are two types of ubiquitous goblet cells (B7 and B8). Their responses to all the histochemical methods used are the same as in other areas, although the intensity of staining differs slightly (Table 10). The goblet cells with the neutral mucoprotein type secretion (type B8) are more common at the mantle edge (Plate 41C and D).

3.3 Littorina littorea

3.3.1 The dorsal mantle epithelium:

The dorsal mantle epithelium in Littorina differs from Pomatias and Bithynia, being made up of columnar epidermal cells with a height of about 20-25µm. These cells contain a basally placed nucleus, but the pigment granules which are found in Bithynia are not present here. In Littorina, in addition to these simple epidermal cells, some secretory

subepidermal cells (L11) are also present at the dorsal surface of the mantle (Figure 18 and Plate 42C).

Cell type L11:

These subepidermal cells are generally distributed on the dorsal surface of the mantle but occur more frequently further away from the mantle edge. They are flask-shaped gland cells with a distinct cell body and an extremely long, thin duct leading to the epidermis (Figure 19 and Plate 43B). These cells are about 60-70μm in length and the ovoid cell body is 5-8μm in width. They have reticular contents which sometimes may appear granular.

The results from the histochemical tests applied show that these cells mainly contain acidic mucopolysaccharide, particularly when considering the toluidine blue method. The reaction with Alcian Blue at low pH in Alcian Blue pH0.5/eosin and Alcian Blue pH0.5/Alcian Yellow pH2.5 methods show that the acidic mucopolysaccharides are composed of only sulphated groups (Table 12 and Plate 43B).

The purple staining obtained with the Alcian Blue/PAS sequence suggests that in addition to alcinophilic substance some periodate-reactive groups are also present which possibly could be neutral mucopolysaccharide or mucoprotein. However, the latter can be dismissed as the contents of the cells did not respond to the tests used for protein (Table 12) and the brown colour obtained with periodic acid paradiamine method confirms that neutral mucopolysaccharides are responsible for the periodate reaction (Plate 43C).

Calcium and lipid were not found. Therefore, the secretion of these cells appears to consist of sulphated and neutral mucopolysaccharides (Table 13).

3.3.2 Mantle edge:

In Littorina two types of mantle edge glands are present at the dorsal side of the mantle edge and these glands are distinguished by their internal structures and staining properties.

Mantle edge gland type A (L12)

At the mantle edge in between the dorsal and ventral epithelium, most of the portion (about 210 x 315μm) is occupied by this large, bulky gland. The gland is composed of clusters of cells which lie embedded in the connective tissue. The cells are elongated, glandular and flask shaped with the long cell processes extending from the cell body and within this cell body is a large ovoid and often central nucleus with a nucleous. All the cell processes group together and open at the dorsal aspect of the mantle edge. These cells are arranged in bundles in which the lower half of the individual cell bulged (Figure 18 and Plate 42).

The gland cells have reticulate contents and their contents stained red with PAS and Alcian Blue/PAS (Plate 43A) which suggests that periodate-positive material is present. The strong reactions obtained with mercuric bromophenol blue and the diazo coupling method confirmed that protein is present in the secretion (Plate 42A and B). A slight positive reaction is shown with periodic acid

paradiamine method (Table 12) which may suggest that some neutral mucopolysaccharide is present. The contents did not respond to the tests for acidic mucopolysaccharides, calcium and lipid and therefore these can be dismissed from consideration (Table 12). Thus, the secretion of this gland is probably a mucoprotein containing some neutral mucopolysaccharide (Table 13).

Mantle edge gland type B (L13)

This is a slender gland occupying a small portion about 50 µm x 20 µm at the extreme edge of the mantle. The gland consists of an elongated array of gland cells with ovoid or slightly elongated cell body and the ducts which open in between the epidermal cells (Figure 18 and Plate 42C). A nucleus can sometimes be visible but it was not possible to identify a nucleolus. Negative reactions with PAS and the black colour obtained with the periodic acid paradiamine method indicate that periodate-reactive material is not present, which in turn confirms that neutral mucopolysaccharide is also not present in the secretion (Table 12). The presence of an acid mucosubstance either containing a sulphate group and/or hyaluronic acid is indicated by the bright blue staining obtained with the Alcian Blue pH2.5/PAS technique (Plate 43A). The presence of sulphate groups in the secretion is further confirmed by the reaction with Alcian Blue pH0.5/eosin (Plate 42C), and Alcian Blue pH0.5/Alcian Yellow pH2.5 techniques, and these sulphate groups are stacked in such a way as to give β

metachromasia in toluidine blue (Table 12). However, there is no evidence of protein, calcium or lipid in the secretion. Therefore, these results indicate that the secretion of this gland consists of sulphated mucopolysaccharides (Table 13).

3.3.3 Ventral mantle epithelium:

In Littorina, the ventral mantle epithelium is made up of tall columnar epidermal cells with a height of about 25-30μm and these cells have a basal, oval nucleus. Interspersed amongst them are ubiquitous goblet cell types L9 and L10 (Figure 18 and Plate 42B and C). All the responses of these goblet cells to the histochemical procedures used are the same as in other areas, however the goblet cells with an acidic mucopolysaccharide type of secretion are more common at the mantle edge (Plate 43A).

4. Hypobranchial gland

Mucus production is a characteristic secretory activity of the molluscan epidermis. Activity of this type reaches a high level in the case of the hypobranchial gland of prosobranch molluscs, with the production of a glairy and complex secretion from a modified epithelium on the inner surface of the mantle wall. This gland is present in almost all the prosobranch gastropods; but its morphology and cytology may vary (Fischer et al. 1968) as do the secretory products. This section describes the structure of the secretory epithelium and a histochemical study of the different cell types present in the hypobranchial gland of Pomatias elegans, Bithynia tentaculata and Littorina littorea.

4.1 Pomatias elegans

In Pomatias elegans although the branchial, central and rectal areas can be distinguished the hypobranchial gland does not take up the whole extent of the mantle but only a part of the central zone and a very small part of the rectal area (Figure 20 and Plate 44). The gland was not observed in the branchial area. The hypobranchial gland in the rectal area is short and thick and its glandular epithelium consists of a few tall cylindrical supporting cells and large secretory cells which include the ubiquitous goblet cell types P9, P10 and P12 (Plates 44C and 45A). These goblet cells have been described already. In both areas the

same cell types are present together with the secretory cells with the large globular content (Type P12), which are more numerous (Figure 20B and C and Plates 44 and 45). All the histochemical responses of these goblet cell types are the same as in the other regions of the body (Tables 8 and 9). However, the intensity of staining may differ slightly.

In the hypobranchial gland of Pomatias elegans no specialized types of secretory cells are present in addition to the goblet cells described.

4.2 Bithynia tentaculata

In Bithynia tentaculata the hypobranchial gland is not as wide as the mantle itself and it is shifted towards the branchial region. The rectal and branchial areas have a simple columnar epithelium and the only secretory cells in these two lateral areas are the ubiquitous goblet cell types B7 and B8 (Figure 21 and Plate 46A). In the central region, however, the hypobranchial gland is formed from the general inner mantle epithelium, which forms a modified epidermis made up of a single layer of very tall secretory cells. In this glandular area four types of secretory cells are present (Figure 21B) amongst which are interspersed a few supporting cells and conical ciliary cells, which taper rapidly away from the epithelial surface. In Bithynia, the gland is mainly dominated by the two types of secretory cells (B11 and B12) which are specialized for this area and the goblet cell types B7 and B8 occur only sparsely (Figure 21 and Plate 47B and C). These two hypobranchial gland cell

types are distinguished by their internal structures and staining properties.

Hypobranchial gland cell type A (B11)

These gland cells are the most common feature of the hypobranchial gland. They are about 25-35 µm in length and 8-10 µm in width. The secretion-filled portion of these cells usually occupies a peripheral region with the remainder of the cell body tapering down to the basement membrane where the large oval nucleus with its nucleolus lies (Figure 21B and Plate 46B). The contents of these gland cells change their appearance from being fibrous to granular in their responses to the different histochemical methods used.

Results from Table 10 indicate that the contents of these gland cells respond with only slight reactions to the techniques applied for acidic mucopolysaccharides. They stained blue with Alcian Blue pH0.5/eosin and yellow/green with Alcian Blue pH0.5/Alcian Yellow pH2.5 methods (Plate 47C and D) indicating that sulphate and carboxyl groups are present. However, it is uncertain if the weakness of these reactions indicate the weak nature of these components or the reactions for these end groups are suppressed by the other substances present in the secretion (Table 10). Results from PAS and the Alcian Blue pH2.5/PAS sequence indicate the presence of peridoate-reactive material in the secretion (Plate 47A). The strong positive reactions obtained with mercuric bromophenol blue and the diazo

coupling method (Plate 46 B and C) and the bright brown colour shown with the periodic acid paradiamine technique (Plate 47B) confirm that protein and neutral mucopolysaccharides are present. Neither calcium nor lipid were evident in the secretion (Table 10). Thus most of these results seem to indicate that the secretion of these gland cells is mucoprotein containing neutral mucopolysaccharide associated with some sulphated and carboxylated groups (Table 11).

Hypobranchial gland cell type B (B12)

Gland cells of this type are few in number, compared to type A, but these cells are very distinct and can be differentiated from other gland cells because of their large globular contents (Figure 21B and Plate 46C). These globules are spherical and are about 2 to 6µm in size. These cells are about 25-35µm in length and 10-12µm in width. The contents of these gland cells did not respond to the stains used for acidic mucopolysaccharides but stained bright blue with the mercuric bromophenol blue method and purple with the diazo coupling reaction (Plate 46 B and C) which confirms that protein containing tyrosine is present in the secretion of these cells. A slight reaction was obtained with the periodic acid paradiamine method which indicates that neutral mucopolysaccharide is also present (Plate 47B). However, negative reactions were obtained for the tests used for the detection of calcium and lipid (Table 10). Therefore, the globules of these gland cells appear to contain protein and neutral mucopolysaccharide (Table 11).

4.3 Littorina littorea

In Littorina, the ventral surface of the mantle that forms the roof of the mantle cavity has a highly glandular area forming the hypobranchial gland. The gland itself is formed from a much folded region of the inner mantle epithelium (Figure 22A and Plate 48A). The secretory epidermis of the gland consists of what is essentially a single layer of very tall cells about 35-40μm (Figure 22B and Plate 48B). Three specialized types of secretory cells are apparent in the hypobranchial gland and sandwiched between these secretory cells are the ciliated cells which are a conspicuous feature of the secretory surface of the gland. These ciliated cells are shaped like inverted cones tapering towards the basement membrane (Plate 48B) and have spindle shaped nuclei located in a region approximately one third of the distance from the epithelial surface to the basement membrane (Figure 22B). Three secretory gland cell types found in the hypobranchial gland are:-

Hypobranchial gland cell type 'A' (L14)

These are large gland cells with a height of 35-40μm and the width about 8-10μm. The contents of these cells appear frothy and granular and occasionally the nuclei can be seen located in the basal half of the gland cell.

The material in these cells stained strongly with toluidine blue (Plate 48C) indicating the presence of acid mucosubstances in the secretion and the blue colour obtained with Alcian Blue pH0.5/eosin (Plate 48B) further confirms

that sulphate groups are indeed present. Negative reactions obtained with mercuric bromophenol blue and the diazo coupling technique and the black colour shown with the periodic acid paradiamine method (Plate 49C) confirmed that neither protein nor neutral mucopolysaccharides are present. Calcium and lipid were not found (Table 12). Therefore, the contents of these cells appear to be acidic mucopolysaccharides.

Hypobranchial gland cell type B (L15)

These cells are less bulbous than cell type L14 and the contents appear more vacuolated in the form of either membranes or bubbles stretching from wall to wall of the cell and presenting a reticulate appearance (Plates 48 and 49 B and C). Both types are of approximately the same length and width. The nuclei of these cells are usually located in the basal region of the cell. The responses of these cells to the histochemical procedures used are the same as those of cell type L14 (Table 12), indicating the presence of acidic mucopolysaccharides in the secretion. However, the contents of these cells always stain less intensely than those of cell type L14 (Plates 48 and 49B and C), and this may be because of the vacuolated nature of the contents.

Occasionally the contents of these cells can be seen being discharged at the gland surface apparently by bursting the limiting membrane of the cells. However, it seems

unlikely that Littorina would possess two different types of cells which produce an identical secretion for lubrication and cleansing the mantle cavity. It is suggested therefore, that the cell types L14 and L15 represent the same cell type in slightly different stages of secretion. A similar suggestion has also been made by Ronkin (1952) in describing the hypobranchial gland of Busycon canaliculatum and by Hunt (1967) for the hypobranchial gland cell types of Buccinum undatum.

Hypobranchial gland cell type C (L16)

Relative to cell types L14 and L15 these cells are few in number, much narrower and are about 4 to 8µm in width although they all are of the same length (Table 13 and Plate 49B and C).

Some of these cells have a classic goblet cell-like appearance (Plate 50A) while others extend somewhat more deeply into the epithelium and have a club shape with the expanded end at the epithelial surface, and like the ciliated cells these cells taper sharply down into the epithelium (Figure 22B and Plate 49B). It is difficult therefore to trace the course of any single cell of this type through the epithelium from the secretory surface to the basement membrane. It seems likely that these variations in morphology result from constraints placed on the cells by the crowding in of adjacent cell types L14 and L15.

The contents of these gland cells are granular and the histochemical results shown in Table 12 indicate that a

detectable amount of protein is present in their cytoplasm. Their contents reacted negatively to all the stains for acidic mucopolysaccharides, calcium and lipid so the presence of these can apparently be discounted. However, the purple colour obtained with the diazo coupling reaction (Plate 50B) and the blue with mercuric bromophenol blue (Plate 50C) suggest that protein containing tyrosine is present and the positive reaction with the periodic acid paradiamine method (Plate 49C) shows that neutral mucopolysaccharide is also present. Thus, the granules of these cells are composed of a mucoprotein containing neutral mucopolysaccharide.

Lastly, it is suggested that these 3 cell types in the hypobranchial gland of Littorina are nothing but the ubiquitous goblet cell types found in other areas as their responses to most histochemical procedures used are the same and the only variations are those of size, which is expected in view of the change in size of the epidermis of the hypobranchial gland.

Pulmonate

Limax pseudoflavus

The gross anatomy and histochemistry of the mucus producing glands of the pulmonate slug Limax pseudoflavus Evans were investigated. This animal can be considered to have four different areas as far as its mucus is concerned, that is, the dorsal and lateral body surfaces down to the peripodal groove, the peripodal groove itself, the sole and the leading edge of the foot. There are also three specialized glandular areas - the pneumostome, the suprapedal gland and Semper's organ in the face. These areas are all indicated in Figure 23.

Fourteen different secretory cell types can be distinguished using the histochemical tests described (Table 1). These cells vary in content, position, shape and size. Table 14 gives their staining reactions and Table 15 their position, size, shape and content.

1. Dorsal body surface

In Limax the general dorsal body surface is thrown into short folds which in section appear as short humps. The mantle of the animal appears to be lightly wrinkled but this pattern is not noticeable in sections. An active, hydrated animal is swollen and mucus tends to accumulate between the body folds giving the whole animal a glossy appearance. A dehydrated inactive animal is contracted and has little mucus between these folds and appears velvety.

The mantle shield and general dorsal body surface contain five main cell types (Figure 23B). The largest of these is the type 1 cell which is widespread over the exposed surface of the animal (Figure 24 and Plate 51A).

Gland cell type 1:

It is a goblet-shaped cell with a basal nucleus that is small and flattened against the cell wall (Plate 52A) but the nucleus is often not visible at all in these cells. The large cell body is embedded in the connective tissue and the musculature of the dorsal and lateral body surfaces, and the secretion of the cell is discharged to the exterior through a single, straight duct. Ducts filled with the secretion are visibe passing between the epidermal cells. The cell contents which occupy the bulk of the cell body and duct, are fibrous in appearance (Plate 51B).

Results from the histochemical tests applied (Table 14) show that acidic mucopolysaccharides are present in these gland cells, particularly when considering that Alcian Blue produces a strong reaction at pH2.5 when presumably both sulphate and carboxyl groups are staining (Plate 53A). The blue staining obtained with the Alcian Blue pH0.5/eosin technique confirms that sulphate groups are present (Plate 53B). The presence of sulphate and carboxyl groups in the secretion is further confirmed by green staining obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique (Table 14) and these end groups display strong β metachromasia in toluidine Blue (Plate 53C). There is no evidence of

neutral mucopolysaccharide or lipid or calcium in the secretion and none of the tests used for protein was positive (Table 14). Therefore, the secretion of cell type 1 is sulphated and carboxylated mucopolysaccharides.

Cell type 7 can easily be mistaken for the ducts of type 1 cells (Plate 51B). The staining reactions of type 7 are very similar (Table 14) but these cells occur in much greater number and also in areas devoid of type 1 cells, that is the sole and the folds near the peripodal groove, and these cell types also differ in the texture of their cell contents (Table 15).

Cell type 10 can also rarely be seen in the dorsal surface, however these two cell types will be considered with the sole glands. In addition to these, three other secretory cell types are also widespread on the dorsal surface (Figure 24).

Cell type 2:

These cells are widespread but only rarely found in the epidermis of the head. The cells are strap-like, with contents that are smooth in texture but which tend to shrink and split horizontally during preparation (Figure 25 and Plate 52B). They are about 200-600μm in length and 30-60μm in width. Gland cells type 2 tend to occupy a slightly more superficial position in connective tissue than cell type 3, that is they lie nearer the epidermis.

Cell type 3:

These cells are about 150-650μm in length and 35-80μm

in width and are deeply embedded in the connective tissue. They have a spatulate shape and the contents are finely granular (Figure 25 and Plate 51A). The responses of these two gland cell types (2 and 3) to most histochemical procedures used are the same and their contents reacted negatively to all the tests for carbohydrates and calcium (Table 14). However, the red staining obtained with Alcian Blue pH0.5/eosin (Plate 53B) suggested that protein could be causing this staining and the bright blue colour obtained with mercuric bromophenol blue (Plate 52B) and the purple colour obtained with the diazo coupling reaction (Table 14) confirm that protein containing tyrosine is present in the secretion.

The results of the staining of cell types 2 and 3 with the Sudan Black B method for lipids is confusing. These cells stained with Sudan black B after Elftman's fixative (Plate 54D) but this reaction persists after chloroform/methanol extraction and after overnight treatment with a 5% solution of Lipase at 37°C. These cells do not stain at all with Sudan black in frozen sections though their staining with mercuric bromophenol blue persists.

From these results it seems that the secretion of cell types 2 and 3 is proteinaceous.

Cell type 4:

These cells are visible after fixation with Zenker's or Elftman's fixative but not after formaldehyde. They are elongated with a granular cytoplasm, which displays a yellow

colour whether stained or not, so this would appear to be the colour of the cell cytoplasm after fixation. These cells generally have no obvious cell neck distinct from the cell body, the cell simply tapers slightly towards the epidermis (Figure 25 and Plate 54A and B). They are widespread on the dorsal surface and are found lying amongst the other cell types. They also occur very infrequently in the sole. The contents of these cells are negative to all the stains tried (Table 14 and Plate 54). So the presence of mucopolysaccharides, protein, calcium and lipid can apparently be discounted.

The dorsal mucus of L. pseudoflavus is yellow and the pigment concerned is soluble in water and absolute alcohol. Therefore, it is suggested that the yellow granular cell may be the source of the yellow colouration.

In L. pseudoflavus, the general body mucus therefore consists mainly of acidic (both sulphated and carboxylated) mucopolysaccharides with the addition of protein and the products of the granular yellow cells.

2. The peripodal groove

This groove may be viewed as being continuous with the duct of the suprapedal gland which flattens out at the leading edge of the foot (Plate 55A). Some of the cell types found in this groove have affinities with both those on the leading edge of the foot and with elsewhere in the sole. These gland cells are often deeply embedded in the dermis of the sole but tend to adopt a more superficial

position in the region of the peripodal groove and they are often found in clusters, particularly in this last region (Plate 56A).

Only cell type 8 is found exclusively in the groove. In any one 7μm section there are only approximately a dozen cells of this type but they occur regularly down the length of the groove (Figure 26A and Plate 56). These gland cells have an oval cell body and a long duct which is much narrower than the cell body and within the latter is a large, ovoid and often central nucleus, although it was not possible to identify a nucleolus (Figure 26B). Their contents are finely granular and stained blue at low pH in Alcian Blue pH0.5/eosin technique (Plate 56B) which indicates the presence of sulphate groups; and the yellow colour obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 sequence suggests the presence of carboxyl groups in the secretion. However, the result with the Alcian Blue/Alcian Yellow technique is unusual as if both the sulphate and carboxyl groups are present as is suspected, then it would be expected that the staining would show a combination of blue and yellow colour and this did not happen, but whichever end groups are present they are stacked in such a way as to give β metachromasia in toluidine blue (Table 14).

In addition, there is some periodate-reactive substance present which may be a neutral mucosubstance as evidenced by the brown staining obtained with the periodic acid

paradiamine method (Table 14). Neither protein or calcium nor lipid was found in these cells. Thus, the contents of these cells are acidic and neutral mucopolysaccharides and the mucus in the peripodal groove therefore contains a diversity of mucus.

3. The sole

In Limax the central band of the tripartite foot is ciliated and four types of mucus-secreting cells are present in the sole (Figure 27). Cell types 9 and 10 occur in the sole and the peripodal groove. Cell type 9 forms a subepidermal layer in which occasional type 10 cells occur (Plate 57).

Cell type 9:

It has a somewhat round cell body but it may also show rather distorted shapes and a long duct. The bulk of the cell lies in the dermis with the duct passing between the epidermal cells (Plate 57A). These cells are found throughout the length and breadth of the foot sole, and the largest numbers are found in the first third of the body. Occasionally the small nucleus located in the basal half of the cell body can be seen (Plate 57A). The contents of these cells may appear reticulate or granular (Table 15). The histochemical results indicate that a mixture of substances is present in these cells (Table 14).

The bluish/pink staining obtained with the combined Alcian Blue/PAS sequence indicates that alcinophilic and periodate-positive substances are present. The cytoplasm of

these cells displays β metachromasia in toluidine blue. The yellow staining obtained with Alcian Blue pH0.5/Alcian Yellow pH2.5 technique indicates the presence of carboxyl groups and the light blue colour obtained with Alcian Blue pH0.5/eosin suggests that some sulphate groups may be present. The reaction with PAS and the brown staining obtained with periodic acid paradiamine method (Plate 57B) indicates that neutral mucopolysaccharide is also present. However, protein, calcium and lipids were not evident in these cells (Table 14). Thus, the secretion of these cells appears to be mainly carboxylated and neutral mucopolysaccharides.

Cell type 10:

They have a similar distribution to type 9 cells but large numbers are also present in the region of the head (Plate 58). These cells occur deeply embedded and are composed of a distinct cell body and an extremely long and thin duct leading to the epidermis. They have a quite large nucleus which tends to be central and the cytoplasmic contents are granular. These granules are large and generally appear spherical (Figure 28 and Plate 58B).

The granular contents of these cells display metachromasia in toluidine blue though no other methods for acidic mucopolysaccharides gave positive results (Table 14). The strong reaction with Schiff's reagent in the PAS and Alcian Blue pH2.5/PAS techniques (Plate 58) suggests that periodate-positive material is present which possibly could be neutral mucopolysaccharide or mucoprotein. However, this

last possibility can be dismissed as none of the tests used for protein gave positive results but the bright brown colour shown with the periodic acid paradiamine method indicates the presence of a neutral mucopolysaccharide in the secretion (Plate 57B).

The PAS and periodic acid paradiamine methods produce stronger colouration in these cells than in cell type 9, which probably would suggest that more neutral mucosubstance is present in these cells assuming that stronger colouration can be interpreted to mean a quantitative increase. Neither calcium nor lipid was found. Thus, most of the histochemical results seem to indicate that the granules of these cells are composed of neutral mucopolysaccharide (Table 15).

In addition to cell types 9 and 10 the most superficial and most common of the mucus-producing glands of the sole region are the type 7 cells (Plate 57A).

Cell type 7:

These cells tend to be elongated and narrow, the neck not being obviously distinct from the cell body (Plate 51B). They are about 20-150μm in length and 3-7μm in width. These gland cells are widely distributed on the dorsal and lateral body surfaces (Plate 51) and the sole of Limax (Plate 57) but the largest numbers are found just behind the head (Plate 58A) and gradually these drop in number, with some fluctuations, towards the tail. The nucleus of these cells is small, occupying a basal position (Figure 24), and the

contents of the cells generally have a smooth texture.

Most of the staining responses of these cells are the same as for cell type 1 and indicate the presence of sulphated and carboxylated groups, and these groups display strong β metachromasia in toluidine blue (Table 14 and Plate 53C).

Periodate-reactive substance, protein, calcium and lipid were not evident in the secretion. Thus, these cells contain the sulphated and carboxylated mucopolysaccharides.

This shows that the sole therefore contributes to the pedal mucus a range of mucopolysaccharides from highly sulphated to neutral. However, the major contributor to the mucus found under the sole is the suprapedal gland.

4. The suprapedal gland

This is the largest mucus-producing gland in the body and the only one with a discrete duct. The suprapedal gland is embedded in the inner tissues of the foot, between the viscera and the sole. In fact, it lies in the foot musculature from just behind the head to about three quarters of the way down the body. There are very few muscle strands in the gland itself and the duct is separated from the body cavity by a thin dorsal membrane. The duct is bounded by an epidermis which displays three cell types (Figure 29 and Plate 59A). The upper epidermis of the duct is composed of flattened cells. The duct has a central groove on its ventral floor and here the epidermis is made up of large columnar cells which occasionally appear to

have basal nuclei and bear long cilia. This central groove is bounded by two densely staining lateral prominences and their epidermis is composed of compact columnar cells which possess central or basal nuclei and short cilia. No glandular tissue is found above the upper epidermis but mucocytes are present in bulk at the lateral prominences and below the central groove.

Only one cell type is present (type 6) in the suprapedal gland. These are large and round and each appears to have an extension to the central groove of the duct of the gland. These gland cells have a nucleus which is often central and spherical (Figure 29 and Plate 60A).

The duct of the suprapedal gland changes its shape and extent of ciliation down its length (Figure 30 and Plates 59 and 60). In the head the duct broadens and the ciliated lateral prominences and the central groove are replaced by a more generally ciliated ventral floor. This floor is raised in the centre (Figure 30B1 and C1).

The histochemical results show that the contents of these gland cells reacted slightly to the Alcian Blue pH0.5/eosin technique which suggests that some sulphate groups are present (Plate 59A) and these groups display slight β metachromasia in toluidine blue (Table 14). However, the contents of these cells show a strong reaction with PAS stain which indicates the presence of periodate-reactive material in the secretion (Plate 59B) and the dark brown staining obtained with the periodic acid paradiamine method confirms

that the periodate-reactive groups represent neutral mucopolysaccharides (Table 14 and Plate 59C). These pedal gland cells reacted negatively to the tests used for protein, calcium and lipid; therefore these can apparently be dismissed from consideration.

The secretion of the suprapedal gland is, therefore, a neutral mucopolysaccharide, possibly associated with some sulphate groups (Table 15).

5. The leading edge of the foot

As would be expected, there are more mucus gland cells present at the anterior end of the foot as more mucus would be required anteriorly, since the animal moves over the trail of slime principally produced at the anterior end.

In L. pseudoflavus, this area contains three cell types in three discrete blocks. For convenience they will be called the superior gland (type 14) the median gland (type 12) and the inferior gland type 13 (Figure 23 and Plate 61A).

The inferior gland (type 13):

The largest of these three glandular areas in median longitudinal sections is the inferior gland. The ducts of these cells open on to the foot just before it would normally make contact with the ground (Plate 61A).

These cells have an ovoid or somewhat elongated cell body with a large spherical centrally placed nucleus, often containing a nucleolus, together with granules of chromatin. Their ducts are extremely long and thin, leading to the

epidermis. The duct 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 (Figure 31B and Plate 61B).

The granular contents did not respond to the tests used for acidic mucopolysaccharides, calcium and lipid and therefore the presence of these components can apparently be discounted. However, the strong reactions with mercuric bromophenol blue and diazo coupling (Plate 61C) indicate that protein containing tyrosine is present and the brown colour shown with the periodic acid paradiamine method confirms the presence of neutral mucopolysaccharide in the secretion (Table 14).

Therefore,these results indicate that the granules of these cells are composed of a mucoprotein containing neutral mucopolysaccharide (Table 15).

The median gland (Cell type 12):

This is a medium sized gland which lies at the centre in between the inferior and the superior glands (Figure 31 and Plate 61). The cells of this gland are similar to type 9 cells of the foot in their contents and morphology. However, there are a few differences in the intensity of staining by the PAS and by toluidine blue (Table 14) but it is questionable whether these represent a significant change in their contents. Probably type 9 cells of the foot are replaced by the median gland cells in this area of the foot producing carboxylated and neutral mucopolysaccharides

(Plate 61A).

The superior gland (Type 14):

The superior gland is present on the dorsal surface of the foot, at the exit of the suprapedal duct. It is the smallest gland at the leading edge of the foot (Figure 31A and Plate 61A). The gland cells have a distinct oval cell body and ducts filled with the secretion are visible in between the epidermal cells. Occasionally the large, centrally placed nucleus can also be seen, although it was not possible to identify a nucleolus (Figure 31 and Plate 61B).

These cells have reticular contents that stained bluish-purple with the Alcian Blue pH2.5/PAS sequence (Plate 61B), this suggests that the alcinophilic and periodate positive components are present. The presence of some sulphate groups is indicated by the blue staining in Alcian Blue pH0.5/eosin technique and these groups display metachromasia in toluidine blue. However, the periodate positive-material in the secretion appears to be neutral mucopolysaccharide as indicated by the brown colour obtained with the periodic acid paradiamine method (Table 14). None of the tests used for protein, calcium and lipid was positive (Table 14). From these results, it seems that the secretion of the superior gland contains acidic and neutral mucopolysaccharides.

Thus, the mucus produced under the foot is highly complex being composed of the products of at least seven

cell types (i.e. 6,7,9,10,12,13 and 14).

6. The pneumostome

The external structure of the pneumostome is shown in Plate 55B. The area around the aperture is equipped with the normal complement of mucus-producing cells found on the dorsal surface, with the addition of cells of type 10. These occur sporadically over the dorsal surface but are concentrated in the area of pneumostome. Type 11 cells are found in the pneumostome (Plate 62).

Cell type 11:

They are subepidermal and are found commonly in the lung itself (Plate 62B) and deep in the channel leading from the lung to the exterior (Plate 62A). They have a distinct cell body about 15μm in size and generally spherical. The cell body and the ducts are filled with the secretion, which is reticulate in appearance (Table 15). The histochemical results show that some sulphate groups are present as indicated by the blue staining obtained with the Alcian Blue pH0.5/eosin technique. However, only a yellow colour is obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 technique which indicates the presence of carboxyl groups and suggests that no sulphate groups are present in the secretion (Table 14). The only possible explanation for these unusual results is that possibly the sulphate groups are present but the reaction of these groups in Alcian Blue pH2.5/Alcian Yellow pH2.5 technique is suppressed by the strong carboxyl groups in the secretion. Whatever end groups are present

they show metachromasia in toluidine blue and the presence of neutral mucopolysaccharide is evidenced by the brown colour obtained with the periodic acid paradiamine method. Neither protein, calcium or lipid were found in the secretion (Table 14). Most of these results therefore indicate that the secretion of type 11 cells is acidic and neutral mucopolysaccharides.

Near the exit of the pneumostome, these type 11 cells are replaced by type 10 cells lying under a layer of type 7 cells (Plate 62A), a configuration which also occurs on the head (Figure 32 and Plates 58 and 62A and C). The epidermal surface around the pneumostome is ciliated (Plates 55B and 63A). In transverse section the pneumostome is surrounded by an annulus of large cells which are generally ovoid but they may adopt unusual shapes. They have a large spherical or ovoid centrally-placed nucleus (Figure 33 and Plate 63A). Their contents are granular and stain positively for protein and with Sudan black B in both frozen and wax sections indicating that proteins are associated with some lipids. These cells do not possess ducts and therefore do not appear to discharge. However, it is suggested that their function may be to serve as an elastic annulus around the pneumostome.

7. Semper's organ

This area is in the 'face' of the animal and the ducts of its cells (type 5) empty on to an area roughly bounded by the two pairs of tentacles (Plates 61A and 63B). This

gland consists of a large bulk of gland cells which are grouped together and most of the time it is not possible to make out the boundary of the individual gland cell completely. However, it is clear that they have a polygonal shaped cell body and a large centrally placed nucleus. The contents of these cells are reticulate in appearance but sometimes may appear granular (Plate 63B).

Histochemical results indicate that a complex type of mucosubstance is present in the secretion of this gland (Table 14). The yellow/green staining obtained with the Alcian Blue pH0.5/Alcian Yellow pH2.5 method indicates the presence of sulphated and carboxylated mucopolysaccharides. The staining with the Alcian Blue pH0.5/eosin method further confirms that sulphate groups are indeed present and these groups are stacked in such a way as to give metachromasia in toluidine blue. Moreover, the strong reactions for neutral mucopolysaccharides as indicated by the results of applying the PAS stain and the bright brown colour obtained with the periodic acid paradiamine method, confirm that neutral mucopolysaccharide is also present. However, none of the tests used for protein, calcium or lipid gave positive results.

From these results it appears that the secretion of this gland is composed of sulphated, carboxylated and neutral mucopolysaccharides (Table 15). This gland stains in a mottled, inconsistent fashion probably indicating

considerable diversity of mucus production.

8. Ciliated areas

Figure 23A shows the areas of the body which are ciliated. The duct of the suprapedal gland is also partially ciliated (Figure 29). Figure 34 shows the movement of small particles of chalk blown onto the surface of the slug. The pneumostome is the only part of the exposed body to be ciliated (Plates 55B and 63A) and it is only in this area that small particles are moved in the mucus. Irritants (e.g. 0.5% Methylene Blue) applied to the surface of the body result in an increased mucus production and writhing movements culminate in the washing off of the irritant. When applied to the head, irritants are removed by the withdrawal of the head under the mantle and the inversion of the tentacles. On re-extension the Methylene Blue irritant is confined to a small blob of mucus which is moved down below the peripodal groove and left behind.

SECTION B

MODE AND MECHANISM OF DISCHARGE

From the study of the various types of glands and the gland cells found in the different regions of three prosobranchs Pomatias, Bithynia and Littorina and the pulmonate slug Limax pseudoflavus, it is suggested that the mode of discharge in these molluscs may be an apocrine or merocrine rather than holocrine type.

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. Therefore, if this type of discharge is to happen in the gland cells and the glands then firstly the nuclei and membranes should be detectable externally and secondly as this type of discharge necessarily involves the death of the cell there should be a continuous supply of young cells in the process of maturation to replace those lost.

However, in the present study there is no evidence of any discharged cell organelles or young developmental stages and therefore it seems that a holocrine mode of discharge is not adopted by either the cells or the gland cells in these four species.

The apocrine mode of discharge involves the ejection of the secretion from the apical portion of the cell and presumably this type of discharge would be adopted by the cells without the ducts. For this reason, it is suggested

that the epidermal goblet cell types found in Pomatias, Bithynia, and Littorina would display an 'apocrine' mode of secretion. However, the various subepidermal cell types in Pomatias, Bithynia and Litorina and the mucocytes of the suprapedal glands in Pomatias and Limax pseudoflavus exhibit well formed ducts and therefore would adopt a merocrine mode of secretion.

What initiates the discharge is not clear from light microscopy. However, there are two other alternatives for the mechanism of discharge. Agreeing with Lawrence (1972) the first is that once the cell has ceased to increase in size there will be continuous production of the secretion and the pressure of newly produced secretion will force the mucus out of the cell. This might keep the skin of the animal continuously moist in normal conditions; but it would not provide the large quantities of mucus needed, for instance when the animal is under attack or the operculum and/or shell being formed or repaired. This ability of gastropods to produce various amount of mucus as required suggests that some form of neuromuscular or endocrine system is involved.

The second alternative relates to the fact that these subepidermal gland cells and mucocytes of the suprapedal gland are found lying 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 these

gland cells and thus forcing out the secretion to be discharged and indirectly by exerting pressure on the haemocoelic fluid which in turn would push against the gland cell wall affecting discharge. Presumably such muscular contraction is under nervous control.

This last mechanism seems to be more likely for the subepidermal gland cell types found in Limax, Pomatias, Bithynia, and Littorina and the mucocytes of the suprapedal glands found in Pomatias and Limax pseudoflavus.

SECTION C

FUNCTIONAL ASPECTS OF THE MUCUS

The term mucus is commonly applied to any epithelial secretion of a slimy, tacky and viscous nature. Throughout the animal kingdom the main lubricating agents are mucopolysaccharides. They are found in the lining of the alimentary canal and genital tracts from worms to mammals and in the integument from Cnidaria to Amphibia.

In gastropods mucus has been specifically implicated in functions such as locomotion (Denny, 1980); trail following (Townsend, 1974; Cook, 1977; Chase et al., 1978); courtship (Quick, 1960; Cook, 1985); homing (Funke, 1968; Cook et al., 1969; McFarland, 1980); aggregation (Lowe & Turner, 1976; Chase et al., 1980) and feeding (Fretter and Graham, 1978).

It would be reasonable to assume from the areas of the animals' body on which the secretions are found that the dorsal surface mucus has a protective and cleansing function; that the pedal mucus has a locomotory and communicative function; that the mucus secretion from the opercular gland cells may be participating in the formation of the operculum and the mantle edge glands in the formation of the shell; and that the function of the hypobranchial mucus is a sanitary one acting to trap and purge the mantle cavity of undesirable dirt and particulate matter. Further possible minor functions of the mucus include cryoprotection, the prevention of desiccation and excretion.

1. Dorsal surface mucus

One general feature of the dorsal surface mucus is the participation in the production of a protective slime layer covering the body surface. Mucus secretions often function as antipredator devices by rendering the animal distasteful (Simkiss and Wilbur, 1977); fouling the predator's feeding apparatus (Richter, 1980) or simply making the animal too slippery to pick up (Wilbur, 1983). However, this function is of particular importance to the naked varieties (slugs) compared to the shelled molluscs. The molluscs with shells have an advantage over naked varieties in that they can protect themselves by retreating into their shells. Therefore one would expect to find numerous and more specialized mucus gland cells over the dorsal surfaces in the slugs and more simple epidermal structures in the shelled molluscs. The observations in the present study agree well with this theory.

For a mucus to protect a surface against accidental mechanical damage it should have a high viscosity and to protect from predators, both large and small a repulsive taste and a low surface tension.

In Limax pseudoflavus, a terrestrial pulmonate slug the dorsal mucus consists of a highly sulphated acid mucopolysaccharide from type 1 cells with the addition of protein from the type 2 and 3 cells. Therefore, the dorsal mucus of Limax pseudoflavus will be a viscous fluid and the addition of protein may provide a matrix to lend coherence.

The high viscosity and cohesion of the dorsal mucus may be seen as a water saving device, in the sense that it does not flow off the animal rapidly and therefore does not need frequent replacement. There is, however, some virtue in a continuous evaporation from the moist skin, as this will reduce the danger of overheating in all conditions except those of fully saturated air. Evaporative water loss from terrestrial animals takes place not from the skin directly but from a superficial layer of secreted mucus. Machin (1964) pointed out that severe desiccation may result in permanent damage to H. aspersa. Therefore superficial drying out is a real danger to the animal, and thus a highly efficient protective mechanism of mucus secretion has been evolved in the skin of snails and other terrestrial pulmonates. The high observed viscosity may also act as a good protection against accidental damage as the mucus is very difficult to penetrate and the low surface tension would lead to any object coming in contact with it becoming quickly covered with this sticky thick mucus.

The type 4 cells in Limax pseudoflavus have an unknown product but could be the source of the pigment seen in the dorsal mucus of agitated animals. The dorsal mucus produced in response to mechanical and/or chemical stimulation is very watery and will favour the removal of foreign bodies from that surface. In a normal unirritated animal the dorsal mucus hardly moves at all (Figure 34) and since the dorsal surface is not ciliated any ejection of matter from

this area must be accomplished by flooding rather than by discrete rejection currents.

The dorsal surface mucin may also act to protect one slug against another slug of the same species. This is supported by the work of Meredith (1972) who removed the dorsal surface mucin from the slugs and then returned them to vivarium. Next morning the dorsal surfaces of these treated slugs were extensively damaged and the source of the damage was found to be the other slugs in the container which ate through the dorsal skin of these animals very rapidly. Cannibalism was not observed when all the slugs had their mucus. It seems that the viscous mucus on the dorsal surface of the slug protects them against being eaten by another slug by fouling up the radula with a thick covering of mucus. The mucus probably acts as a protection against other small predators in a similar way.

In Limax pseudoflavus the area around the pneumostome is ciliated and it contains the cells which produce a neutral and carboxylated mucopolysaccharide (pneumostome type 10 and 11). The area round the pneumostome is cleaned in two ways. First, if large irritants are placed in the pneumostome the area will be flushed out with pallial water. Second, small particulate matter is moved radially out from the pneumostome either to end up lodged in the dorsal mucus or to be moved down on to the foot mucus. Cell types 10 and 11 presumably provide the mucus for this rejection current. This is supported by the observation that cell type 10 is

more frequent on the right side (pneumostome) than on the left side (non-pneumostome) between the bottom of the mantle and the peripodial groove. In the channel of the pneumostome, type 10 cells form a deep subepidermal band beneath a layer of type 7 cells. This is a configuration which also occurs on the dorsal surface of the head (Plates 58 and 62). The mucus from type 7 cells may provide for adhesion between two areas (edges of the pneumostome or between head and lower mantle). Adhesion between adjacent areas of the body will prevent excessive water loss.

Semper's organ is a large gland present in Limax pseudoflavus pouring a mucus on to the front of the head. The contents of this gland are diverse and therefore any functional interpretation is necessarily speculative. Mucus on the face of the animal may assist in feeding by sticking together particulate food but this would be a function more normally associated with salivary secretions (Moreno et al., 1982). It is likely that it serves to clean the head and the sensory epithelia of the tentacles during their withdrawal. Rapid production of large quantities of mucus on the head forms a blob of mucus containing any irritants which is then removed by wiping the head on the underside of the mantle. Treating the head with methylene blue produces a response like this but, of course, it is not known from where the mucus comes.

Although slugs are more dependent on their mucus, having no shell into which to withdraw and more dependent on

behaviours such as huddling (Cook, 1981) and homing (Cook, 1979) which have as their basis the pheromonal qualities of their mucus, the mucus layer is without doubt also very important for terrestrial and aquatic snails.

It is rather difficult to determine the specific function of each gland cell type found on the dorsal surface of Pomatias elegans (terrestrial); Bithynia tentaculata, (freshwater) and the Littorina littorea (marine). One general feature in the terrestrial snail is the participation of the mucus in keeping the skin moist for the purposes of respiration and to hinder dehydration (ef. Herfs, 1922; Machin, 1964). But this function is of secondary importance for the aquatic forms. A more general function for this mucus layer may be to serve as a barrier for the passage of water and specific ions and it may also have a more specific function in binding or absorbing particular ions (Scott, 1968). In fresh water snails the external body surface is almost certainly a major site of ion uptake (van der Borght and van Puymbroeck, 1964; van der Borght, 1963; Greenway, 1971b; Thomas et al., 1974). This was suggested for instance, by Bolognani-Fantin (1967) for calcium ions and by van der Borght and van Puymbroeck, (1970) for rubidium. The selective absorption of calcium ions by the epidermis appears to occur with a comparatively low energy requirement (Greenway, 1971).

Another important aspect in the function of the dorsal mucus gland cells is the lubrication of surfaces which make

a great deal of contact. This is supported by the observation that in Pomatias elegans, Bithynia tentaculata, and Littorina littorea a large number of goblet cells are present in folds between the tentacles and the epidermis joining the head and foot. Chase and Tolloczko (1985) also suggested the same function for the mucus gland type 3 in the tentacle of Achatina fulica, as these cells were found abundantly along the shaft of the tentacle.

Another disputed function of the dorsal mucus is that it may have antiseptic properties since the molluscan body is free of bacterial and fungal growth but Campion (1961) found this not to be true in the case of Helix aspersa. However, Pickering (1974) suggested that continued replacement of mucus from the animal's body may discourage adhesion of parasites and other disease organisms.

2. Pedal mucus

The structure of the feet and associated glands in Pomatias elegans, Bithynia tentaculata, Littorina littorea and Limax pseudoflavus is clearly adapted to their different modes of life and their consequent physiological and environmental requirements.

The main difference found between the foot structures of terrestrial and aquatic forms is the absence of the compound suprapedal gland in the latter. In both Bithynia and Littorina the mucus producing glands are distributed all over the foot but in Pomatias the mucus is delivered to the longitudinal furrow. However, this may not be true of all terrestrial prosobranchs but may be associated with the somewhat idiosyncratic mode of locomotion adopted by Pomatias (Lissmann,1945).

Lubrication of a particularly well controlled kind is essential on the sole of gastropods because of their method of locomotion. Cilia cannot beat without fluid over them and one might expect that the muscular contractions which move the creature would result in a far less smooth kind of progression without the aid of some kind of mucopolysaccharide on the surface. As gastropods actually move over a trail of mucus it protects the sole against the sharp soil particles and thus reduce the risk of the mechanical damage.

The physical properties of mucus coating are an

integral part of the locomotory system. In gastropods the locomotion is determined by at least three factors. First the presence of acid mucopolysaccharides is indicative of a high viscosity (Hunt, 1973). Second, the presence of protein will provide a matrix conferring some solid properties of mucus (Denny and Gosline, 1981; Hunt, 1973). Finally, the presence of calcium ions will increase the viscosity of protein/polysaccharide matrices (Gray, 1926). Of these factors, the localized production of calcium appears to play no great part in Limax pseudoflavus and Bithynia tentaculata as none was found in them and very little was found in Littorina littorea. However, a substantial amount of calcium is present in the foot of Pomatias.

The use of a flat pedal sole for locomotion and adhesion is common in the gastropods and requires the presence of mucus for lubrication and adhesion. The presence of several types of mucocytes in the foot suggests the possibility that products of these cells are responsible for more than one function.

Clearly the neutral mucopolysaccharide produced by the suprapedal gland (cell type 6) in Limax pseudoflavus will be involved in locomotion. The mucus of the suprapedal gland must have two types of properties; those suited to free movement down the duct and those of a diphasic gel under the foot. These two requirements may not be compatible. There are few muscles in the suprapedal gland of Limax

pseudoflavus and only a thin membrane separating the duct and the body cavity (Figure 29 and Plates 59 and 60). Movements within the duct therefore, must be by the activity of the cilia on the ventral floor. The neutral contents of the suprapedal gland cells (type 6) indicate that the contents of the suprapedal duct will not be highly viscous. Further the lack of protein in the secretion will not allow the sort of sulphydryl cross bonding envisaged by Denny and Gosline (1981) as being involved in the sol/gel properties of the pedal mucus of Ariolimax columbianus (Gould) (an arionid slug). If there is a protein in the pedal mucus of Limax pseudoflavus then it must be added to the suprapedal gland secretion after it leaves the duct. The type 13 cells in the leading edge of the foot contribute a mucoprotein (Table 15) to the suprapedal secretion as it moves over this area of the foot. , It may be the products of these gland cells which give the pedal mucus the properties described by Denny and Gosline (1981). Chetail and Binot (1967) described the suprapedal gland of Arion rufus. Neutral mucopolysaccharide is secreted in the posterior part of the gland and is joined by a more acid secretion from the front. The leading edge of the foot has not been examined. This may possess a similar system to that proposed for Limax pseudoflavus where an initially free flowing mucus receives additives at the front of the animal in order to acquire the properties required under the foot. Cellular separation of the synthesis of a protein/polysaccharide complex in the

hypobranchial gland of Buccinum undatum L. has also been demonstrated (Hunt, 1973).

The suprapedal duct of Limax broadens out as it approaches its exit, and the central area also rises (Figure 30). This will tend to spread the mucus out over the dorsal surface of the anterior foot by pushing it towards the edges. This area is also covered with cilia and receives the secretion of the type 14 cells in the superior gland. These produce a neutral and weakly acidic mucopolysaccharide which should therefore have a low viscosity. A mucus of this type at this place may assist in the spreading of the products of the suprapedal gland over the foot. The type 12 cells lie immediately below these type 14 cells. Type 12 cells secrete a carboxylated and neutral mucopolysaccharides and since both in their position and their product they are closely allied to the type 9 cells of the sole their role may not be specifically associated with being located at the leading edge of the foot. Despite the minor differences in staining properties, this median gland might be better viewed as an extension of the layer of type 9 cells in the sole. This layer is penetrated by the large inferior gland (type 13 cells) giving the impression of two separate cell populations (Plate 61).

In Limax pseudoflavus, the whole of the leading edge of the foot is ciliated and so is the central band of the tripartite foot (Figures 27 and 31). During locomotion the pedal waves are also confined to this central band. The

cilia in the peripheral bands peter out at a level corresponding to the back of the head. The wide distribution of cilia on the anterior foot probably serves to further distribute the mucus to the edges. The presence of cilia in the central band may also be associated with the distribution of mucus by moving it backwards whilst it is in its sol phase during locomotion, thus countering the peristaltic effect of the pedal waves moving it forward. The lack of cilia in the peripheral bands of the foot may therefore, be associated with the lack of pedal waves in these areas.

The peripodal groove is ciliated and produces a weakly acidic and neutral mucopolysaccharide (Cell types 8, 9, and 10). This area has to be viewed in the context of the mass mucus movements in the crawling animal. The peripodal groove is an extension of the suprapedal duct and all mucus above it is therefore stationary relative to the animal and below it stationary relative to the ground. To facilitate the movement of one mass of mucus relative to the other it is reasonable to suggest that a fluid mucus is inserted between them. The cilia in the groove do not provide a rejection current since particles in that area of the foot remain stationary in one or other of the mucus sheets. The function of the cilia in the groove may be to aid in the insertion of the fluid mucus between the more viscous dorsal and pedal mucus sheets (Figure 35B).

In a stationary animal the mucus must allow both for

adhesion and for detachment of the foot from the substrate. Gastropods adhere to their substrate by Stefan type adhesion (Grenon and Walker, 1978). This form of adhesion depends upon the opposition of two flat surfaces separated by a thin layer of fluid. The strength of the adhesion increases with the viscosity of the fluid. Type 7 cells are widespread over the body but specially common in the sole and produce the most highly sulphated products found in this region. It would be expected, therefore, that the secretion of these cells would increase the viscosity of the pedal mucus and thereby promote adhesion. Type 9 cells on the other hand secrete neutral mucopolysaccharide which would be expected to be less viscous and therefore aid in releasing the foot from the substrate. Similarly the type 12 cells at the leading edge of the foot also secrete a neutral mucopolysaccharide and might therefore be involved in unsticking the anterior foot. This interpretation of the formation and function of the pedal mucus is summarized in Figure 35A.

In Pomatias elegans four cell types are found in the suprapedal gland and it is presumably these cells which give the pedal mucus the properties required for locomotion. Its delivery to the longitudinal furrow rather than to an arc around the leading edge of the foot is an obvious adaptation to its peculiar stepping form of locomotion. The suprapedal gland produces a mixed neutral/acidic mucoprotein which will mix with the secretions of the sole gland types P5 and P6 on

the ventral surface of the foot. Adhesion to the substrate would be promoted by the sulphated mucopolysaccharide from cell type P6 whilst release might be achieved by the neutral mucopolysaccharide from cell type P5.

Recently Bensalem and Chetail (1982) described the unusual adaptation of Pomatias elegans in employing the whole glandular equipment of its foot for the physiological maintenance of its hydrocalcic meta-bolism in the conditions of the biotope. They also proposed an additional role for the sole gland, that is to trap calcium ions in the secretion which spreads out on the absorbing sole surface.

In aquatic forms the anterior glands are present around the leading edge of the foot, secreting the neutral mucopolysaccharide and protein. These glands appear analogous in function to the multicellular suprapedal gland which is present in many of the terrestrial gastropds and serves to form the mucus track on which these snails move (Hyman, 1967). In Bithynia tentaculata cell type B2 is distributed all over the ventral surface of the foot, produces a sulphated mucopolysaccharide and is therefore, presumably associated with adhesion. Cell types B3 (in the sole) and B1 (in the anterior gland) produce acidic and neutral mucopolysaccharide. Since Bithynia uses ciliary locomotion it is unlikely that the properties to be found in this mucus will be the same as found by Denny and Gosline (1981) for a slug using muscular creeping.

In Littorina, two clusters of cells are present at the

leading edge of the foot secreting protein and neutral mucoprotein. These secretions together with the mucopolysaccharides from cell types L3, L4 and L5 form the pedal mucus which will presumably have similar properties to the pedal mucus of other gastropods using muscular creeping.

In Bithynia and Littorina, the cilia on the dorsal surface of the anterior part of the foot (Figure 5) probably aid in bringing the mucus from the anterior pedal gland cells to the front and ventral surfaces of the foot.

In addition to the use of pedal mucus in locomotion and adhesion it is used extensively by many gastropods as a means of communication and navigation. The "slime trail" of pedal mucus left behind as the animal crawls is often important in behaviour, used either as a means of "homing" or for following conspecifics (Trott and Dimock, 1978; Cook, 1979; McFarland, 1980).

3. Mantle secretion

It is generally accepted that the shell is to be considered as a secretion product of the mantle, especially the mantle border and that each layer of the shell is secreted by a definite region of the underlying mantle.

The periostracum is the uncalcified outer organic covering of the molluscan shell. The nature of the periostracum is related to the environment. Its thickness varies with habitat and is said to be greatest in the fresh water species and least in species from warm seas (Fretter and Graham, 1962). V.R. Meenakshi et al. (1969) examined the periostracum of twelve genera of gastropods from different habitats and found that the fresh water species had two layers, the terrestrial had a single layer and the marine gastropods had a single or incomplete layer depending upon species. In relation to the histochemical study of the periostracum they pointed out that the periostracum is not simply tanned protein but also includes neutral mucopolysaccharides in all species and traces of lipids in some. Secondly, the acid mucopolysaccharides commonly associated with calcification were found to be absent in this uncalcified portion of the shell.

The present investigations on the histology and histochemistry of the mantle of three prosobranch species from different habitats agree well with the findings of Meenakshi et al. (1969) as far as the periostracum is

concerned.

In Pomatias elegans, the cells of the mantle edge gland (P15) open by the long neck processes on to the outer surface of the mantle edge. From the histochemical results for these gland cells it can be concluded that the protein and neutral mucopolysaccharide produced by them may help in the formation of the periostracum.

Bevelander and Nakahara (1970) mentioned that in Littorina the dorsal and ventral gland cells of the mantle edge gland cluster were structurally similar and contributed towards the formation of the inner and outer layers of the periostracum respectively. The histochemical study of the nature of the contents of these gland cells (L12) confirms that the neutral mucoprotein secreted by them participates in the formation of the periostracum.

In Bithynia tentaculata, a cluster of the gland cells (B10) is present at the mantle edge producing a protein type of secretion and this secretion may be responsible for the formation of the periostracum. However, Bithynia differs from Littorina and Pomatias as this mantle edge gland cluster of cells does not appear to be secreting a neutral mucopolysaccharide and did not respond to the periodic acid paradiamine technique, however the slight reaction with PAS suggests the possibility of its presence in the secretion. Alternatively, the only source for the neutral mucopolysaccharide which happens to be present in most of the periostraca would be from the goblet cells (B8)

secreting neutral mucopolysaccharide and this view is supported by the observation that a greater number of them are at the mantle edge.

In Pomatias and Littorina a second cluster of the gland cells producing an acidic mucopolysaccharide type of secretion is present at the mantle edge. Apparently their role in the formation of the periostracum is doubtful as an acidic mucopolysaccharide component was found to be absent in all the periostraca studied by Meenakashi et al. (1969). However, the acid mucopolysaccharides are known to play an active role in the calcification process by binding the calcium ions, probably as a metachelate complex (Sobel and Burger 1954), Simkiss and Tyler (1957, 1958). Kapur and Gibson (1968) explained the role of the sulphated mucopolysaccharide in Helisoma and such a method of calcium incorporation has been suggested by Kado (1960) in other molluscs. It is postulated that the material in question may play the same role in the calcification of the shell.

Beedham (1958) suggested that although the mucus secreted on the outer mantle surface may be involved in the transfer of calcium salts from the epithelial cells to the calcareous layers of the shells, the presence of numerous mucus cells indicates that this is not the only function. The abundance of mucus at the highly muscular mantle edge suggests that the mucus secreted between the mantle and the shell supplies necessary lubrication for the withdrawal and re-extension of the mantle margin.

Saleuddin (1979) proposed an interesting role for the mucus gland cells which are present at the mantle edge in Helisoma and secrete acidic mucopolysaccharides. He proposed that this mucus secretion might help in the movement of the developing periostracum along the groove. However, this function would not be appropriate in the case of Pomatias and Littorina as the periostracal groove is not present in these species and the gland cells that form the periostracum open directly on the outer surface of the mantle.

The inner calcareous (hypostracal) layers of the shell are said to be secreted by the snail's entire dorsal epidermis (Durning (1957), Lemche and Wingstrand (1959), Abolins-Krogis (1963) and Saleuddin (1970, 1979)).

Nakahara and Bevelander (1967) studied the ingestion of particulate matter by the outer mantle fold in Macrocallista maculata and Isognomon alatus (Pelecypoda) and concluded that the apparent normal function of the mantle cell is the removal of particulate matter from the fluid by means of pinocytic activity which may be helpful in normal shell and regenerating shell (Chan and Saleuddin 1974).

In addition to this shell secreting activity, the mantle secretion is also said to be repugnant and also to act as an antiseptic and as a defensive adaptation in many molluscs (Fretter and Graham (1962), Thompson (1960), Edmund (1968)). The glandular ventral surface of the mantle is associated with the production of mucus and several other

substances including repellent or toxic secretions. In some gastropod species these mantle glands produced a toxic proteinaceous secretion after rather drastic stimulation and it was therefore assumed that these glands are repugnant in function. Thompson (1960) observed that in some gastropod species, the mantle produced strong acidic secretions after an abrupt disturbance and thought it to be as a defensive adaptation.

In Bithynia, Littorina and Pomatias a large number of goblet cells secreting highly sulphated mucopolysaccharides and proteinaceous secretion are present at the ventral epidermis of the mantle. However, any functional interpretation of their role as a repugnant or in a defensive adaptation will be necessarily speculative.

4. Hypobranchial secretion

The function of the hypobranchial secretion is not known with certainty, but a considerable amount of effort has been put into attempts to prove that it produces a toxic secretion and can be poisonous to some animals when injected (Dubois, 1909). However, it has been shown in experiments with fish that secretions of the hypobranchial gland do not diffuse freely into the water round an uninjured dog whelk and therefore, cannot act as an external poison (Jullien, 1948).

Recently, Houbrick (1980) observed that when Modulus modulus (prosobranch) was violently disturbed or if the hypobranchial gland in this snail was stimulated with a probe, it exuded copious strands of tiny, globular, mucus-like structures. The animal continued to exude these globular particles whenever stimulated until the gland was spent. Houbrick thought that the exudate was probably used defensively by the snail when under attack. However, the composition and nature of the exudate was not studied.

Fischer (1925) claimed that the hypobranchial secretion in Nucella has sexual significance in that it leads to an aggregation of animals attracted by its odour (cited in Fretter and Graham 1962). However, the most reasonable assumption as to the significance of the hypobranchial secretion in the life of the gastropod mollusc is that with the aid of ciliary activity, it keeps the surface of the

mantle clear of debris and thus prevents mechanical fouling of the ctenidium and the osphradium, the two pallial organs adjacent to the gland (Ronkin, 1955).

The function of the hypobranchial mucus in marine molluscs is thought to be for the consolidation and removal of sediments that enter the mantle cavity and which if not removed would obstruct or impede the organs of the pallial complex (Yonge, 1947). Hunt and Jevons (1965) suggested that a consideration of the requirements of a material performing such a function show that it should possess both the properties of a viscous liquid and of a fibrous semisolid matrix, the former property promoting a slow dragging flow over the internal surfaces and organs of the mantle cavity and facilitating the removal of particles of detritus. The semisolid matrix would promote the consolidation of suspended particles into a solid mass before it is voided." Furthermore, Hunt (1973) pointed out that in the hypobranchial secretion of Buccinum undatum, a marine gastropod, the structural viscosity would result from the molecular interactions between the two secretion products, the acid mucopolysaccharide and glycoprotein moieties, as they do not have these properties when separated, although the acid mucopolysaccharide does have a high classical viscosity in aqueous solution. Hunt (1973) suggested that the ciliated cells which are present in this area probably serve the dual function of mixing the glycoprotein and the mucopolysaccharide moieties of the secretion after their

release at the gland surface, coupling this minor role with the major one of moving the viscoelastic sheet of secretion over the epithelial surface.

In the present study the histological observations and the histochemical results on the hypobranchial gland of Littorina littorea closely resembled those of the hypobranchial gland of Buccinum undatum (Hunt 1967, 1973). In Littorina also the hypobranchial secretion is mainly acidic mucopolysaccharides secreted by the gland cell types L14 and L15 and the neutral mucoprotein type of secretion produced by the gland cell type L16. Interspersed among these cells are the ciliated cells (Figure 22). A small quantity of sulphated mucopolysaccharide is present in the hypobranchial secretion of Pomatias elegans, a terrestrial prosobranch. Bolognani-Fantin and Ottaviani (1981) suggested that this may reflect both a reduced need to decontaminate the mantle cavity with a thixotropic mucin in a terrestrial species as well as the need to economise on sulphate metabolism in the same environment.

However, in Bithynia tentaculata, a fresh water gastropod, this glandular area is dominated mainly by gland cells producing a neutral mucoprotein.

What is involved in changing the appearance and the histochemical nature of secretion in the hypobranchial gland cells is not clear, but the most reasonable assumption may be that it is related to evolutionary specialization requiring adaptation to different habitats.

5. Other functions:

Furthermore, in different species of gastropods a number of other diverse functions have evolved for the mucus secretion. The mucus secretions are used in various ways in reproduction by forming the "slime threads" to which the terrestrial slug Limax maximus hangs during copulation (Rollo and Wellington, 1977 and in the mucus coating of the egg of the snail Assiminea californica (Fowler, 1980), which causes a camouflaging layer of mud to adhere to the eggs.

The capacity of mucus as cryoprotectant has been observed by Hargens and Shabica (1973) in the antarctic limpet, Patinigera polaris and the mucus in this animal helped it to survive exposure to temperatures down to -10°C.

Therefore, it is evident that these mucus secretions, far from being simple slime, are a diverse, highly functional and important group of biomaterials.

VIII COMPARISON

1. Comparison of the pedal gland cell types in the prosobranchs Pomatias, Bithynia and Littorina, and the pulmonate slug Limax pseudoflavus.

The gastropod foot subserves the double function of attachment and locomotion. This requires the presence of mucus for lubrication during locomotion and adhesion. The presence of several types of mucocytes in the foot suggests the possibility that the products of these cells are responsible for more than one function and the variations in the form and distribution of these mucocytes on the surface of the gastropod foot is probably related to the habitat and the type of locomotion.

Gainey (1976) suggested that taking into consideration the size, form, habitat and phylogeny of the gastropods several generalizations can be made concerning the occurrence of particular modes of locomotion. He claimed that ciliary locomotion predominates in small aquatic gastropods, while the terrestrial gastropods generally employ pedal waves during locomotion and this is probably related to the weight of the animal since a given mass weighs more on land than in water and thus would reduce the effectiveness of cilia as a means of terrestrial locomotion. The gastropods living in the rocky intertidal zone often have retrograde waves, and this is probably related to the greater reliance of these animals upon adhesion in agitated

water, where muscular waves enable a snail to keep a portion of its foot firmly attached to the substratum while at the same time moving forward, thus increasing its adhesion during locomotion.

Bithynia tentaculata is a comparatively small fresh water gastropod and adopts the ciliary mode of locomotion. Littorina littorea is a large, marine species living in the rocky intertidal zone and adopts muscular creeping with retrograde waves. In Pomatias elegans, a terrestrial gastropod, the foot is structurally and even functionally divided longitudinally and it moves forward in alternate "steps". Even though the general functional anatomy of these three prosobranchs Pomatias, Bithynia and Littorina is similar, the morphology and distribution of the gland cells on the surface of the foot is very different.

In Pomatias, a suprapedal gland is present on the foot musculature in the anterior third of the foot and the delivery of the pedal secretion from the suprapedal gland to the longitudinal median furrow rather than to an arc across the leading edge of the foot is an obvious adaptation to its peculiar stepping form of locomotion.

In both aquatic forms studied (Bithynia and Littorina) the anterior pedal glands are present at an arc across the leading edge of the foot (Figure 37, B1 and Figure 38, L1 and L2). These glands may be homologous to the suprapedal gland in Pomatias (Figure 36A) but morphologically they are very different.

The location of these anterior pedal glands at the leading edge of the foot and the movements of the anterior margin of the foot during locomotion leaves little doubt that these cells secrete lubricating mucus, and as the snail moves the mucus from these glands spreads a broad path of slime over which the animal makes its way. Littorina is said to be more reliant upon adhesion in agitated water (Gainey, 1976) and therefore, besides the musculature, mucus would be expected to play some part in this adhesion. This might explain the presence of two types of anterior pedal glands in Littorina littorea.

These glands may be different morphologically but in general the nature of their secretion is similar, containing mainly neutral mucopolysaccharide and protein associated with some acidic mucopolysaccharides.

On the sole of the foot also the morphology and distribution, and the nature of the secretion of the subepidermal gland cell types in Pomatias, Bithynia and Littorina differ considerably.

In Pomatias, two types of cells , P5 and P6, are present in the region of the median furrow (Figure 36). In Bithynia also, two types of cells, are present but type B2 cells are generally distributed over the sole whilst type B3 cells are confined to a transverse band in the middle region of the foot (Figure 37). In Littorina, however, cell types L3, L4 and L5 are generally distributed over the surface of the foot, but have a tendency to be accumulated at the front

end of the foot (Figure 38). These variations in the distribution of mucus gland cells on the sole of the foot in these three species are probably related to the different modes of locomotion adopted by these species.

Cell type P6 in Pomatias would probably be equivalent to cell type B2 in Bithynia and cell type L3 in Littorina as they all contain acidic mucopolysaccharides and are located in the sole (Table 16A). Cell type P5 in Pomatias could be equivalent histochemically and to some extent physically to the cell type L5 in Littorina. Both of these are flask shaped with a distinct cell body and the duct leading through the epidermis; and their contents are of a similar mucoprotein type containing neutral mucopolysaccharide (Table 16B).

In Bithynia, however, no protein cells are generally distributed on the sole. Furthermore, Bithynia again differs from Pomatias and Littorina in that the cell types P5 in Pomatias and L5 in Littorina both contain neutral mucopolysaccharides associated with protein whereas in B3 cells of Bithynia neutral mucopolysaccharide is combined with acidic mucopolysaccharides.

In general, the nature of the secretion over the sole is the same in Pomatias and Littorina, containing mainly protein and neutral mucopolysaccharide associated with acidic mucopolysaccharides. As proposed by Cook (1987) it can be concluded therefore, that these components are essential for the gastropod creeping system.

The pedal mucus in Pomatias, however differs from Bithynia and Littorina as far as calcium is concerned as none was found in Bithynia and very little is present in Littorina but in Pomatias a substantial amount of calcium is present in the gland cell types P2, P4, P5 and P6. Clearly the role of calcium in Pomatias is very different from that in Littorina or Bithynia as calcium is very important in Pomatias for the physiological maintenance of its hydrocalcic metabolism (Bensalem and Chetail, 1982).

Comparison of these three prosobranchs with the pulmonate slug Limax pseudoflavus is extremely difficult. First, the functional anatomy of the animal is different and secondly the distribution and role of the mucus is different.

However, as far as the pedal glands are concerned the anterior pedal glands in Bithynia and Littorina could be equivalent to the three cell types present at the leading edge of the foot in Limax pseudoflavus (Figures 37, 38 and 39). These all are present in clusters and are located at the leading edge of the foot. Histochemically, their secretion is mucoprotein mainly containing neutral mucopolysaccharide and protein combined with some acidic mucopolysaccharides (Table 16C) and probably all serve the same function.

The suprapedal glands of Pomatias and Limax (terrestrial gastropods) are different morphologically and histochemically. Nevertheless, these two glands could be

comparable. In both species they are present in the foot musculature and in terms of their histochemistry, even though the secretion of the suprapedal gland in Pomatias is complex, the main secretory component in the suprapedal glands of both is neutral mucopolysaccharide (Table 16D).

Cell type B2 in Bithynia, L3 in Littorina and P6 in Pomatias may be equivalent to cell type 7 in Limax (Figures 36, 37, 38 and 39). All are sole gland cells with fibrous/reticular contents and histochemically all contain acidic mucopolysaccharides. However, cell type 7 in Limax is present in tissues other than those of the sole, while cell type B2 in Bithynia, L3 in Littorina and P6 in Pomatias are confined to the sole (Table 16A).

Lastly, cell type P13 in Pomatias may be equivalent to cell type 10 in Limax pseudoflavus (Figures 36 and 39). These cells are distributed on the foot and the dorsal and lateral body surfaces and their granular secretion is neutral mucopolysaccharide (Table 16E).

2. Comparison of the cell types on the dorsal body surface of the prosobranchs Pomatias elegans, Bithynia tentaculata, Littorina littorea and the pulmonate slug Limax pseudoflavus.

In general, the distribution and the cell types present on the dorsal and lateral body surfaces of Pomatias, Bithynia and Littorina are very similar. These all are goblet cell types. In Pomatias four types of goblet cells are present while in Bithynia and Littorina there are two

types (Figures 36, 37 and 38).

Goblet cell type B7 in Bithynia and L9 in Littorina are almost identical in terms of their appearance and the histochemical nature of their secretion. The contents of these cells are reticulate in texture and are acidic mucopolysaccharides. Cell type P10 in Pomatias is probably equivalent to these gland cell types, but their contents appear to be smooth in texture (Table 17A).

The goblet cell type B8 in Bithynia, L10 in Littorina and P11 in Pomatias would seem to be identical at least in terms of histochemistry, since all contain the neutral mucoprotein type of secretion (Table 17B). In histological appearance, however, they are different, the contents of the cells B8 in Bithynia being reticulate whilst the contents of cell L10 in Littorina and P11 in Pomatias are granular.

The gland cell types present on the general dorsal body surface of Limax pseudoflavus, are entirely different from those in Pomatias, Bithynia and Littorina. This is to be expected since these three latter prosobranchs are shelled molluscs; whilst the pulmonate slug Limax pseudoflavus is a naked species and the functional anatomy of Limax is very different. Again, the role of the dorsal mucus would apparently be different in a slug from that in snails.

Limax pseudoflavus is devoid of the epidermal goblet cells and all the cell types on the dorsal body surface are large and are present beneath the epidermis (Figure 39). The general body mucus in Limax mainly consists of sulphated

and carboxylated mucopolysaccharides associated with some protein but the neutral mucopolysaccharide component found in the goblet cells of Pomatias, Bithynia and Littorina is absent in Limax.

The cells identical to the pigmented, yellow granular cells (type 4) in Limax were not found in Pomatias, Bithynia or Littorina.

Bensalem and Chetail (1982) observed two types of cells in the outer part of the suprapedal gland in Pomatias. In both the present study and Delahaye's work (1974) three types of gland cells are apparent (Figure 36B). Bensalem and Chetail's types a1 and a2 agree well in terms of appearance and histochemical nature with those of the P3 and P2 gland cell types respectively in Pomatias . In both cases, a1 and P3 cells contain acidic mucopolysaccharides and a2 and P2 produce a neutral mucoprotein. However, in Bensalem and Chetail's investigations there appeared to be no equivalent to type P1 cells of Pomatias with their proteinaceous secretion.

On the basis of an ultrastructural study, Bensalem and Chetail revealed that a1 cells showed different features when young and when fully grown. This does not permit a full explanation since a1 cells contain acidic mucopolysaccharides while P1 and P2 contain proteinaceous and neutral mucoprotein types of secretion respectively. Therefore, even if P1 and P2 may be considered as different developmental stages, these would be the different stages of

a 'protein type' cell and not with one cell having an acidic mucopolysaccharide type of secretion. So, regarding the number of different cell types present in the outer part of the suprapedal gland no definite conclusion can be reached.

Another point of difference is that in comparing Bensalem and Chetail's findings on the inner part of the suprapedal gland with those in the present study, they are found to be descriptively similar but different in their histochemistry.

Bensalem and Chetail found that the inner part of the suprapedal gland contained neutral mucopolysaccharide, protein with sulphide groups and a substantial amount of calcium. In the present study this part appeared to contain both acidic and neutral mucopolysaccharides associated with calcium.

However, there is a possible explanation for these differences in histochemical results. Bensalem and Chetail found protein with sulphide groups in the inner part of the suprapedal gland but this part showed negative responses to all the protein tests used in the present study. Bensalem and Chetail, however, used a more extensive range of protein tests in their work and the inner part of the suprapedal gland reacted positively either with the Chevremont and Frederick or the Barrnett and Seligman reactions for protein with S-S groups. So that a protein with sulphide groups could possibly be associated with the acidic and neutral mucopolysaccharides in the inner part of the suprapedal

gland. However, these authors did not find acidic mucopolysaccharides in this part of the suprapedal gland, whereas the histochemical results in the present study show that acidic mucopolysaccharides with both sulphated and carboxylated groups are present. In the present investigation the contents of the inner part of the suprapedal gland stained blue with Alcian Blue at pH0.5 (Plate 5B) and this indicates the presence of sulphate groups in the secretion. Furthermore, the yellow-green staining obtained with Alcian Blue pH 0.5/Alcian Yellow pH2.5 method (Plate 4B) shows that both sulphate and carboxyl groups are present. However, these groups do not show metachromasia in toluidine blue but as mentioned already this could be because the neutral mucopolysaccharides and also the protein (Bensalem and Chetail's result) present in the secretion are suppressing metachromasia.

In their study, Bensalem and Chetail also used the toluidine blue method for the detection of acidic mucopolysaccharides and apparently got negative results. The difference in the result with Alcian Blue can be explained as the fixatives used are different, and if they used the Alcian Blue pH2.5/PAS sequence the results could be confusing as the inner part of the suprapedal gland stained bluish/purple with this method and here the purple colour with PAS stain suppresses the blue staining with Alcian Blue. Therefore, one could interpret the result as the

presence of neutral mucopolysaccharide while being ignorant of the presence of acidic mucopolysaccharides.

In conclusion, the contents of the inner part of the suprapedal gland are probably a mucoprotein type containing protein with sulphide groups associated with neutral and acidic mucopolysaccharides with both sulphated and carboxylated groups and calcium.

However, the main disagreement with the findings of Bensalem and Chetail is that in Pomatias, the sole glands were interpreted as containing developing stages of mucocytes, whilst in the present work they are believed to contain two different gland cell types.

Bensalem and Chetail mentioned that young mucocytes were present at depth in the gland and reacted lightly to the histochemical procedures used, whilst the mature mucocytes were found near the surface with their ducts reaching to the epidermis and they contained the acidic and neutral mucopolysaccharides associated with proteins and calcium ions. The present study however, reveals that the sole glands in Pomatias are not two developmental stages but in fact two different kinds of mature cells. As shown in Figure 36B and Plate 8, the ducts of P5 and P6 cells can be seen reaching to the epidermis and in terms of histochemistry both types show strong but different reactions with all the histochemical procedures used.

The contents of the gland cell type P5 are mucoprotein and contain neutral mucopolysaccharide whilst cell type P6

contain acidic mucopolysaccharides and calcium.

Therefore, the secretion which Bensalem and Chetail found lining the whole sole with a thin film of mucus consisting of acid and neutral mucopolysaccharides, protein and calcium ions is not the product of the mature sole gland cells but is a mixture of the substances secreted by the cell types P5 and P6.

A possible explanation for the histological results obtained by Bensalem and Chetail might be that they studied the sole glands in the longitudinal sections, in which cell type P6 appears to be present laterally to the cell type P5 lying deeper in the musculature and therefore it is difficult to see the ducts of P6 cells reaching to the epidermis, since in longitudinal section complete type P5 cells apparently mingle with the ducts and cell bodies of cell type P6 (Figure 36A and Plate 1A).

3. Comparison of the findings on the pedal and dorsal surface glands of the gastropods in the present study with those of other gastropods.

3.1 Comparison with other prosobranchs.

Gainey (1976) described the pedal glands in Neritina reclivata and Thais rustica and anterior mucocytes were found to be present in both species. These anterior mucocytes could be equivalent to the anterior pedal glands in Bithynia and Littorina since all these are located at the anterior part of the foot. However, histochemically, the anterior mucocytes in Neritina and Thais are different from

those in Bithynia and Littorina. In Bithynia and Littorina the secretion of the anterior pedal gland is always a mixture of carbohydrates combined with protein but the anterior mucocytes in Neritina stained positively with Alcian Blue and those of Thais stained with PAS. However, the exact equivalence of the secretion in these glands cannot be established since very few histochemical techniques were available for the study on Neritina and Thais (Table 18).

Gainey also described the subepidermal mucocytes in Neritina and Thais and these were found to be distributed on the ventral surface of the foot. These subepidermal mucocytes may be equivalent to the sole glands in Pomatias, Bithynia and Littorina. Subepidermal mucocytes in Neritina stained with Alcian Blue and therefore these mucocytes may be histochemically identical to cell types P6 in Pomatias, B2 in Bithynia and L3 in Littorina since these all are pedal gland cell types and contain acidic mucopolysaccharides.

However, the subepidermal mucocytes in Thais stained positively with PAS which indicates the presence of periodate-reactive substance which could be neutral mucopolysaccharide or mucoprotein. Therefore, in terms of histochemistry these subepidermal mucocytes are identical to cell types P5 in Pomatias and L5 in Littorina as these are present on the sole and contain neutral mucopolysaccharide and protein (Table 18).

In Neritina and Thais Gainey described the epidermal mucocytes which are probably similar to the goblet cell types found in Pomatias, Bithynia and Littorina. The epithelial mucocytes in Neritina, however, did not stain with Alcian Blue or PAS and therefore the nature of their contents is not known. In Thais the epidermal mucocytes stained with Alcian Blue and PAS but Gainey did not explain if these two are the same or different types of mucocytes. However, on the basis of the staining properties it seems that the Alcian Blue-positive epidermal mucocytes may be equivalent to goblet cell types P10 in Pomatias, B7 in Bithynia and L9 in Littorina since all contain acidic mucopolysaccharides. The PAS-stained epidermal mucocytes in Thais are probably identical to the goblet cell type P11 in Pomatias, B8 in Bithynia and L10 in Littorina as these are PAS-positive and contain neutral mucopolysaccharides (Table 18).

Grenon and Walker (1978) described the pedal glands in two limpets, Patella vulgata and Acmaea tessulata. Comparison with these marine prosobranchs is rather difficult since the functional anatomy and the distribution of mucus glands is different in these species from Pomatias, Bithynia and Littorina.

However, the gland cell types P2 in Patella and A2 in Acmaea could be equivalent to cell type B3 in Bithynia since all these are sole gland types with agranular contents and

produce acidic and neutral mucopolysaccharides; but in A2 cells of Acmaea a neutral mucopolysaccharide fraction was not present (Table 18).

The gland cell types P6 in Patella and A4 in Acmaea are very much like the cell type L5 in Littorina in appearance, and all contain a granular secretion. In terms of histochemistry, all produce a proteinaceous secretion but in L5 cells neutral mucopolysaccharide is also present (Table 18).

The epithelial mucocytes described by Grenon and Walker (1978) in Patella and Acmaea are very much like the goblet cell types P10 in Pomatias, B7 in Bithynia and L9 in Littorina in appearance and all contain acidic mucopolysaccharides (Table 18). The distribution of these mucocytes in Patella and Acmaea however differs from those in Pomatias, Bithynia and Littorina. In Patella and Acmaea these epidermal mucocytes are present only in the epithelium of the sole, but in Pomatias, Bithynia and Littorina they are usually absent from the sole epithelium and are generally distributed on the dorsal and lateral surfaces.

3.2 Comparison of Limax pseudoflavus with other Pulmonates

3.2.1 Terrestrial pulmonates

Whilst there appears, at first, to be a large volume of information available on which to base comparisons, many studies are not strictly comparable. Previous studies on pulmonates have either been conducted on a narrow range of tissues (Ghose, 1963; Dalal and Pandya, 1976) or have been

conducted with the electron microscope with little regard to the chemistry of the contents of the cell or to their distribution over the surface of the animal (Newell, 1973 and 1977; Wondrak, 1969). Where detailed work has been carried out some comparisons are possible but it must be stressed that because of variations in techniques exact correspondence of staining properties is unlikely.

Bolognani-Fantin and Bolognani (1964) studied Helix pomatia and their description of mucus and protein cells resembles that of L. pseudoflavus (cell types 1,7,2 and 3) except that in H. pomatia all these cells are present on the dorsal surface and the foot but in L. pseudoflavus only cell type 7 is present in the sole (Figure 39). The mucus gland cells were found to contain a sulphate radicle, which is also present in the mucus gland cell types 1 and 7 in L. pseudoflavus.

The protein cells of H. pomatia were found to contain tyrosine and thiol groups. In L. pseudoflavus no protein cells are present in the sole but those on the dorsal surface contain tyrosine (Table 19).

Calcium gland cells which were found in H. pomatia are not present in L. pseudoflavus.

The most interesting point of comparison between the two gastropods lies in what Bolognani-Fantin (1965) called the 'Phenolic cells' in the sole. The phenolic cells could be equated to the yellow granular cells (type 4) in L. pseudoflavus. As in L. pseudoflavus, Bolognani Fantin also

found these cells to be yellow after fixation and their contents reacted negatively to the general protein and mucopolysaccharide tests. However, these authors found them only on the sole whereas in L. pseudoflavus the yellow granular cells are generally distributed (Figure 39 and Table 20).

Campion's work on Helix aspersa (1961) shows that the mucus gland cell type A is comparable with cell type 1 in L. pseudoflavus in their appearance, and histochemically both contain sulphated mucopolysaccharide.

The B mucus gland cells of Helix aspersa (Campion 1961) may be equivalent to type 10 cells in L. pseudoflavus as both are present on the mantle and are periodate-reactive (Figure 39 and Table 19). However Campion also described the pigment cells in Helix aspersa, the pigment being flavone; this may be equivalent to the yellow granular cells (type 4) found in L. pseudoflavus, but the nature of the contents in the type 4 cell of L. pseudoflavus is not known. Therefore, although type 4 cell in L. pseudoflavus resembles in appearance the phenolic cells in Helix pomatia (Bolognani-Fantin and Bolognani, 1964 and Bolognani-Fantin, 1965) and pigment cells in Helix aspersa (Campion, 1961) an exact comparison cannot be made as the phenolic cells in Helix pomatia were found to contain di or poly-phenol (ortho or para) and the pigment in Helix aspersa was found to be a flavone.

Type C gland cell in the foot of Helix aspersa would seem to be equivalent to cell type 9 in L. pseudoflavus.

Histochemically both of these cells show slight reactions with Alcian Blue and toluidine blue; however type 9 cells in L. pseudoflavus are also PAS positive and contain neutral mucopolysaccharide.

The gland cell type D (Campion 1961) in Helix aspersa could be equivalent to the type 10 cell in L. pseudoflavus as both are present on the sole and contain periodate-reactive material (Figure 39, Table 20).

Campion demonstrated lipid in one of the mantle cell types in frozen dried material but this was not detected after formaldehyde-calcium fixation, which normally preserves fats. These findings must be of dubious validity since the tests were not accompanied by adequate controls (e.g. lipase or chloroform/methanol extraction). The results regarding the presence of lipid in L. pseudoflavus are also in some doubt as staining of cell types 2 and 3 is obtained with Sudan Black after Elftman's fixative but not in frozen sections.

Only one cell type described by Campion is radically different from those in L. pseudoflavus. These are the calcic glands in Helix aspersa. Clearly the role of calcium in Helix is different from that in L. pseudoflavus. The dorsal mucus of H. aspersa is milky due to presence of calcium whilst that of L. pseudoflavus is never milky.

Elves's (1961) general description of the foot sole of Discus rotundatus is much like that of L. pseudoflavus, though 'amoebocytes' described by him are not observed.

Also he believed that some of the mucus gland cells discharged into intercellular spaces in the foot before final discharge, whilst those nearest the surface discharged through ducts. Only discharge through ducts is seen in L. pseudoflavus.

The aggregation of mucus gland cells in the vicinity of the peripodal groove is present in both animals and Elves thought that peripodal mucus gland cells resembled the pedal gland mucocytes (Figure 41). In L. pseudoflavus however, these two (cell types 6 and 8) are entirely different (Figure 40A).

The suprapedal gland in Discus is composed of two cell types, one type being in the discharged state and the other in a developing or discharging state, but in L. pseudoflavus only one cell type is present.

The suprapedal gland in L. pseudoflavus is unlike that in Milax sowerbii described by Barr (1926). In the latter the gland is free in the body cavity and anchored to the foot by only a few small muscles, whilst in L. pseudoflavus the gland is embedded in the tissues and musculature of the foot. Moreover, the gland duct in M. sowerbii shows secondary branching which is not seen in L. pseudoflavus.

One last point of difference lies in the finding of crystalline concretions in the gland duct of M. sowerbii which were believed to be an excretory product, but these are not present in L. pseudoflavus.

In comparing the pedal glands of Arion ater (Barr

1928) and L. pseudoflavus, there are five points of difference. One is that Barr found some glandular cells in the upper epidermis of A.ater, whilst the upper epidermis in the pedal gland of L. pseudoflavus does not appear glandular (Figure 40B). The second difference is that the contents of the gland mucocytes are described as either granular or vacuolated whilst in L. pseudoflavus the pedal gland mucocytes have a reticular appearance.

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 L. pseudoflavus, but others discharge their secretion into intercellular spaces and thence into the gland duct.

The fourth point of difference is that Barr described the whole of the foot sole and foot fringe as being ciliated whereas in L. pseudoflavus only a median band of cilia appears along the foot sole. Nor are calcic gland cells present in L. pseudoflavus, as they are in A. ater.

Arcadi's (1963) description of the basket cell complexes in Lehmania poirieri appear to be similar to the type 1 cell in L. pseudoflavus but their histochemistry differs to some extent (Table 19). The mucus was found to be intensely PAS positive in L. poirieri whereas in L. pseudoflavus the dorsal mucus gland cells (cell type 1) are only slightly PAS-positive. However, both cells are alcinophilic.

The second cell type described was the granular cell

complex of the sole and again in appearance it is much like the type 10 cell in L. pseudoflavus. In terms of histochemistry both types are periodate-reactive. Moreover, these two cell types are similar in distribution as both are present in tissues other than those of the sole (Figure 39).

Chetail's and Binot's (1967) description of the pedal gland of Arion rufus is similar to some extent to that of L. pseudoflavus in the present study. They divided the gland into anterior lateral, anterior median and posterior regions. In L. pseudoflavus, although lateral and median regions are present no distinction in cell types can be made. Correspondingly, they described three cell types in A. rufus a1, a2 and b. Type a1 cells in A. rufus can be easily identified as equivalent to the suprapedal gland mucocytes (cell type 6) in L. pseudoflavus (Figure 40B). The contents of both types are periodate-reactive and contain neutral mucopolysaccharide but the lipid component which was found in a1 cells is not evident in the cell type 6 of L. pseudoflavus (Table 20). No equivalents to types a2 and b appear to be present in L. pseudoflavus.

In this same paper, they also described the gland cells of the sole. The type I mucus gland cell appears to be similar to cell type 10 in L. pseudoflavus in structure and to some extent in histochemistry (Table 20), since both contain neutral mucopolysaccharide, but again lipid was found to be present in the type I cell in A. rufus but not in type 10 cells in L. pseudoflavus. Type II mucus gland

cells are similar in distribution, physically and to some extent histochemically to the cell type 9 in L. pseudoflavus (Table 20). Both are the most numerous cell types in the sole and are present throughout the foot and these types show less intense staining compared to other cell types in the foot. The type III mucus gland cells of A. rufus are very similar to the cell type 13 in L. pseudoflavus. Both types are present at the anterior end of the foot (Figure 39) and have granular contents that are composed of protein. However, there appears to be no equivalent in L. pseudoflavus to the cell type IV mucus gland cells of A. rufus which contains protein and metachromatic component.

In the mantle of A. rufus the M2 mucus gland cell appears similar to the cell type I in L. pseudoflavus but its histochemistry is different. Whilst both types contain acidic mucopolysaccharides there is no evidence of protein, neutral mucopolysaccharide or lipid in type 1 cells of L. pseudoflavus (Table 19).

Another point of difference is that Chetail and Binot (1967) demonstrated lipid in the pedal gland cell types a1, a2 and sole types I and III though only after wax impregnation and the tests were not accompanied by adequate controls (e.g. lipase or chloroform/methanol extraction). However, no lipid is evident in cell types of L. pseudoflavus equivalent to these cell types in A. rufus.

Wondrak (1969) also studied the gland cell types in Arion rufus and though the descriptions of the cells in Wondrak's

and Chetail and Binot's work agree to some extent, there are however, a few differences. Wondrak described only two types of gland cells in the sole of A. rufus whilst Chetail and Binot described four types and again Wondrak noticed the concentration of gland cells in the region of the peripodal groove but Chetail and Binot failed to do so.

Lawrence's (1972) description of the type M cell in Arion hortensis is almost identical to cell type 1 in L. pseudoflavus, both being present at the dorsal and lateral body surfaces and containing sulphated mucopolysaccharide (Table 19). The m cells in A. hortensis appear physically similar to the type 7 cells in L. pseudoflavus, but their distribution and histochemistry differs. Type 7 cells are distributed on dorsal and lateral body surfaces and the sole in L. pseudoflavus (Figure 39) but m cells were never found in the sole. M and m types in A. hortensis were believed to be different stages of one cell type.

The protein gland cells in A.hortensis appear fundamentally similar to the type 3 cells in L. pseudoflavus and both contain tyrosine. In A. hortensis, however, Lawrence noticed the presence of yellow pigment in the matrix of the protein cells whereas in L. pseudoflavus protein and yellow pigment are present in two different cell types.

In the sole Lawrence found 3 cell types A, B and C but again she believed that A and B were different stages of the same types of cells. However, no equivalent to A/B cell

types is present in L. pseudoflavus. Cell type C in A. hortensis could be equivalent to cell type 10 in L. pseudoflavus, both types are less numerous in the sole and have granular contents. However, the type C cell in A. hortensis has a mucoprotein type of secretion whilst cell type 10 in L. pseudoflavus produces neutral mucopolysaccharide (Table 20).

Lawrence also noticed the concentration of gland cells in the region of the peripodal groove which would seem to be similar to the cell type 8 in L. pseudoflavus (Figure 40A) but their histochemistry differs. Both contain acidic mucopolysaccharides, but in L. pseudoflavus neutral mucopolysaccharide is also present whilst in A. hortensis the acidic mucopolysaccharide is combined with protein (Table 20).

In comparing the suprapedal gland of A. hortensis and L. pseudoflavus the general description is similar, however two cell types are found in A. hortensis containing metachromatic and nonmetachromatic components but only one cell type is present in L. pseudoflavus and produces weakly acidic and neutral mucopolysaccharides (Figure 40B).

The suprapedal gland in Veronicella floridana (Cook, 1987) contained two cell types. V1 cells secreted the protein and showed slight metachromasia in toluidine blue. No equivalent to this cell type is present in Limax pseudoflavus. However, cell type V2 resembled to some extent morphologically and histochemically cell type 6 of

Limax pseudoflavus and both contain neutral mucopolysaccharide (Table 20).

In Veronicella, there is no specialization at the leading edge of the foot as in Limax pseudoflavus but the cell types V1 and V2 were present dorsally in this area.

The foot structure in Veronicella is unlike that of other pulmonate slug species including Limax pseudoflavus. In Veronicella the foot possesses transverse ridges and four types of gland cells are associated with each ridge. V3 and V4 gland cell types contained carboxylated mucopolysaccharides and Cook suggested the possibility that the apparent V4 cells might be the inflated ducts of cell type V3. These gland cells could be, to some extent, equivalent histochemically to cell type 9 in Limax since these cells contained carboxylated mucopolysaccharides (Table 20) but in Limax pseudoflavus carboxylated groups are associated with neutral mucopolysaccharide and in terms of morphology, type 9 cells exhibit long and thin ducts in contrast to V3 cells which possess thick ducts.

Cell type V5 in Veronicella would seem to be equivalent to cell type 10 in Limax histochemically, but differs in distribution as type 10 cells in Limax are present in the tissues other than those of the sole and in appearance . However, both contain neutral mucopolysaccharide (Table 20). No equivalent to cell type V6 in Veronicella with glycoprotein appears to be present in the sole of Limax.

At the foot margin in Veronicella cell types V6, V7 and V8 were present. V6 and V7 produced glycoprotein and neutral, carboxylated mucopolysaccharides respectively. However, the nature of the contents in V8 cells is unknown as they failed to stain with any procedures used. These foot margin gland cells in Veronicella could be equated to the cell types 7, 8, 9 and 10 in Limax, since they all are present in the equivalent area of Limax. However, the histochemical nature of the pedal mucus in this area differs considerably. In Limax the glycoprotein component which is found to be present in Veronicella is not evident, and in addition to neutral and carboxylated mucopolysaccharides, sulphated mucopolysaccharides are also present in cell type 7 of Limax (Table 20).

In conclusion, it seems that the pedal mucus in Limax and Veronicella is a mixture of protein and neutral mucopolysaccharide associated with acidic mucopolysaccharides. In Limax the acidic mucopolysaccharide is composed of both sulphated and carboxylated groups but in Veronicella only carboxylated groups are evident. In this respect however Veronicella differed from all the other gastropods compared as in them sulphated mucopolysaccharide is always found to be present in the pedal mucus.

Comparing the mucus cells on the dorsal body surface in Limax and Veronicella, it appears that these cells are different in number, size, organization and the

histochemical nature of their contents.

In Limax mainly four cell types are present on the dorsal surface (cell types 1, 2, 3 and 7; Figure 39), whilst in Veronicella there were three cell types (V9, V10 and V11), but Cook (1987) believed that V9 and V11 were the same cell types but in different stages of secretion. In both cases, cell types V9 and V11 produced carboxylated mucopolysaccharide and these cell types could be equivalent to cell type 1 in Limax. However, Limax cell type 1 contains both sulphated and carboxylated groups (Table 19).

Cell type V10 in Veronicella would seem to be to some extent equivalent to cell types 2 and 3 in Limax histochemically. In all cases the secretion is proteinaceous; however, Limax cell types 2 and 3 contain protein with tyrosine whilst the tyrosine groups are not evident in V10 cells of Veronicella since they failed to stain with the diazotization coupling reaction but stained with PAS and toluidine blue (Table 19).

Therefore, in terms of histochemistry the dorsal mucus in these two terrestrial slugs is composed of carboxylated mucopolysaccharide and protein but in Limax sulphated groups are also present. However, these variations in number, size, cellular organization and the histochemical nature of the contents in the gland cell types present on the dorsal surface of Limax pseudoflavus and Veronicella would

seem to be related to the fact that in Limax the dorsal, viscous mucus is freely distributed over the exposed surfaces, but in Veronicella the comparatively small amount of mucus is held in reserve in the pits giving the surface generally a dry and leathery appearance.

It can be concluded therefore, that whilst Limax and Veronicella are both terrestrial slugs, their mucus-producing glands are very different both in their organization and in the type of mucus produced on the dorsal and pedal surfaces. The sulphated mucopolysaccharide component and tyrosine containing protein which are present in the pedal and dorsal mucus in Limax were not evident in the mucus of Veronicella.

Thus, Limax pseudoflavus appears to resemble closely the other pulmonate slug Arion in terms of the distribution of mucus gland cells and the histochemical nature of their secretion.

3.2.2 Comparison with the fresh water pulmonates

It was thought that the cell types in Lymnaea stagnalis could be comparable to the gland cell types in Limax, Pomatias, Bithynia and Littorina. Therefore as Zylstra (1972) used relatively few techniques for his investigations on Lymnaea, the cell types in Lymnaea were studied again using the same histochemical techniques which have been applied in the present investigations in order to get an equivalent picture of Lymnaea (Tables 21 and 22).

The epidermal goblet cell type 1 in Lymnaea would seem to be equivalent to the goblet cell types P11 in Pomatias, B8 in Bithynia and L10 in Littorina, morphologically and to some extent histochemically (Figures 36, 37, 38 and 42, Plate 64A). Zlystra suggested that the secretion of the goblet cell type in Lymnaea is of a nonmucin type, but the tests in the present study confirm that it is proteinaceous, containing tyrosine and associated with calcium (Tables 21, 22 and 23). In the goblet cells of Pomatias (P11), Bithynia (B8) and Littorina (L10) the protein component is combined with neutral mucopolysaccharide.

Zylstra named the two ubiquitous subepidermal gland cell types as 'muciparous gland cell' and 'nonmuciparous gland cell' but the histochemical tests applied to these gland cells in the present study show that the 'nonmuciparous cell type' is actually 'muciparous' as the protein in this cell is associated with carboxylated and neutral mucopolysaccharides. Therefore here it is named as 'muciparous cell type II' (Tables 21 and 22 and Plate 64B and C). No equivalent to the ubiquitous muciparous cell types I and II is found in Pomatias, Bithynia or Littorina.

However, the ubiquitous muciparous type I cell in Lymnaea could be equated to the cell type 7 in Limax (Figures 39 and 42), since these cells are generally distributed and produce sulphated and carboxylated mucopolysaccharides (Plate 65A). In Lymnaea, however, the muciparous type 1 cells are also PAS-positive and contain

neutral mucopolysaccharide (Table 23 and Plates 64 and 65B).

As far as pedal glands in Lymnaea are concerned they show some similarity with Bithynia and Littorina. Two types of anterior pedal glands are present in Lymnaea which may be equated histologically to the anterior pedal glands found in Bithynia and Littorina (Figures 37, 38 and 42).

One of the anterior pedal glands in Lymnaea is composed of cells with an oval cell body and the long ducts and their contents are reticulate (type 11); this gland may be identical to the anterior pedal gland (cell type B1) in Bithynia. The other anterior pedal gland cell type in Lymnaea (type 10), with granular contents, appears to be similar to the anterior pedal gland cell type L2 in Littorina.

Histochemically, however, they are different. In Bithynia and Littorina the anterior pedal glands contain mainly protein and neutral mucopolysaccharide with some acidic mucopolysaccharide. In Lymnaea, however, the anterior pedal glands contain mainly acidic mucopolysaccharide with some neutral mucopolysaccharide but no protein fraction is present (Tables 21, 22 and 23).

The lateral foot edge gland cells in Lymnaea could be equivalent to the B3 cell type in Bithynia, at least in terms of histochemistry, since in both cases sulphated, carboxylated and neutral mucopolysaccharides are present.

4. Comparison of the mantle edge structure of the prosobranchs Pomatias elegans, Bithynia tentaculata and Littorina littorea.

In gastropods the mantle edge is most often a simple, perhaps swollen projection in cross-section. This projection may be separated from the shell-secreting dorsal surface either by a supramarginal/periostracal groove, the epithelium of which may or may not be glandular in appearance; or by a shallow groove and rows of ducts issuing from large subepithelial cells; or simply by the rows of ducts (Stasek and McWilliams 1973). Hyman (1967) summarized information relating to the mantle edge of prosobranchs and concluded that a supramarginal groove occurs in relatively few taxa, but was also found in the primitive opisthobranchs and in shelled pulmonates. This broad taxonomic distribution among the more primitive taxa suggests that the presence of a defined supramarginal groove is not necessarily common among the gastropods but is an original feature of the class.

However, the supramarginal groove is not present in the mantle of the three prosobranchs considered in the present study, Pomatias, Bithynia and Littorina. There is a general similarity but also some variation is present in the structure of the mantle edge of Pomatias, Bithynia and Littorina. However, these are to be expected since they come from different habitats and taxa.

The dorsal mantle epithelium in Pomatias and Bithynia is made up of small, cuboidal epithelial cells and is devoid of any type of secretory cells (Figures 43 and 44). In Littorina, however, the dorsal mantle epithelium is made up of tall columnar cells and interspersed between these are subepidermal mucus cells (type L11) which produce sulphated and neutral mucopolysaccharide (Figure 45, Table 24A).

However, the structure of the ventral mantle epithelium in Pomatias, Bithynia and Littorina is similar. In all cases, the ventral mantle epithelium is made up of tall, columnar epithelial cells, amongst which are the ubiquitous goblet cell types, four types in Pomatias, two types in Bithynia and two types in Littorina. Although the mantle edge glands all open at the dorsal aspect of the mantle edge in Pomatias, Bithynia and Littorina their number, morphology and the chemical nature of their secretions differ (Figures 43, 44 and 45 and Table 24 A, B, and C).

In Pomatias, two types of glands open at the dorsal side of the mantle edge. The type P14 mantle edge gland could be comparable to some extent physically and histochemically to the mantle edge gland cell type L13 in Littorina. In both cases, the gland cells have a reticulate appearance and produce acidic mucopolysaccharides (Table 24B).

The mantle edge gland cell type P15 in Pomatias could be equated to the cell type L12 in Littorina and B10 cells of the mantle edge gland in Bithynia. The cells in

all these glands are somewhat oval and have reticular contents. In terms of their histochemistry, all produce proteinaceous secretions. In Pomatias, and Littorina the protein is combined with neutral mucopolysaccharide. In Bithynia neutral mucopolysaccharide was not evident, but the reaction with PAS suggests the possibility of its presence in the secretion (Table 24C).

Bevelander and Nakahara's (1970) general description of the mantle edge glands in L. littorea resemble that of the present study. However, they divided the mantle edge gland type L12 into two gland types and called them the dorsal and ventral glands. Bevelander and Nakahara mentioned that these glands were structurally similar and contributed towards the formation of inner and outer layers of the periostracum respectively, but these authors did not report any differences in morphology especially in vesicular secretory contents.

In the present study, however, these two gland types could not be differentiated either structurally or histochemically and cells of L12 responded similarly to all the histochemical procedures used.

Bevelander and Nakahara did not give any evidence or suggestion for the role of the gland cell type 3 (type L13 in the present study) nor did they mention the subepidermal cells (type L11) on the dorsal mantle epithelium of Littorina littorea (Figure 45).

5. Comparison of the mantle edge structure of Pomatias, Bithynia and Littorina with other gastropods.

5.1 Comparison with other prosobranchs.

Walsh's (1981) description for the subepidermal gland cells on the outer mantle epithelium of Busycon canaliculatum and Busycon carica is almost identical to the cell type L11 on the dorsal mantle epithelium of Littorina littorea. In all cases, they are subepidermal and produce sulphated mucopolysaccharides (Figure 45, Table 25A). In Littorina, however, the neutral mucopolysaccharide is associated with sulphated mucopolysaccharide.

The ventral mantle epithelium in B. carica and B. canaliculatum closely resembles the ventral mantle epithelium in Pomatias, Bithynia and Littorina (Figures 43, 44 and 45). In all these prosobranchs the goblet cells are present on the ventral mantle epithelium and contain PAS-positive substance and acidic mucopolysaccharides. In Pomatias, Bithynia and Littorina, however, these two components are present in separate goblet cell types. Whether that is also the case in B. canaliculatum and B. carica is not made clear by Walsh. The goblet cells with acidic mucopolysaccharides could be equivalent to the goblet cell types P10 in Pomatias, B7 in Bithynia and L9 in Littorina. The PAS-positive goblet cells appear to be similar to the goblet cell types P11 in Pomatias, B8 in Bithynia and L10 in Littorina since these cells produce

neutral mucopolysaccharide. The supramarginal glands in B.canaliculatum and B. carica could be equated to the gland type P15 in Pomatias and type L12 in Littorina (Figures 43 and 45). In all cases these glands are present at the mantle edge and produce a neutral mucoprotein type of secretion. The distal supramarginal gland at the mantle edge in B. canaliculatum and B. carica could be comparable to the mantle edge gland type P14 cells in Pomatias and type L13 in Littorina. In terms of histochemistry, in both Busycon species the sulphated mucopolysaccharide is combined with protein (Table 25B).

However, no equivalent to the subepidermal cells in the inner mantle epithelium of B.canaliculatum is present in Pomatias, Bithynia or Littorina.

The general morphology and the histochemistry of the mantle edge in Pila virens (Umadevi et al. 1984) differ from that of Pomatias, Bithynia and Littorina (Figures 43, 44, 45 and 46, Table 25A). Firstly, the supramarginal groove was found to be present in Pila but is not present in Pomatias, Bithynia and Littorina. Secondly, the epidermal mucocytes with highly sulphated and weakly carboxylated mucin were found to be present on the dorsal and ventral epithelium of the mantle, whilst in Pomatias, Bithynia and Littorina the epidermal mucocytes (goblet cell type P10, B7 and L9) with an acidic mucopolysaccharide type of secretion occurring only on the ventral epithelium (Figures 43, 44 and 45). Lastly, no equivalent to the goblet cells in Pomatias, Bithynia and

Littorina with the mucoprotein type of secretion (cell type P11, B8 and L10) was found in Pila.

Umadevi et al.'s description of the shell gland in Pila closely resembled that of the mantle edge gland (B10) in Bithynia (Figures 44 and 46) but the histochemistry differed. In both cases these glands produce a proteinaceous secretion but in Pila the shell gland also secreted the highly acidic mucopolysaccharides, lipid and calcium (Table 25B).

These authors demonstrated the presence of lipid and calcium in the shell gland, though only after wax impregnation. In neither case, however, were the tests accompanied by adequate controls (e.g. lipase or chloroform/methanol extraction for lipids and HCl extraction for calcium) nor was the recommended fixative for calcium (95% Alcohol) used.

In Pomatias and Littorina the mantle edge glands produce acidic mucopolysaccharide and protein but in both cases they are secreted by different types of glands and not in a mixture from the same glands as in Pila.

The most interesting point is that protein and neutral mucopolysaccharides are said to be the important components of the periostracum secreted by the mantle edge (Meenakshi et al. 1969). However, in Pila neutral mucopolysaccharide was not evident in the secretions at the mantle edge (Table 25B).

5.2 Comparison of the mantle edge structure of Pomatias, Bithynia and Littorina with shelled pulmonates.

5.2.1 Terrestrial pulmonates

Saleuddin (1970) studied the structure and histochemistry of the normal mantle in Helix pomatia and in 1976 he carried out ultrastructural studies on the formation of the periostracum in Helix aspersa. In both cases he described briefly the structure of the mantle edge.

The general morphology and histochemistry of the mantle edge in these two species of Helix seem to be different from those of Pomatias, Bithynia and Littorina (Figures 43, 44, 45 and 47, Table 26). The periostracal groove (supramarginal groove) was found to be present in both Helix species but it is apparently not present in Pomatias, Bithynia and Littorina. In Helix pomatia and Helix aspersa many mucus cells were found to be located beneath the ventral mantle epithelium, whereas in Pomatias, Bithynia and Littorina all the mucocytes on the ventral mantle epithelium are goblet cell types (Figures 43, 44 and 45).

The morphology of the periostracal gland in Helix is very different from that of the mantle edge glands in Pomatias, Bithynia and Littorina.

The periostracal gland at the mantle edge in Helix pomatia and Helix aspersa is made up of pyramidal-shaped epithelial cells (Saleuddin 1976) (Figure 47). The mantle edge glands in Pomatias, Bithynia and Littorina are compound

glands which are composed of numbers of subepidermal cells with a distinct cell body and a long duct leading to the epidermis (Figures 43, 44 and 45). In terms of histochemistry, the periostracal gland of Helix pomatia stained positively only for RNA, but tests for RNA have not been employed in the present study.

5.2.2 Freshwater pulmonates

Zylstra (1972) described four types of gland cells (types 4, 5, 6 and 7) at the inner mantle epithelium of Lymnaea stagnalis, and two types in Biomphalaria pfeifferi. Again, histologically these gland cells are different from the epidermal goblet cell types at the ventral mantle epithelium in Pomatias, Bithynia and Littorina, since the gland cells in Lymnaea and Biomphalaria were embedded in the connective tissue beneath the ventral mantle epithelium (Figure 48A). In terms of histochemistry, however, these gland cell types would seem to be comparable (Table 27). The mantle muciparous gland cells in Lymnaea and Biomphalaria secreted sulphated and carboxylated mucopolysaccharides and therefore may be equivalent to the goblet cell types P10 in Pomatias, B7 in Bithynia and L9 in Littorina which produce acidic mucopolysaccharides. The mantle nonmuciparous cell types in Lymnaea and Biomphalaria produced a proteinaceous secretion and would probably be similar to the goblet cell type P11 in Pomatias, B8 in Bithynia and L10 in Littorina which secrete protein and neutral mucopolysaccharide.

The morphological features of the mantle edge in Lymnaea and Biomphalaria (Zylstra et al. 1978) differed from those of Pomatias, Bithynia and Littorina (Figure 48B and Plate 66). The periostracal groove was found to be present in these two fresh water pulmonates and seven different gland cell types in Lymnaea and 3 types in Biomphalaria were located below the periostracal groove. Zylstra et al. mentioned that although these gland cells showed only a few histochemical differences they differed ultrastructurally. The gland cell types g3, g4, g5 and g6 at the periostracal groove in Lymnaea, could be equivalent to the mantle edge gland type P14 in Pomatias, and L13 in Littorina. Since all are present at the dorsal aspect of the mantle edge and in terms of histochemistry, the secretion produced by all these gland cells is acidic mucopolysaccharide (Table 27).

At the mantle edge in Helisoma the mucus cells were found to be present at the base of the periostracal groove (Saleuddin, 1979). These mucus cells would seem to be comparable to the mantle edge gland cell types P14 in Pomatias and L13 in Littorina (Figures 43, 45 and 49, Table 27) since all mucus cells are located at the mantle edge region and in Pomatias and Littorina they produce acidic mucopolysaccharides.

In conclusion, it seems that the mantle edge structure of the three prosobranch species Pomatias, Bithynia and Littorina is very similar with only very few differences and closely resembles the mantle edge structure of Busycon

canaliculatum and Busycon carica. However, the mantle edge structure of Pomatias, Bithynia and Littorina differed considerably from that of Pila virens and is very different from that of the shelled pulmonates.

All these variations would seem to be related to the fact that in Pomatias, Bithynia, Littorina, Busycon canaliculatum and Busycon carica the supramarginal groove is not present at the mantle edge. In contrast, however, in Pila and in all the shelled pulmonates the supramarginal groove was found to be present at the mantle edge.

6. Comparison of the hypobranchial glands of Pomatias, Bithynia and Littorina with other prosobranchs.

In comparison with the foot and mantle relatively very few studies have been carried out on the histology and histochemistry of the hypobranchial gland in gastropods. This gland is present in almost all prosobranchs but its morphology, cytology and the nature of its secretion varies betwen species.

Ronkin's (1952) description of the two cell types in the hypobranchial gland of Busycon canaliculatum may be comparable histochemically to cell types L14 and L15 in the hypobranchial gland of Littorina littorea. All are epidermal goblet cell types and secrete acidic mucopolysaccharides (Table 28).

In many respects the morphology and histochemistry of the hypobranchial gland in Littorina closely resembles the hypobranchial gland in Buccinum undatum (L). Hunt's (1967)

description of the morphology of the hypobranchial gland cell types A and B in Buccinum is almost identical to the hypobranchial gland cell types L15 and L14 respectively found in Littorina (Figures 52 and 53). In terms of histochemistry, however, they differed slightly (Table 28). Although the contents of all these cells stained strongly with Alcian Blue and toluidine blue and thus indicated the presence of acidic mucopolysaccharides, cell types A and B in Buccinum also reacted slightly to tests for protein.

The goblet cells (type C) in the hypobranchial gland of Buccinum undatum (Hunt, 1973) appear to be similar to the hypobranchial gland cell (type L16) in Littorina. In both cases the secretion is proteinaceous but the contents of the goblet cells in Buccinum also stained with Alcian Blue (Table 28), indicating the presence of acidic mucopolysaccharides.

In the hypobranchial gland of Paludina vivipara (Ottaviani, 1978) the mucocytes were found to be present in the rectal, branchial and central parts of the hypobranchial gland. These mucocytes seem to be comparable in distribution and appearance to the ubiquitous goblet cell types (B7 and B8) which occur sparsely in the hypobranchial gland of Bithynia tentaculata (Figure 51). However, they differ histochemically. In Paludina vivipara these mucocytes stained with PAS and with Alcian Blue pH2.5. In

Bithynia, however, alcinophilic and PAS positive material are present in two different goblet cell types.

Two cell types which were found to be present in the central area of Paludina vivipara appear comparable to the hypobranchial gland cells types B11 and B12 in Bithynia. Cell type 1 in Paludina and type B12 in Bithynia produce a proteinaceous secretion combined with neutral mucopolysaccharide. The second type found in Paludina may be equivalent to the hypobranchial gland cell type B11 in Bithynia, since in both cases their proteinaceous fraction is combined with acid mucopolysaccharide (Table 28) but in Bithynia neutral mucopolysaccharide is also present.

Bolognani-Fantin and Ottaviani (1981) described four secretory cell types in the central part of the hypobranchial gland in Murex brandaris. These appear to be comparable to the hypobranchial gland cell types in Pomatias, Bithynia and Littorina.

The cells with the large granular secretion (type 1) in Murex could be equivalent to the cell type P12 in Pomatias and the cell type B12 in Bithynia (Figures 50 and 51). In terms of histochemistry all are PAS-positive and produce a proteinaceous secretion (Table 28). In Bithynia and Pomatias however, the protein is combined with neutral mucopolysaccharide.

The fine granular cells in Murex (type 2) may be identical to cell type L16 in Littorina. Both are PAS-positive and contain protein. In Littorina, however,

neutral mucopolysaccharide is also present.

Mucocytes in the hypobranchial gland of Murex may be equivalent to cell types L14 and L15 in Littorina since all are alcinophilic and produce acidic mucopolysaccharides (Figure 52; Table 28).

In the same paper Bolognani-Fantin and Ottaviani described the hypobranchial gland in Viviparus viviparus. The hypobranchial gland cell type B12 in Bithynia containing protein and neutral mucopolysaccharide would seem to be equivalent to cell type 1 in Viviparus viviparus which contained glycoprotein, whilst the second type could be equated to the ubiquitous goblet cell type B7 in Bithynia. In both cases, the secretion is acidic mucopolysaccharide (Table 28).

These authors described only the central part of the hypobranchial gland of Pomatias elegans and found one cell type. In the present study, however, the hypobranchial gland was found to be present at the centre and also towards the rectal area of the mantle cavity of Pomatias and three ubiquitous goblet cell types are present (Figure 50). The cell type P12 with the globular contents are PAS-positive and their secretion is a mucoprotein. This cell type P12 could be the equivalent of the hypobranchial cell type which Bolognani-Fantin and Ottaviani identified, as their cells were also PAS positive, but in contrast the cells they described had finely granular contents. However, this could result from the use of different fixatives, except that

unlike cell type P12 these cells also reacted negatively for protein tests and positively for lipid tests. It is necessary to add that controls for lipid tests were not used by these authors (Table 28).

As seen from the comparison of the results of the present study with those of past workers, some types of cells are found to be comparable, whilst others are different to varying extents. Thus, there are some differences of opinion concerning the findings of both Chetail and Binot (1967) and Wondrak (1969) on Arion rufus, Bensalem and Chetail (1982) and the present study on Pomatias pedal glands and Bolognani-Fantin and Ottaviani (1981) and the present study on Pomatias hypobranchial gland. It can be difficult to make comparisons when different terminologies, fixatives and histochemical tests are used by various authors, and adequate controls are not used.

IX DISCUSSION

This study has attempted to examine the glandular cells that contribute to the mucus covering of the foot, the subopercular cleft, the mantle and the hypobranchial gland of prosobranchs from different habitats, i.e. Pomatias elegans (terrestrial), Bithynia tentaculata (freshwater) and Littorina littorea (marine) and to define the nature of their secretion using the same histochemical techniques on each in an attempt to relate the different gland cells to the varying physiological and environmental requirements of each species.

The structure and histochemistry of the gland cells of the terrestrial pulmonate slug Limax pseudoflavus have also been investigated and the glandular cells associated with the epithelium of the fresh water pulmonate Lymnaea stagnalis were re-examined using the same histochemical techniques in order to compare these terrestrial and freshwater pulmonates with the prosobranchs. Whilst Pomatias has attracted considerable attention, there were no comprehensive studies of Littorina, Bithynia or Limax pseudoflavus. The functions of the mucus produced by these gastropods have already been considered (Chapter VII, Section C).

There is no obvious and consistent difference in the literature concerning the nature of slug and snail mucus. However, the present study reveals that there are variations

in the form and frequencies of the glandular cells distributed on the dorsal body surface of the gastropods studied and the nature of their secretion also differs considerably.

In the terrestrial pulmonate slug Limax pseudoflavus the large and numerous subepidermal gland cells are present on the dorsal body surface, whilst in the same area of the freshwater pulmonate snail Lymnaea stagnalis the subepidermal gland cells are still present but less numerous. In contrast, the three prosobranch species studied have only epidermal goblet cells which are generally distributed on the dorsal body surface.

In terms of its histochemistry, the general body mucus in Limax mainly consists of sulphated and carboxylated mucopolysaccharides combined with protein, whilst in Lymnaea the sulphated, carboxylated and some neutral mucopolysaccharide is associated with protein. The secretion of the goblet cells of the prosobranchs consist of acidic and neutral mucopolysaccharide combined with protein. Thus, the neutral mucopolysaccharide component that is found to be present in the dorsal 'snail' mucus is absent from Limax body mucus.

It seems likely that these variations in the form and frequency of the cell types and the nature of their secretion in these gastropod species would be related to their structural variations and the difference in the role of the mucus in these molluscs.

The role of the dorsal body mucus in most of the terrestrial pulmonate slug species including Limax pseudoflavus (Order Stylommatophora) would probably differ from that of the terrestrial slug Veronicella floridana (order Systellommatophora). The main difference between the dorsal body surface of Limax and Veronicella (Cook 1987) is that the dorsal mucus of Limax is viscous and freely distributed over the exposed surfaces, whilst that of Veronicella is held in reserve in pits giving it a dry and leathery appearance. The histochemistry of the dorsal mucus in these two slugs also differs. In Limax the general body mucus consists of sulphated and carboxylated mucopolysaccharides associated with protein but in Veronicella the dorsal body mucus was composed of only carboxylated mucopolysaccharides and glycoprotein while the sulphated groups were not found in Veronicella. This difference in the histochemistry of the dorsal mucus in Limax and Veronicella would seem to be related to the different functions performed by the mucus in these two molluscs.

In Limax, the dorsal viscous body mucus may keep the skin moistened and clear of debris as well as allowing some cooling of the body by the latent heat of evaporation, and the viscosity of the dorsal mucus in Limax is presumably due to the presence of acidic mucopolysaccharide in the mucus as the viscosity of mucus is said to be related to its acidity (Hunt, 1973). Whereas in Veronicella (Cook, 1987) a special

type of cellular organization is present on the dorsal body surface and most of the mucus empties onto the lateral aspects of the dorsal and ventral surfaces. Therefore very little mucus is deposited on the dorsal surface giving Veronicella a dry and leathery appearance. Evans (1953) mentioned that the leathery skin of Alderia modesta (Loven) is probably an adaptation to prevent desiccation. This may also be true in the case of the tropical slug Veronicella. It might explain as well why Veronicella need not be covered by a large amount of viscous mucus as in Limax, and again this will justify the absence of sulphated mucopolysaccharides in Veronicella dorsal mucus.

Cook (1987) noticed that in a stimulated Veronicella a copious and highly viscous mucus was produced and he further mentioned that the physical properties of gastropod mucus cannot be predicted by considering their staining properties alone. Sulphated mucopolysaccharides were not found to be present in the dorsal mucus glands of Veronicella and according to Cook therefore the dorsal mucus should not have been of a viscous nature. However, the following suggestions would probably explain the source of 'viscous' mucus in a stimulated Veronicella.

Firstly, Thompson (1960) noticed that in a number of prosobranchs and pulmonates a strong acid was secreted by the animals after an abrupt disturbance, as a defensive adaptation.

Secondly, the properties of viscous liquids are said to

arise because of the presence of highly charged linear macromolecules, e.g. hyaluronic acid with uronic acid carboxyl groups or the epithelial sialomucins (Hunt and Jevons, 1965; Hunt, 1970). The histochemical tests carried out on the dorsal mucus glands in Veronicella showed that they contained only carboxylated mucopolysaccharide (Cook 1987) and their strong positive reactions with Alcian Blue at pH2.5 indicates the presence of hyaluronic acid.

It seems therefore that Veronicella produces copious, viscous mucus only when stimulated and this might be as a defensive adaptation. Hence the structural viscosity of the mucus would appear to result from molecular interactions between the carboxylated mucopolysaccharides, that is, hyaluronic acid and the glycoprotein secreted by the dorsal surface mucus glands. This difference in the cellular organization and the nature of the secretory products of the dorsal body mucus glands in Limax and Veronicella would probably be related to their different environmental, ecological and physiological requirements.

Thus, it seems that the structure of the foot and its associated glands in the gastropods studied is also clearly adapted to their different modes of life and their consequent physiological and environmental requirements.

The main difference found between the foot structures of terrestrial and aquatic forms is the absence of compound suprapedal glands in the latter. The histochemical nature of the pedal mucus also varies in the different gastropod

species and this could be related to the different modes of locomotion adopted by these gastropods.

In Limax, Littorina and Pomatias the pedal mucus consists of a mixture of protein associated with neutral and acidic mucopolysaccharides. Acidic mucopolysaccharide in these species is composed of both sulphated and carboxylated groups. The pedal mucus of Veronicella (Cook, 1987) however, differs from these and other gastropod species in that sulphated mucopolysaccharide was not evident in Veronicella mucus. It may be concluded therefore that the mixture of protein and neutral mucopolysaccharide associated with sulphated or carboxylated groups is an essential feature of the gastropod creeping system. This agrees with Denny and Gosline's (1981) findings that the thixotrophic qualities of Ariolimax mucus that are essential for locomotion were attributed to protein. Protein also seems to be essential for the ciliary mode of locomotion as it is present in the pedal mucus of Bithynia (secreted by the anterior pedal gland cell type B1) and Lymnaea (secreted by the ubiquitous muciparous gland cell type II). Elves (1961) observed the ciliary locomotion of Discus rotundatus (a terrestrial pulmonate) and found mucus glands in the foot but preliminary observations have also shown the presence of some protein secreting glands in the foot of Discus (personal observation). However, it is important to remember that the proportion of protein and acidic and neutral mucopolysaccharides in the pedal mucus of gastropods

would vary according to the physical and chemical properties of mucus necessary for the different modes of locomotion. The absence of protein in the sole glands of Bithynia and in the pedal glands of Lymnaea would seem to indicate that protein may not be the major component required for the ciliary type of locomotion adopted by these fresh water gastropods.

Histochemical studies of opercula have shown the presence of both protein and mucopolysaccharides (Vo\velle 1969a,b; Vo\velle et al., 1977) and this corresponds to the staining reactions of the cell types found in this study. It has been suggested that the conchiolin and varnish layers of the operculum are secreted by the epidermal cells in two different areas of the foot, the former by the transverse dorsal opercular groove and the latter by a subopercular cleft under the posterior and lateral margins of the operculum (Fretter and Graham 1962); but the examples studied here do not conform to that pattern. In Littorina the tall epidermal cells at neither the transverse dorsal opercular groove nor the subopercular cleft are secretory and therefore cannot contribute to operculum formation. The major secretory cells in the area are the subepidermal ones (L6 and L7) in the opercular groove and the subopercular cleft and these produce protein and sulphated mucopolysaccharides. Goblet cells (L9 and L10) are also concentrated in these areas and tend to stain more strongly than elsewhere. There is no apparent difference in the cell

types at these two locations and therefore no evidence to support two distinct functions.

In Littorina most of the secretory activity associated with operculum formation is in the opercular groove. The secretion of the opercular material must proceed at a greater rate on the right than on the left side. The result of this is that the operculum is pushed gradually round clockwise on the opercular disc of the foot (Fretter and Graham 1962). This corresponds to the relatively large opercular groove. Whilst this description may also be appropriate in Pomatias it is not adequate for Bithynia. In Bithynia the secretory cells are distributed evenly round the edge of the disc where the operculum is free from the foot. At the anterior opercular groove the secretory cells produce a limited amount of material giving the operculum its angular shape and contributing to the asymmetrical concentric pattern of growth. The opercular secretory cells of Bithynia are epidermal (B4, B5, B6 and B8) as opposed to the subepidermal ones found in Littorina and Pomatias.

After a consideration of the amino acid composition of opercula in 13 species of prosobranch, Hunt (1976) concluded there was a marked similarity between conchiolin and varnish layer composition and that at least two proteins were present, one glycine-rich and the other rich in acidic amino-acids. He suggested that there were three possibilities as to their origin: (1) there may be two closely related proteins, one in each layer, (2) there is

only one protein secreted, the differences between the conchiolin and varnish layers being brought about after secretion and (3) two proteins are secreted both of which contribute to the conchiolin and only a glycine-rich one forms the varnish layer. The two protein-secreting cells in the opercular gland types of Bithynia responded differently to the same staining procedures (Table 4), so do those of Pomatias (Table 2). In Littorina two cell types are again found but they differ in texture rather than in the staining properties of their contents. With the restricted range of protein-specific stains used in this study conclusions concerning the precise number of different proteins secreted are impossible, but the indications are that in each of the prosobranch species studied at least two different secretory cell types are involved.

Hunt (1976) also claimed that there was no appreciable difference between the amino acid composition of uncalcified opercula in species from different habitats. The present study, however, indicates that there is a considerable variation in the location and histochemical properties of the gland cells involved in the formation of the operculum in Pomatias (terrestrial) Bithynia (fresh water) and Littorina (marine).

A histochemical study of the periostraca of 12 genera of gastropods by Meenakshi, Hare, Watabe and Wilbur (1969) showed that protein and neutral mucopolysaccharide were present in all cases whilst there was no evidence of acid

mucopolysaccharide; and traces of lipid were found in some species. The periostracum is secreted by the mantle edge gland cells and the results of the histochemistry of gland cell types at the mantle edge in Pomatias, Bithynia and Littorina confirm the findings of Meenakshi et al. (1969) as protein and neutral mucopolysaccharide are the secretory products at the mantle edge in these species.

It is generally accepted that the shell is a secretory product of the mantle edge and calcium is the main component of the shell. It therefore appears to be rather surprising that positive reactions for calcium were not obtained for any cell type at the mantle edge in Pomatias, Bithynia and Littorina. In Lymnaea stagnalis, however, the goblet cells reacted positively to tests for calcium (Tables 21 and 22). Timmermans (1973) also found these cells responded to calcium tests, but mentioned that epidermal calcium cells do not provide the calcium for the shell of fast growing snails, and that the necessary calcium is supplied by the surrounding water or by food. However, Durning, (1957); Guardabassi and Piacenza, (1958); Tsujii, (1960); and Abolins-Krogis, (1963), found that the calcium necessary for shell repair had been provided by calcium cells situated in the connective tissue of the mantle.

It seems therefore, that the calcium necessary for shell formation and shell repair in case of Pomatias, Bithynia and Littorina would be supplied either by the calcium cells located in the connective tissue or by direct

uptake.

Interestingly, it has been noticed that the number of mantle edge gland clusters appears to be more in species from marine and terrestrial habitats compared to the freshwater one (Table 29). Three histologically different clusters opening at the mantle edge are found in Acmaea (Haller, 1894), Lottia (Fisher, 1904) and Scurria (Thiem, 1917). Again in Patella vulgata three sets of histochemically different gland cells are found to be present at the mantle edge (personal observation, Plate 67). Thiem (1917) and Davis and Fleure (1903) seem to have noticed only one set of marginal glands in Patella vulgata. They however, applied only routine histological staining methods. Although Tullberg (1882) and Dakin (1912) in Buccinum and Kleinsteuber (1913) in Calyptraeids found only one cluster opening at the mantle edge, these species were studied long ago and probably also used general histological staining methods. In Littorina littorea also two sets of histochemically different glands are present at the dorsal aspect of the mantle.

Nevertheless, only one cluster opening on the dorsal side of the mantle edge is a common occurrence in most of the fresh water forms such as Viviparus (Annandale, 1921; Starmuhlner, 1952); Neritina (Andrews 1935); Bithynella (Bregenzer, 1916); Valvata (Starmuhlner, 1952); Pila virens (Umadevi et al, 1984) and Bithynia tentaculata.

A relationship between the habitat and the number of

periostracal layers in gastropods has been shown by Meenakshi et al. (1969). Freshwater species had two layers; the terrestrial had a single layer and the marine gastropods had a single layer or an incomplete layer. As the periostracum is secreted by the mantle edge there could possibly be some relationship between the habitat, the number of mantle edge glands and the number of periostracal layers in the prosobranchs.

Hunt (1973) described three cell types in the hypobranchial gland of Buccinum as acid mucin cells, goblet cells and ciliated cells . The acid mucin cells secreted the acidic mucopolysaccharides whilst the goblet cells secreted the glycoprotein. Previously, Hunt and Jevons (1965) had been able to separate an acid and a neutral mucopolysaccharide from the hypobranchial secretion of Buccinum undatum. Hunt (1973) offered no evidence for the presence of neutral mucopolysaccharide in his histochemical results but it would appear that very few histochemical techniques were applied in his study.

The hypobranchial gland of Buccinum undatum closely resembles histologically and histochemically the hypobranchial gland of Littorina littorea and with the histochemical procedures applied in the present study it was possible to detect neutral mucopolysaccharide in the hypobranchial gland of Littorina littorea.

In all the marine forms the mucocytes (possibly containing acid mucopolysaccharide as in Littorina littorea

and Buccinum undatum) form the main constituent of the hypobranchial gland (Table 30). The clear cells mentioned by several authors were probably mucocytes as the cells that secrete acid mucopolysaccharides in the gastropods have a characteristically clear or empty appearance (Thompson and Slinn, 1959; Thompson, 1960a, 1961, and 1965) when not specifically stained.

However, cells secreting protein material are the common type of cell in the hypobranchial gland of most fresh water species and mucus cells occur very sparsely. What is involved in changing the proportion of acid mucopolysaccharide to protein in the secretion of the hypobranchial gland cells is not clear but the possibility of a relationship between the habitat and the nature of the hypobranchial gland secretion cannot be completely dismissed. However, much more work on the hypobranchial gland cells of the gastropods from different habitats needs to be conducted in order to reach such a conclusion.

Fretter and Graham (1962) mentioned that hypobranchial gland cells tend to contain more mucus in gastropods that have a ctenidium in the mantle cavity than in animals where the ctenidium has been replaced by ciliated stripes. The present study shows that in the case of Bithynia tentaculata, however, although the ctenidium is present the hypobranchial gland cell contents are highly proteinaceous. In Bithynia it may be so, because the mucus from this gland (presumably) is used for webspinning to trap particulate

food matter.

The regular form of necks in all the subepidermal gland cell types in Pomatias, Bithynia, Littorina and Limax pseudoflavus indicates that discharge is merocrine. The merocrine method would be advantageous since it presumbly causes less disruption of the epidermis and is less wasteful in terms of cell organelles. Since there is no evidence of developmental stages it seems likely that these gland cells function throughout the life of the animal. The epidermal goblet cell types in Pomatias, Bithynia and Littorina are apparently without ducts and therefore would adopt an apocrine mode of secretion.

Until recently the interpretation of histochemical slides of gastropod tissue has been largely based on previous studies of the staining reactions of material from mammalian tissues. However, the gastropod material may, as in the case of mucopolysaccharide, be utterly unlike the apparently equivalent mammalian material and the slides may therefore, be interpreted incorrectly. Histochemical study of the gastropod mucopolysaccharides therefore allows us to find out which gastropod material will be stained or not stained by the all histochemical techniques used in this work.

The usual methods of characterising the different secretions is to use stains which have 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 pH levels to improve staining specificity. When staining mucopolysaccharides the tests available simply indicate that such a substance is present but do not characterise the type of group involved. The use of different pH media however to some extent makes stains like Alcian Blue more specific for acidic mucopolysaccharides and the use of the periodic acid-paradiamine method more specific for neutral mucopolysaccharide, as has been demonstrated by the results obtained in this work. By comparing autoradiographs with the results of various staining techniques on mouse tissue, Curran and Kennedy (1956) were able to claim that Alcian Blue (pH 1.00) was a highly specific stain for sulphated mucopolysaccharides. Toluidine blue on the other hand stains both sulphate and carboxyl groups but it is possible to differentiate between these two sorts of acids by staining in Alcian Blue at various pH values. However, it remains true that all the experimental work on the interpretation of these two stains has been based on mammals.

Meredith (1972) in his studies on molluscan mucins has shown that his results of the chemical analysis of the mucin match up very well with the results of histochemical examination of the epithelial glands responsible for the mucin. Furthermore, he claimed that although the PAS technique is considered by many people to be unreliable,

when dealing with molluscan mucins the PAS stain is a reliable technique.

When using a range of tests on a complex substance such as a gastropod mucus however conflicting and unexpected results may be obtained simply because methods of detection are too crude. Many mucus secretions are composed of a combination of mucopolysaccharides and protein and the proteinaceous part may go undetected since its normal staining properties are masked by the acidic groups. For instance, in the present study it was not possible to identify the protein in the inner part of the suprapedal gland of Pomatias. However, Bensalem and Chetail (1982) detected protein with sulphide groups in this part.

Although histochemical methods can provide evidence for some of the types of units present within a secretion of a single cell type 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 in these studies the source of secretion remains unknown and so may present a problem of linkage. If a better understanding is to be obtained about the nature of secretions produced by these gastropod species then it will be necessary to subject them to such methods although the difficulty of obtaining adequate, pure samples would be considerable.

Thus, the results of the present study have provided some problems, which might be solved by use of such

techniques as those outlined above. However, it has still been possible to a considerable degree to examine the structure and to characterise the secretions of the gland cells which contribute to mucus covering in the prosobranchs, Pomatias elegans, Bithynia tentaculata, Littorina littorea and the pulmonate slug Limax pseudoflavus and to relate the difference in the structure and the nature of secretion of these gland cells to the varying functional, environmental, behavioural and physiological requirements of each species. It is hoped that the concepts which have emerged will provide a basis for further research and the questions broached stimulate new discussion about the complex problem of understanding the chemistry of mucus in gastropods and its use in their mode of life.

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ADDENDUM

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APPENDIX I

TABLE I : Showing the routine instituted for each fixative used

Fixative	Fixation time	Washing under running tap water	Alcohol %					Clearing Agents						Wax under vacuum			
			70% Alc.	80% Alc. brown with Iodine	80% Alc.	90% Alc.	Absolute alcohol	Methyl benzoate	Benzene			or	Toluene	I	II	III	IV
Zenker's fixative	24 hrs.	24 hrs.	24 hrs	24 hrs	24 hrs	12 hrs	12 hrs. (2 changes)	5-6hrs. (2-3 changes)	1 hr. (3 changes) 15 min.	15 min.	30 min. in Benzene saturated with paraffin wax		12 hrs. over-night	1hr	1hr	1hr	1hr
95% alcohol	36 hrs. (3 changes)	-	-	-	-	-	12 hrs. (gen. over-night)	"	"	"	"		"	"	"	"	"
10% formaldehyde	2 - 3 days	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-
Baker's formaldehyde calcium	2 - 3 days	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-

APPENDIX 2

MALLORY'S TRIPLE STAIN (Grimstone and Skaer 1972)

Method

1. Dewax and bring sections to water.
2. Stain for 15 seconds in Mallory's triple stain.
3. Wash in running tap water for 3-5 seconds.
4. Dehydrate rapidly in alcohols, clear in xylene.
5. Mount in balsam or DPX.

Results

Nuclei red, nucleoli yellow, collagen blue, mucus blue, cytoplasm pink or yellow.

APPENDIX 3

MODIFIED ALCIAN BLUE/ALCIAN YELLOW TECHNIQUE FOR DIFFERENTIATION OF ACID MUCOPOLYSACCHARIDES

This method uses Alcian Blue at low pH(0.5) to stain sulphated compounds and Alcian Yellow at pH2.5 to stain carboxylated mucosubstances.

Preparation:

1. 1g. Alcian Blue 8 Gx in 100 ml. of N/5 hydrochloric acid.
2. 1g. Alcian Yellow in 100 ml. of 3 percent acetic acid.

Method:

1. Bring sections to water and rinse in N/5 hydrochloric acid.
2. Stain in Alcian Blue solution for 30 minutes.
3. Rinse in N/5 HCl and wash in water.
4. Blot dry with filter paper.
5. Rinse in pH 2.5 buffer (3% acetic acid).
6. Stain in Alcian Yellow Solution for 30 minutes.
7. Wash in water.
8. Dehydrate and bring to xylene.
9. Mount in Balsam or DPX.

Result:

Sulphated mucosubstances - blue.

Carboxylated mucosubstances - yellow.

Mixture of the two - shades of green.

APPENDIX 4

ALCIAN BLUE pH 0.5/EOSIN

STAINING TECHNIQUE FOR SULPHATED MUCOPOLYSACCHARIDES

1. Dewax and bring sections to water.
2. Rinse in pH 0.5 buffer (N/5 HCl).
3. Stain for 10-30 minutes in Alcian Blue 8GX (1g. Alcian Blue in 100 ml. of N/5 hydrochloric acid).
4. Wash for 10 seconds in pH 0.5 buffer.
5. Wash well in water.
6. Dehydrate till 90 per cent alcohol.
7. Stain 10-15 seconds in eosin (0.5 per cent in 90 per cent alcohol).
8. Dehydrate in absolute alcohol.
9. Bring to xylene.
10. Mount in Balsam or DPX.

Result:

Sulphated mucopolysaccharides -blue.

APPENDIX 5

DIAZOTIZATION COUPLING TECHNIQUE FOR TYROSINE

(Glenner and Lillie, 1959)

Solutions required

1. Diazotizing agent

   $NaNo_2$ 6.9 g

   Glacial acetic acid 5.8 ml.

   Distilled water to 100 ml.

2. Alkaline coupling reagent

   70 per cent Alcohol 100 ml

   8-amino-1-naphthol-5-sulphonic acid (S-acid) 1 g

   Potassium hydroxide 1 g

   Ammonium sulphamate 1 g

   Dissolve in order and chill to 3°C in refrigerator.

Method

1. Bring sections to water.
2. Diazotize overnight at 3°C in strict darkness.
3. Rinse in four changes of distilled water at 3°C for 5 seconds each.
4. Place in alkaline coupling reagent for 1 hour at 3°C also in the dark.
5. Wash in 3 changes of 0.1 N hydrochloric acid for 5 mins. in each.
6. Wash in running water for 10 mins.
7. Dehydrate, clear and mount.

Results

Tyrosine sites are coloured red-purple to pink.

APPENDIX 6

MERCURIC BROMOPHENOL BLUE (Bonhag 1955).

Bromophenol blue 0.1g

10% Mercuric Chloride 100 ml.

Method

1. Bring sections to water.
2. Stain for 15 minutes in above solution.
3. Differentiate in 0.5% acetic acid.
4. Wash in water.
5. Dehydrate rapidly.
6. Clear in Xylene.
7. Mount in Balsam or DPX.

Result

Proteins stain a deep blue colour.

APPENDIX 7

PERIODIC ACID SCHIFF (P.A.S.) REACTION (McManus 1946)

Method

1. Bring sections to water.
2. Oxidise in 1% Aqueous Periodic Acid for 10 mins.
3. Wash in running water for 5 mins. and rinse in distilled water.
4. Place in Schiff's reagent for 10 - 20 mins.
5. Wash for 10 mins. in running water.
6. Dehydrate in alcohol, clear in xylene and mount in Balsam or DPX.

Results

P.A.S. Positive substances - red or magenta.

APPENDIX 8

PERIODIC ACID PARADIAMINE METHOD (PAD) (Spicer 1966) FOR NEUTRAL MUCOPOLYSACCHARIDE

Reagents

Add 50 mg N,N-dimethyl-p-phenylenediamine HCl, just before use, to 50 ml. citrate-phosphate buffer (0.1 m-Citric acid, 4.8 ml., 0.2m disodium phosphate, 7-2 ml, distilled water 38 ml)

Alternatively, dissolve 100 mg. paradiamine in 50 ml. distilled water and adjust pH to 5.0 with 0.4M-Na_2HPO_4

Method

1. Bring slides to water.
2. Oxidize in 1 per cent. aqueous periodic acid for 10 mins.
3. Rinse in running water for 10 mins.
4. Immerse in paradiamine solution for 7, 24, or 48 hrs.
5. Differentiate in 1 per cent. HCl in 70 per cent. alcohol for 8 seconds (after 24 hours' stain) or for 10 seconds (after 48 hours' stain).
6. Wash in water for 5 mins.
7. Dehydrate through the alcohols, clear in xylene and mount in Balsam or DPX.

Results

Neutral mucopolysaccharides, brown; periodate-reactive polymers, purple or grey-brown; periodate unreactive mucosubstances, black.

APPENDIX 9

THE COMBINED ALCIAN BLUE pH2.5 - PAS REACTION (Mowry 1963) FOR THE DIFFERENTIATION OF ACIDIC AND NEUTRAL MUCOPOLYSACCHARIDES

Method

1. Bring sections to water.
2. Rinse briefly in 3 per cent aqueous acetic acid.
3. Stain for 30 mins. in 1 per cent Alcian Blue 8GX in 3 per cent acetic acid (pH 2.5).
4. Rinse briefly in water and then in 3 per cent acetic acid, running water and distilled water.
5. Oxidise for 10 mins. in 1 per cent periodic acid (aqueous) at room temperature.
6. Wash in running water for 5 mins.
7. Immerse in Schiff's reagent for 10 mins.
8. Wash in running water for 2 mins.
9. Rinse in 3 changes of 0.5 per cent sodium bisulphite, 1 min. in each.
10. Wash in running water for 5 mins.
11. Dehydrate, clear and mount.

Results

Acid mucosubstances - blue

Neutral mucosubstances - magenta red.

Mixtures of both will produce a mixed colour depending upon the predominant type.

APPENDIX 10

TOLUIDINE BLUE METHOD (Standard-Pearse 1980)

Method

1. Bring sections to water.
2. Stain in 0.5 percent aqueous toluidine blue for 10-15 minutes.
3. Rinse in distilled water.
4. Dry on hot plate.
5. Clear in xylene and mount in Balsam of DPX.

Result

β-metachromasia purple; γ metachromasia red or pink.

Comment

Air dry to reserve metachromasia.

APPENDIX 11

THE VON KOSSA METHOD FOR "CALCIUM" DEPOSITS

Method

1. Bring sections to distilled water and rinse thoroughly.
2. Immerse in 0.5-1 percent aqueous $AgNO_3$ for 10-15 minutes. (This procedure shold be carried out in sunlight or ultraviolet light).
3. Rinse in distilled water.
4. Immerse in 5 per cent aqueous sodium thiosulphate, 30 seconds.
5. Wash in tap water.
6. Dehydrate in alcohol, clear in xylene and mount in Canada Balsam.

Result

Phosphates and carbonates appear black.

APPENDIX 12

ALIZARIN RED S METHOD FOR CALCIUM (Dahl 1952)

Preparation of solution

Stir 0.5g of the dye in 45 ml. distilled water and add 5 ml. of a 1:100 dilution of 28 per cent, NH_4OH, with constant sirring. The pH should be between 6.3 and 6.5 and the solution keeps for at least a month.

Method

1. Dewax in xylene.
2. Wash in absolute alcohol and 95 percent alcohol (two changes of each).
3. Drain for a few seconds on a paper towel.
4. Stain with Alizarin solution for two minutes.
5. Wash with distilled water for 5-10 seconds.
6. Dehydrate in 95 percent alcohol and absolute alcohol.
7. Clear in xylene and mount in cedar wood oil.

Result

Calcium deposits appear orange red.

APPENDIX 13

SUDAN BLACK B METHOD FOR LIPIDS (Ann Preece 1972).

Preparation of solution

Dissolve 0.7gm of Sudan Black B in 100 ml. of absolute propylene glycol by heating to 100° to 110°C, stirring thoroughly for a few minutes. Do not exceed 110°C, since a useless gelatinous suspension will result. Filter hot through Whatman No. 2 paper to remove excess dye. After cooling to room temperature, filter through a fritted glass filter of medium porosity with the aid of suction. This is a stable solution.

Method

1. Rinse sections in water.
2. Drain, then immerse in Sudan Black B for 8 minutes.
3. Drain well, then agitate in propylene glycol wash (85%) for 3 minutes.
4. Drain again, then agitate in propylene glycol wash (50%) for 3 minutes.
5. Drain, agitate in a beaker of tap water.
6. Drain and mount in glycerol.

Result

Lipids stain black or blue.