STUDIES ON THE CUTICLE AND EPIDERMAL SECRETORY CELLS OF LUMBRICID AND ENCHYTRAEID OLIGOCHAETES

VOLUME I Text

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for the degree of

Doctor of Philosophy, Keele, 1975

VOLUME I

FRONTISPIECE

Dendrobaena
veneta var.
zebra

"... not a worm is cloven in vain,"

Tennyson "In Memoriam" Canto LIV.

ABSTRACT

The histochemistry of the cuticle and epidermal secretory cells of seven lumbricids is recorded and three types of secretory cell delineated. The function of these secretions is discussed and a reassessment of the identity of the so-called albumen cells is made.

At the ultrastructural level eleven lumbricids are studied. Details of the cuticle provide the basis for a possible explanation of the orientation of the collagen fibres within it. The three types of secretory cell are described, one of which shows species variation, the significance of which is discussed.

The epidermis of nine enchytraeid species is examined histochemically and ultrastructurally. Species of the genus Lumbricillus are studied in depth; the remaining species, from differing habitats, are compared with it.

ACKNOWLEDGEMENTS

The encouragement and advice of Dr. P. F. D. Chevins and the continued interest of Professor A. R. Gemmell during the course of this investigation are gratefully acknowledged.

Appreciative thanks are accorded to Dr. M. J. Tynen for confirmation of the identification of certain of the enchytraeids and for the many helpful discussions on this oligochaete family that have taken place.

The assistance of Mr. P. M. Webster during the electron microscope studies is recognised, as is his help and that of Mr. G. Burgess in the preparation of the photographic material.

The Department of Biology, University of Victoria, B.C., Canada is thanked for the provision of facilities for the processing of material collected in British Columbia.

INDEX TO VOLUME I

1 GENERAL INTRODUCTION

The aim of this research was to study, in some detail, the nature and ultrastructure of the secretory cells and the cuticle of the unmodified epidermis of some lumbricid oligochaetes and to compare these results with more limited studies of similar sites in certain microdrile oligochaetes.

Over the last few years numerous fairly sophisticated histochemical techniques have been developed to distinguish between the many types of mucinous secretions in mammalian tissue, but the literature revealed that little detailed attention had been given to oligochaete secretions. An expectation that "further knowledge of this (earthworm) secretion should not be long delayed" occurs in Laverack's Text on the Physiology of Earthworms (1963), and in the Foreword to the collected papers on Mucus in Invertebrates (Annals of the New York Academy of Science, 1965) Jakowska (1965a) underlines the absence of earthworm work by including a frontispiece photograph of lumbricids. In 1970, Hunt compiled a text on polysaccharide-protein complexes in invertebrates which, though wide-ranging, contained no information on earthworm epidermal mucosubstances.

The lumbricids chosen for this study ranged from species known to be restricted to ecological situations rich in organic matter, through ubiquitous forms to species found only in agricultural and horticultural soil and never in manure or compost heaps. Although it is known that over such an ecological range the calciferous glands show variation, it was thought that perhaps the epidermis and its secretory structures might also reflect differences, since the skin

is not only the animal's defence against the environment but also its point of contact with it. In environments as different as a sewage bed and a well limed garden it might be anticipated that the epidermes of the species inhabiting such situations might perhaps show variations.

In contrast to the megadrile lumbricids, which have become fully terrestrial, are the microdrile families, the majority of which are aquatic. Stephenson (1930) argues that their aquatic state is a secondarily acquired one and that their copulation, hermaphroditism and cocoon formation reflect their origins in an environment that was not purely aquatic. Clark (1969) is less convinced that the cocoon should form the basis for phylogenetic speculation concerning the ancestral oligochaetes.

Within the microdrile families tentative relationships have been established for all but the Enchytraeidae (Brinkhurst & Jamieson, 1971), the phylogeny of which remains obscure. Whilst other microdrile families have remained purely aquatic and predominantly freshwater, the enchytraeids show much greater ecological variation. Their distribution is primarily either terrestrial or littoral, with fewer species occurring in natural freshwater situations in temperate zones. If the origins of the oligochaetes lie within a freshwater ancestral form as suggested by Clark (1969) it is of interest that members of this microdrile family are found less frequently in such a habitat than in the more severe environments of the terrestrial habitat and the littoral zone.

It was therefore thought that a study of the epidermis of selected enchytraeids from a range of habitats would provide information that would allow for a more informed discussion concerning the elements present in the skin of oligochaetes.

The research project fell into two sections. Firstly, a general histochemical survey of the epidermis of lumbricid and enchytraeid material was carried out in order to determine the nature of the secretions. This was undertaken using tissue prepared for light microscopy either by paraffin or similar embedding, or by freezing techniques, since the number of histochemical methods available to the electron microscopist is limited. Secondly, parallel studies were carried out at the ultrastructural level, since the literature showed that the secretory cells of lumbricids had received only cursory attention and that some confusion existed with respect to the cuticle ultrastructure. No previous work had been recorded on the enchytraeids chosen for this study.

The results of the lumbricid study have been prepared for publication concurrently with the writing of this text.

2 LUMBRICID MATERIALS AND METHODS

i) Introduction

In order to examine tissue and carry out most histochemical techniques it is necessary to fix the tissue prior to embedment and sectioning. From both the histological and histochemical point of view the choice of fixative can be critical.

Ideally the fixative should diffuse rapidly to combat autolysis, render diffusable substances insoluble, permit the tissue to survive subsequent treatments prior to sectioning and render the tissue available for staining and observation. The best fixatives are those leading to the denaturation of cellular proteins without recognisable precipitation (Ruthmann, 1970), thus providing an "equivalent image" of cell structure at any given level of microscope resolution. However, there is no ideal fixative for all tissues, only the one best suited to the substance to be studied, and even then some changes will occur.

Belanger (1963) pointed out that the end product of the paraffin technique was a microframework of the living substrate in which remained mainly denatured proteins and large molecules of mucopolysaccharides. More or less of these will have been retained depending on the histological treatment. However, comparisons of what is left should be valid within the experimental conditions. Barrett (1971) was less optimistic and stated that as the polysaccharide components of mucosubstances were chemically more inert, they could be expected to be more difficult to fix in an insoluble form than the protein components. Consequently, aldehyde fixatives were likely to be ineffective, and alcohol and acetone would leave the molecules free to dissolve in water as subsequent techniques require hydration. Similarly, heavy metals and acid, normally effective for proteins, were likely to have

little action on many mucosubstances unless the latter were trapped by the fixation of other proteins surrounding them.

A major problem therefore faces histochemists working on mucosubstances, quite apart from certain histochemical techniques requiring specific fixatives, and consequently a wide range was chosen for this study.

For electron microscopy the fixation requirements are even more critical, necessitating not only very rapid but also very good fixation of the organelles, so that the detail can be distinguished easily and accurately without the presence of artifacts.

ii) Materials

(a) Light microscopy

British lumbricids of the following species were used for the light microscope study:

Allolobophora longa Ude 1885

Dendrobaena subrubicunda (Dendrobaena rubida Savigny 1826 f. subrubicunda Eisen 1874)

Dendrobaena (Eisenia) veneta Rosa 1886 var. hibernica Friend 1892

Dendrobaena (Eisenia) veneta Rosa 1886 var. zebra Michaelsen 1902

Eisenia foetida Savigny 1826

Lumbricus rubellus Hoffmeister 1845

Lumbricus terrestris Linnaeus 1758

Specimens were collected from sewage beds, garden and agricultural soil at the University of Keele, Staffordshire, and from compost heaps and rich garden soil at sites in Montgomeryshire, Wales.

(b) Electron microscopy

British lumbricids of the following species were used for the

electron microscope study:

Allolobophora caliginosa Savigny 1826

Allolobophora chlorotica Savigny 1826

Allolobophora longa Ude 1885

Bimastos muldali Omodeo 1956 (Allolobophora minima Muldal 1952)

Dendrobaena subrubicunda (Dendrobaena rubida Savigny 1826 f. subrubicunda Eisen 1874)

Dendrobaena (Eisenia) veneta Rosa 1886 var. hibernica Friend 1892

Dendrobaena (Eisenia) veneta Rosa 1886 var. zebra Michaelsen 1902

Eisenia foetida Savigny 1826

Lumbricus rubellus Hoffmeister 1845

Lumbricus terrestris Linnaeus 1758

Octolasion (Octolasium) lacteum Oerley 1881

Specimens were collected from the sites given under 2 ii a, and in addition some specimens of Eisenia foetida were examined from rich compost heaps at Mill Bay, Vancouver Island, British Columbia, Canada.

iii) Methods

(a) Light microscopy

Pieces of tissue were taken from the post-clitellar region, and for transverse sections units of 4-6 segments were processed. In order to avoid blunting of the microtome knife by the gut soil particles, either the gut was removed after fixation, leaving just the body wall, or the worm was left, prior to fixation, for 2-3 days on damp filter paper to evacuate the gut, in which case the whole body structures were processed, so enabling histochemical comparisons to be made between the epidermal reactions and those of the more deeply seated tissues. Also, long strips of body wall from the post-clitellar region

were taken and rolled around a thin matchstick and secured prior to fixation, sections from this 'swiss roll' enabling any differences along the worm to be examined. Regions anterior to the clitellum were also processed and studied.

The handling of the animals before fixation was kept to a minimum and attempts were made to reduce mucus discharge at the moment of fixation. Plunging into fixative caused violent contraction with discharge of mucus and of coelomic contents through the dorsal pores, especially in E. foetida and the three Dendrobaena forms. Anaesthetics, including alcohol and chloroform vapour and drips, Nembutol and MS 222 (Runham, Isarankura & Smith, 1965) were tried, also prefixation cooling of the worms for 30 min at 2-4^{o}C, with or without a further 10 min at -2^{o}C.

A number of fixatives were chosen from the literature (Gurr, 1962; Humason, 1967; Lillie, 1965; Pearse, 1968) including Zenker, Helly, Bouin and para-toluene sulphonic acid with formalin (Steedman, 1970). Carnoy, 10% neutral buffered formalin and weak Bouin for lipid extraction were employed when the histochemical technique so demanded. All were used at room temperature.

The post-fixation washing of the tissue depended on the choice of fixative, dehydration was by means of an alcohol series and clearing was accomplished either in toluene or benzene prior to wax infiltration and embedment. For material embedded in polyester wax (400-polyethylene glycol distearate) the schedule of Steedman (1957) was followed and the cut sections were mounted using formol-gelatine (0.2% gelatin 98 ml : 40% formalin 2 ml). For the paraffin and ester waxes, the cut sections were mounted on albumenised slides and dried after flattening. The section thickness ranged from 2 µm (400-polyethylene glycol blocks) to 10 µm depending on subsequent technique.

For frozen sections, standard procedures were adopted, taking a small piece of worm tissue, not exceeding 3 mm in any direction, and mounting it on a drop of water on a cork disc, which in turn was mounted on a drop of water on a metal carriage. This was placed in a wire basket and held in liquid nitrogen vapour for a short time to ensure that the tissue became attached to the disc before being lowered into liquid nitrogen for 15 seconds. The knife blade of the Cryostat ("Pearse" Cold Microtome Type H) was kept cool with solid CO_2 and the temperature of the cabinet was maintained at -14°C to -15°C. Sections of 10 µm thickness were collected on a coverslip, allowed to dry for 2-3 min, then stained with or without subsequent fixation of the material.

Preliminary autoradiographic studies, designed for both light and electron microscope investigations, were undertaken on E. foetida, using S^{35} in the form of freeze dried solid $Na_2S^{35}O_4$ made up in earthworm Ringer. 0.1 ml of high dosage 70/µCi or 0.1 ml of low dosage 7/µCi was injected into the coelom of the posterior half of each experimental animal. Animals sampled at 0.5, 1, 2, 4, 6, 12, 24, 48 hours were processed for electron microscopy (Section 2 iii b) and 1 µm and ultrasections taken. The 1 µm light microscopy sections were coated with Kodak NSA 2 emulsion and developed at intervals of 10, 14, 21, 18, 35 days in Kodak D 19.

To check the nature of the secretion of the so-called 'albumen' cells, appropriate portions of oviduct of Rana temporaria and Colomba livia were processed as for earthworm tissue.

The range of histological and histochemical techniques employed included the following:

A. General techniques

1. Heidenhain's haematoxylin and eosin

2. Mallory Triple Stain (Pantin, 1962)

3. Triacid general stain (Steedman, 1970)

B. Polysaccharides and Mucopolysaccharides

1. Alcian Blue (AB)

   i AB 8GX 1% in 3% acetic acid (pH 2.5) (McManus & Mowry, 1964)

   ii AB 8GX 1% pH 1.0 (sections blotted dry) (Pearse, 1968)

   iii AB 8GX 1% pH 0.5 (sections blotted dry) (Pearse, 1968)

   iv AB 8GX, Critical Electrolyte Concentration (CEC) technique (Scott & Dorling, 1965; Quintarelli & Dellovo, 1965):
   0.1M; 0.2M; 0.25M; 0.3M; 0.4M; 0.5M; 0.6M; 0.8M; 1.0M; 1.2M; 1.4M Magnesium chloride

   v AB-Periodic Acid Schiff (PAS) (McManus & Mowry, 1964)

   vi (a) AB.CEC 0.2M $MgCl_2$-PAS (Andersen, Møllgård & von Bülow, 1970)

   (b) AB.CEC.0.5M $MgCl_2$-PAS (Andersen et al., 1970)

2. Azure A - pH extinction (Pearse, 1968)

3. Thionin (Humason, 1967)

4. Toluidine Blue (TB) 1% aqueous (Humason, 1967)

5. PAS (McManus & Mowry, 1964)

6. Aldehyde Fuchsin (Ald.F)

   i Ald.F (Pearse, 1968)

   ii Ald.F-AB (Pearse, 1968; Spicer & Meyer, 1960)

7. Diamines

   i HID & LID, with and without Peracetic Acid (Pearse, 1968; Spicer, 1965)

   ii HID-AB; LID-AB (Pearse, 1968; Spicer, 1965)

8. Geyer's Tetrazonium method (Pearse, 1968) 10% Neutral formalin fixation
9. Ferric alum - Coriphosphine (Pearse, 1968)
10. Acridine orange, CTAC method (Pearse, 1968) Carnoy fixation.

C. Protein

1. Ninhydrin-Schiff (Humason, 1967)
2. Millon's reagent (Humason, 1967)
3. Bromphenol Blue (BPB)
   i BPB
   ii Mercuric BPB (Humason, 1967)
4. DMAB-nitrite (Pearse, 1968), 10% Neutral formalin fixation
5. Performic acid (PFA)-Alcian Blue
   i PFA-AB (Pearse, 1968) 10% Neutral formalin fixation
   ii PFA-AB-PAS (Andersen et al., 1970) 10% Neutral formalin fixation
6. Biebrich Scarlet (BS) - pH range (Spicer & Lillie, 1961; Spicer, 1962a), using various buffers to get pH range from 11 - 1.5
7. Weigert's Commercial Resorcin Fuchsin (WRF) (Puchtler & Sweat, 1960)
8. van Gieson - modification using 1% Acid fuchsin and Saturated Picric Acid in a 1:4 ratio
9. Amido Black for collagen (Lillie, 1965)
10. Chlorazol Black for collagen (Pantin, 1962)
11. Acid Fuchsin - Light Green SY Yellowish (FALG) (Gurr & MacConaill, 1961)
   i FA-LG with acid bath between (Gurr & MacConaill, 1961)
   ii FALG used as a mixture with pretreatment in 3% acetic acid for 10 minutes before stain
12. Naphthol Yellow S (Lillie, 1965).

D. Nucleic acids

1. Methyl Green - Pyronin Y (MG-PY)
   - i MG-PY (Humason, 1967)
   - ii MG-PY.CEC (Scott, 1967) 10% Neutral formalin fixation.

E. Lipids

1. Fettrot technique (Pearse, 1968)
2. Sudan Black (SB) (Barka & Anderson, 1965 after Berenbaum)
3. Luxol Fast Blue
   - i Luxol Fast Blue MBS (Humason, 1967)
   - ii Luxol Fast Blue ARN (Humason, 1967)
4. Lillie's Nile Blue (Humason, 1967)
5. Menschik's Nile Blue Sulphate (Pearse, 1968)

F. Chemical blocking/conversion, used with certain of the above techniques

1. Acetylation (McManus & Mowry, 1964) 45 min - 24 h
2. Saponification
   - i KOH 0.1N 45 min, room temperature (McManus & Mowry, 1964)
   - ii NH_4OH (McManus & Mowry, 1964)
   - iii potassium permanganate, 0.5% for 30 min, room temperature (Fisher & Lillie, 1954)
   - iv at low temperature (Stoward, 1967a)
3. Methylation (McManus & Mowry, 1964)
   - i drastic - 4, 12, 24 h at $60^{o}C$
   - ii mild - 4, 12 h at room temperature
   - iii using Thionyl chloride (Stoward, 1967a)
4. Sulphation (McManus & Mowry, 1964)

5. Acid hydrolysis

   i 0.1N HCl 2 h at 56°C (Andersen et al., 1970)

   ii 0.1N H_2SO_4 1 h at 80°C (Andersen et al., 1970)

6. Deamination: van Slyke's reagent (Humason, 1967)

7. Pyridine lipid extraction (McManus & Mowry, 1964) weak Bouin fixation

8. Permanganate oxidation

   i 0.5% for 20 min at 25°C

   ii 0.5% for 25 min at 20°C.

G. Enzymes

1. Papain 1% for 1.5 h, pH 5.5
2. Pepsin (Lillie, 1965)
3. Trypsin (Lillie, 1965)
4. Diastase (Humason, 1967)
5. Salivary Amylase
6. Neuraminidase - 17 h (Spicer, Horn & Leppi, 1967) 10% Neutral formalin fixation
7. Hyaluronidase - 4-6 h (Leppi & Stoward, 1965) Carnoy fixation
8. ß-Glucuronidase, with or without peracetic acid (Fullmer, 1960)
9. RNase - 2 h (Horn & Spicer, 1964)

Dye batch numbers and Enzyme batch numbers are given in Appendix 1.

(b) Electron microscopy

For the electron microscope study small pieces of body wall were taken from post-clitellar, dorso-lateral sites (above chaeta d) for the bulk of this study, but portions from anterior regions (segments 4-7) were also taken from specimens of Eisenia foetida. Pieces of

worm, 4-6 segments long, were dropped into a petri dish containing buffered fixative and quickly cut open and long strips from the dorso-lateral region transferred to a clean dish of fixative in which the final cutting of the tissue pieces for processing was carried out.

The following standard schedule, including in-block uranyl acetate staining during acetone dehydration, was used for most of the tissue processed:

1.	Primary fixation: 5% glutaraldehyde buffered with 0.1M phosphate at pH 6.3	60-90 min
2.	Buffer rinse	15 min
3.	Post-fixation: 1% osmium tetroxide similarly buffered	45-60 min
4.	Distilled water	2 x 10 min
5.	Dehydration: 30% acetone	10 min
	2% uranyl acetate in 30% acetone	10 min
	60% acetone	10 min
	90% acetone	10 min
	100% acetone	2 x 10 min
6.	Spurr's resin (Spurr, 1969):	
	i Acetone:Resin 2:1	30 min
	ii Acetone: Resin 1:2	30 min
	iii Resin	2 x 30 min
	iv Final Resin	overnight

Stages 1 - 6ii were carried out on a shaker, and stage 6iii and 6iv on a spinner.

Some material was fixed in glutaraldehyde alone (stage 1) and some in osmium alone (stage 3). In addition, material of E. foetida was fixed employing a buffer molarity range of 0.01M - 0.15M, also

using a pH range, at molarity 0.1M, of 3 - 7. In an attempt to overcome problems that might have been associated with dehydration, processing with Durcupan A was carried out on some E. foetida material.

Block treatment with 0.2% pepsin in 0.1N HCl, pH 1.6-1.7 for 1 h at 37°C, with appropriate controls, was carried out on E. foetida material. On E. foetida, A. longa and L. terrestris pronase treatment on ultra-sections (0.25% pronase at pH 7.4, adjusted with 0.01M NaOH, for 30 min at 38°C after prior oxidation with 10% Periodic acid - after Monneron & Bernhard, 1966) was carried out.

Sections showing silver or silver gold interference colours, 600-900Å thick according to Sjöstrand (1967), were cut on a Reichert OMU 2 microtome, using glass knives, and mounted on uncoated Athene 200 copper grids. Gold sections were cut for the pronase digestion experiments.

Sections were stained with 1% aqueous uranyl acetate (10 min) followed by lead citrate (5 min) (Reynolds, 1963) and were examined with a Philips E.M. 200 at 60 kV. Adjacent 0.5 - 1 µm sections were stained with 0.1% Toluidine blue in 1% borax, or by the PAS technique (Weakley, 1972).

Photographs were taken using Recordak Microfile Film Type 5669, developed in Kodak D19 for 5 min at 20°C and printed on to Kodak paper, K2 or K3, developed in Kodak D163 for 4 min.

3 LUMBRICID HISTORICAL BACKGROUND

(LIGHT MICROSCOPY)

The use of histochemical techniques to characterise the nature of the cuticle and secretory products is comparatively recent and the results of the limited number of workers on oligochaetes will be dealt with in the appropriate histochemical sections (Sections 5; 6; 7; 8).

Quite apart from the histochemical nature of the secretory cells they can be described on purely histological grounds and the literature appertaining to the light microscope representation of the cell types and the cuticle will be reviewed here.

The cuticle has not received much attention from light microscopists this century, but earlier workers on lumbricids had presented a detailed report of its striated nature. Claparède (1869) had described two systems of striations crossing each other at an angle of 70-75°, whereas Perrier (1873/74) had suggested an angle of 80° and attributed the iridescence of the cuticle to these striations. Mojsisowicz (1877) reported that the two systems crossed at approximately right angles and Cerfontaine (1890) supported Mojsisowicz (1877) with respect to the angle at which the fibres crossed one another but challenged his description that one system ran longitudinal to the body axis and the other circular to it. Cerfontaine (1890) on L. terrestris (agricola) established that the two systems crossed at right angles and that all the striations ran at an angle of 45° to the longitudinal axis of the worm, and proved, by simple experiments, that the observed iridescence was not primarily due to the striated cuticle. With respect to the orientation of the striations, his observations were to form the basis of descriptions until the advent of electron microscopy, his work being quoted by Schneider (1902;

1908) and Stephenson (1930).

Cerfontaine (1890), using maceration techniques, concluded that the two systems of striations were not in the same plane and proposed, from these studies and from sections cut at 45° to the longitudinal axis, that the two systems lay one above the other, though he failed to see this clearly in his preparations, due partly, he thought, to the failure of ordinary staining techniques other than Mayer's haematoxylin to colour the cuticle. However, in a limited number of his preparations he claimed to detect a thin line towards the middle of the cuticle and presumed this to be the junction between the two layers of the cuticle. Electron microscope studies have shown him to have been wrong in this assumption.

When he examined sections cut at 45° to the long axis, he recorded "an infinity of little lines producing a sort of striation perpendicular to the surface of the body ... allowing the protoplasm of the hypodermal cells to be in rapport with the exterior" (translation), these being distinct from the mucous cell pores. The interesting implications of these observations will be discussed after the electron microscope account of the cuticle (Section 10). Similar extremely fine striations running perpendicular to the body surface were described in the clitellum cuticle of A. caliginosa by Lutfy (1965a).

The secretory cell pores which perforate the cuticle have been described by Cerfontaine (1890), Beddard (1895) and Schneider (1902; 1908). However, Lankester's description (1865) of the pores ("very minute canals") was accompanied with the speculation that they were either respiratory or excretory and a means whereby "water passes to the perivisceral cavity and a dense fluid passes out". Friend (1924) also noted a dense fluid issuing from the pores in the cuticle.

Certain histological aspects of the secretory cells have been

documented for over a hundred years. Leydig (1865) described cutaneous glands with basal nuclei and filled with clear, strictly delimited, refringent corpuscles, though Claparède (1869) considered such glands to be a sort of intercellular substance. Perrier (1873/74) supported Leydig's description (1865) of discrete glands and Horst (1878) described two glandular types, the homogeneous and the type filled with refringent corpuscles or globules. A homogeneous gland type was also described by Vejdovsky (1884). Ude (1886) also described only one type, but this was a cell filled with small globules.

Cerfontaine (1890), in a detailed account of the epidermis (hypodermis) of L. terrestris (agricola), using both sectioned material and macerated tissue, distinguished two types of gland cell. The one type was described as being oval and filled with relatively large corpuscles, more or less deformed by reciprocal compression, and having a basally placed nucleus. The other type was figured as being pear-shaped with the narrow end towards the cuticle and as having a very finely granulated secretion, much finer than the granules of the surrounding supporting cells, and the secretion was described as "probably mucus". He discussed the possibility that, despite the different aspects of the glandular contents, the two types may be one and the same element in different stages of secretion, the fine granular cells representing the empty stage of the corpuscular type, but dismissed it because of the constancy with which the two types were found in the normal hypodermis and in the region of the clitellum. As it is this cell type which has given rise to some confusion in the later literature, a fuller discussion of it and other aspects of Cerfontaine's work (1890) is dealt with in Section 8iv.

Keng (1895) mentioned only one glandular type, in the epidermis of L. terrestris, containing mucin globules and figured it with discrete spheres similar to the corpuscles (large granules) of Cerfontaine (1890).

Schneider (1902; 1908) on A. (E.) rosea described the slime cells as either slim or plump depending on their physiological state and the contents as granular, except when emptying, when the secretion became clumped resulting from the fusion of the granules, or strand-like or forming a fine network. Because the staining reactions were not the same in all the cells he suggested that the contents changed with age and that the network of secretion in some cells represented the empty stages of the granular cells. Herlant-Meewis (1958/59) was later to suggest a similar sequence in which the form and the chemical nature of the secretory product was thought to change whilst in the goblet cavity. Schneider (1902; 1908) also suggested that the slime cells became recharged from the cytoplasmic lining at the base after secretion. In addition to the slime cell type, Schneider (1902; 1908) described another type of gland cell - Eiweisszellen - occurring in smaller numbers than the slime cells. The Eiweisszellen cell type was roughly oblong and without a goblet cavity but filled with a finely granulated secretion the staining properties of which (eosinophilic, green with German Toluidine blue) readily distinguished them from the normal or regenerating mucous cells.

Feldkamp (1924) on L. herculeus established three types of gland cells: large granular, fine granular and network or alveolar cells, but did not comment on the nature of the secretion or the relationship or distinctness of the types. Thomas (1932) on a species of Lumbricus, in one paragraph and without any evidence, claimed that both the large and small granular cells were simply different stages within a sequence of mucigenesis.

Herlant-Meewis (1958/59) on E. foetida reported that the mono-cellular glands of normal skin were of two types: mucous and "albumineuse", and indicated that confusion existed in the literature concerning their

identification. Her reference to earlier work was based mainly on Stephenson (1930) and not on the fuller original descriptions of Cerfontaine (1890) and Schneider (1902; 1908). Again, because of the varying histochemical results, it was suggested that the mucous cell type underwent a sequence of events which affected not only its histology but also its histochemistry. At first the secretion was granular and non acidic, later becoming "spumeuse" (frothy, bubbly) and strongly acidic, all traces of neutral mucosubstance having disappeared. The possibility that two types of mucous cell were present was not considered. The "albumineuse" type of cell described by Herlant-Meewis (1958/59) accords perfectly with the description of the Eiweisszellen of Schneider (1902; 1908).

At the level of the clitellum, normal mucous cells occur as in the unmodified epidermis. Grove (1925) on L. terrestris, and Grove & Cowley (1927) on E. foetida describe them as typical goblet, reticulate cells. Lutfy (1965a) on A. caliginosa recorded the contents as in the form of a wide meshwork in Region A (dorso-lateral) whereas in Region B (ventro-lateral) he described cells in which the contents were "more or less granular, but the granules lack any distinctive individuality" in addition to the reticulate type.

The extrusion of the secretory products received considerably less attention from early workers than did their histological appearance. Schneider (1902; 1908) described fibres, thought to be muscle, penetrating the epidermis and extending almost to the cuticle, also extensions of the lamella (basement membrane and connective tissue) passing between the supporting cells, but did not directly relate either of these to mucus extrusion. Szüts (1911) suggested that the contractile fibrillae in the epidermal cells (non-secretory cells) were capable of altering the shape of these cells and so causing the

extrusion of the mucus from the gland cells. Smallwood (1926) claimed that mucus expulsion was under nervous control, but Coonfield (1932) questioned direct innervation and favoured a contractile mechanism. As early as 1895, Keng had suggested that possibly the coelomic fluid escaping through the dorsal pores could, by reason of its alkaline reaction, stimulate the mucous glands to discharge. This, unsupported by experimentation, must have been an entirely speculative suggestion, but has an interesting parallel in certain human disease situations where, accompanying some types of chronic bronchitis and asthma, there is rise of pH to the alkaline side. This has the effect of producing increased mucus flow, though not by directly stimulating the existing mucous cells but by transforming the normal ciliated epithelium with few goblet cells into a mucous membrane consisting almost entirely of goblet cells (Cardell, 1956).

Schneider (1902; 1908) appears to be alone in recording any observations on the refilling of the empty mucous cells, describing how, after expulsion, the basal nucleus rounds off and projects like a cone into the empty cell space, surrounded by cytoplasm. He described the recharging of the collapsed cell with granules from the "theka" and the base, which became thickened again.

The development and replacement of the secretory cells has received attention and given rise to two conflicting ideas. Vejdovsky (1884) claimed, on D. rubida, that the glandular cells were modified epidermal cells (supporting cells), but Cerfontaine (1890) challenged this and, from studies on macerated tissue, proposed that the large granular cells arose from the transformation of the basal cells of the hypodermis (epidermis). These two contrary views have their modern counterparts. Valembois (1971b), on regeneration of lumbricid body wall grafts (A. caliginosa and E. foetida), also stated that the mucous

cells take their origin from basal cells, but gave no evidence as to how this differentiation was accomplished. The supporting cell origin of mucous cells has been established, however, in other annelid groups, if not in oligochaetes. Thouveny (1967) claimed such an origin in polychaetes and Cornec, Solbani & Turki (1970), on polychaetes and leeches, reported that the polychaete mucous cells differentiated directly from the "cellules piliers" (supporting cells), as does Gondran (1954) for the leech Glossosiphonia.

References to the function of lumbricid mucus are numerically many and physiologically varied. In all cases, no reference is made to any particular cell type, it being assumed, and not contradicted by the literature, that only one type of mucous cell existed in lumbricid skin despite the differing histological and histochemical descriptions. The small granular (albumen) cells had always been regarded as distinct by most workers and no function had been ascribed to them in the literature.

Cerfontaine (1890) stated that the hypodermal glands poured out a substance that was capable of preventing evaporation and maintaining moistness. However, Keng (1895) noted that when worms were placed on a dry surface the body glistened as a result of minute droplets being exuded from the dorsal pores and concluded that the coelomic fluid was used to delay desiccation. Wolf (1940) also stated that the respiratory moisture was derived from the coelomic fluid exudation, perhaps together with the product of the mucous glands, and added that fluid from the nephridia might also contribute to the skin moistness and so play an indirect part in respiration. Such a suggestion is of historical interest in that until 1853 the nephridia were regarded as respiratory organs, Gegenbaur (1853) first suggesting their excretory role. In an earlier paper on the behaviour of

L. terrestris to dehydration, Wolf (1938) characterised stages in drying-out and noted that the main fluid response was in terms of coelomic fluid from the dorsal pores except in the anterior region, where such pores do not exist, and here the apparent wetness was thought probably to be due to mucous cell secretion. Hall (1922) observed that as A. chlorotica approached its vital exsiccation limit a "mucus-like" covering was secreted, but was not more precise than that. Similarly, Grant (1955) working on moisture relationships in earthworms noted that when worms were placed on a dry field of blotting paper they became covered with a "mucous-like" secretion from the dorsal pores. Everywhere else in his paper the moistness of the integument is attributed directly to the coelomic fluid, to which he also attributes locomotory and temperature regulation functions.

Healy (1963) stated that mucus kept the epidermis of earthworms moist so that it could function as a respiratory surface and Negus (1963), in the context of a general discussion of the functions of mucus, noted that the cutaneous respiration of earthworms was facilitated by their covering of mucus. In other oligochaetes more precise statements have been made. In tubificid worms Whitley (1968) has shown how lead and zinc pollutants precipitate the surface mucus, as they do on fish gills, and so disrupt the oxygen exchange by formation of a mucus-metal complex. However, in fully aquatic oligochaetes it might be argued that the mucus may be performing some other function than that of keeping the surface moist for respiration. In the megascolecid study of Varute & Nalavade (1970) on Pheretima elongata it has been suggested that the integumentary mucus, by virtue of the hyaluronic acid component present, acts as a hydrophilic colloid, having the property to bind water and so maintain a thin watery film covering the body.

A lubricative function is often attributed to mucus, but the earliest reference to the need of a lubricant during locomotion relates not to the mucus but to the coelomic fluid. In 1727, the Canon of Windsor, Dr. Derham, in a work entitled "Physico-Theology", described a slimy juice which emitted at certain perforations between the annuli "to lubricate the body and facilitate the passage into the earth". This reference (quoted by Friend, 1924) clearly refers to coelomic fluid exuding through the dorsal pores, structures which, incidentally, were first described in 1672 by a Dr. Willis who thought that they "supplied the place of lungs".

The more specialised function of cementing burrow galleries was first documented by d'Udekem (1862), and in 1949 Michon described the diapause chambers of A. caliginosa as earth capsules lined with a mucous secretion. Grant (1955) suggested that the unusual amounts of mucus secreted by some experimental individuals of A. caliginosa indicated the onset of diapause, and he regarded this as a reaction to unfavourable moisture conditions. Avel (1929) had regarded the summer diapause of A. longa as an obligatory reaction and independent of environmental factors, but Gerard (1960) challenged the idea of an obligatory diapause and concluded that the state was a quiescent one and a behavioural response to the critical physical factor of soil moisture. Either way, it is accompanied by the secretion of mucus which is therefore fulfilling a protective role.

The protective function of mucus is also mentioned by Laverack (1963) and Healy (1963), but Keng (1895) had attributed such a function to the combined complex of the coelomic fluid exudate and the mucin, the former containing phagocytic cells.

The action of mucus as a deterrant to predators is well documented in other animal groups (Jakowska, 1965b), but in worms

the fluid produced on handling, irritating and subjecting to temperature extremes is described as containing both mucus and coelomic components (Keng, 1895; Friend, 1924), the cellular elements present in it confirming the participation of the latter. Lawry (1973) reported that any manipulation necessary to implant intramuscular electrodes caused loss of coelomic fluid, whereas Adolph (1927), in contrast, had stated that neither mucus nor body fluid were expelled upon handling or drying and that after faradic stimulation a cell-less fluid, apparently from the mucous gland cells, was produced, but that after prolonged or repeated stimulation, body fluid containing cells was poured out, and this always issued from the nephridiopores. However, Fujimoto & Adams (1965) recorded jets of mucus being expelled when worms were irritated by being placed in water containing solid carbon dioxide. Notwithstanding, the brandling, E. foetida, got its specific name and its other popular name - the foetid worm - because of the unpleasant odour attached to the turbid yellow fluid which it ejects when irritated, the colouration of which is due to the chloragogen cells from the coelom.

Bahl (1947), Needham (1957) and Haggag & El Duweini (1959) have all implied that the mucus may be a vehicle for nitrogen excretion, Needham (1957) calculating that about 50% of the nitrogen lost from the body of earthworms being in the form of a mucoprotein.

That mucins may act as a buffer defence system in lumbricids was suggested by Laverack (1963) and both Pease (1966) and Pinter (1967) have evidenced that acid mucopolysaccharides can act as protective colloids by providing a buffer defence mechanism in mammals.

Spicer, Staley, Wetzel & Wetzel (1967) and Negus (1963) (who referred to a paper by Fleming in 1928) have attributed bactericidal properties to mammalian mucus and Jakowska (1963) has reviewed the

evidence in fishes. Cooper, Acton, Weinheimer & Evans (1969), during their work on bactericidal response in L. terrestris, isolated many bacterial strains from the gut contents and the exterior body slime, and used 5 of these, all gram-negative rods, together with other bacterial antigens for their experiments. They failed to elicit bactericidal responses in their coelomic assays, though Keng (1895), working at a cruder level, had observed phagocytosis of introduced anthrax by the coelomic cells of L. terrestris. However, the source of the earthworm bacterial antigens from slime in the work of Cooper, Acton et al. (1969) would suggest that earthworm mucus is not itself bactericidal.

Recently an entirely new function has been ascribed to earthworm mucus. Ressler, Cialdini, Ghoca & Kleist (1968) and Ratner & Boice (1971) have described, from behaviour studies, a pheromonal function. As their work is pertinent to the results of the present electron microscope study of the orthochromatic mucous cells in lumbricids, it will be discussed in Section 11.

4 LUMBRICID HISTOLOGY

The various fixation methods produced widely differing representations of the cuticle and the epidermal secretory cells, para-toluene sulphonic acid with formalin (PTSA/f) appearing to result in considerable swelling of both the gland cells and the cuticle (Plate 1a). In so doing it permitted clear observation of the cuticular striations, known in the literature since 1869 (Claparède), particularly in oblique or surface slices of the cuticle. These striations are not claimed to be the individual cross-lamella fibres of electron microscope studies, but merely the gross result of the refractivity of the fibre system. The greatest shrinkage of the cuticle was in Zenker and Helly fixed material.

With respect to the gland cells, chemical anaesthetics before fixation produced drastic emptying of the gland cells and the pre-fixation cooling was found to give the best retention of secretion within the cells. Differences similar to those given for the cuticle were observed in the gland cells, PTSA/f always leaving them with the appearance of bloated sacs only about one to one and a half times as high as wide (Plate 1a). Zenker material, on the other hand, presented them as elongated, almost columnar, cells often four times greater in height than width (Plate 1b). Formalin and Bouin material produced effects somewhere between these two, and frozen material, with or without subsequent fixation, was unsuccessful in terms of preserving any recognisable cell shape, considerable distorsion of the whole epidermis having taken place.

In view of these variations, the presentation of cell measurements cannot be of real value.

The various fixation methods also produced differences in the contents of the secretory cells, three types of which were established. Two of these are describable in broad histological terms: the mucous cells with contents in the form of large, obvious granules lying in the secretory cavity, and the secretory cells where no such goblet sac occurred and the granules were small. The third type was not so histologically uniform in its appearance but was distinguishable histochemically by the metachromasia displayed by the secretory cavity contents (Plate 2a). Such cells will be referred to as the metachromatic mucous cells in contrast to the large granular, orthochromatic, mucous cells (Plate 2a) and the small granular cells (Plate 4e).

Zenker and Helly appeared to be the most satisfactory fixatives for retaining the secretion of the large granular cells, as noted by Pedersen (1963) on turbellarian epidermal secretory cells, the contents appearing as discrete granules with distinct spaces around them (Plates 1b; 2b; 4e). With some histochemical techniques these spaces reacted differently from the granules. Some slight degree of coalescence of the granules was observed in the Helly-fixed lumbricid material. With Bouin and formalin, many more cells showed coalescence of the granules into coarse strands and this was also true to some degree in PTSA/f material. Carnoy fixation tended not to retain the granularity of these cells and in frozen sections there was no trace of granules.

From the histological picture, irrespective of the histochemical nature of the secretion, it has been possible to equate this cell type to earlier descriptions of gland cells in lumbricid epidermis. Particularly in Zenker and Helly material this cell is recognisable as the large granular gland cell of Cerfontaine (1890), Schneider (1902; 1908), Feldkamp (1924), Thomas (1932) and the GM2 granular stage of Herlant-Meewis (1958/59). It also equates, probably, to the

description by Lutfy (1965a) of the ordinary mucous cells that occur in region B of the clitellum where he states that the cells appeared "more or less granular, but the granules lacking any distinctive individuality." As he used both Helly and Bouin and coalescence of granules has been observed in the present study with these fixatives, it could be assumed, on histological grounds alone, that he was referring to the large granular cell type. Defretin & Demailly (1953) did not describe the appearance of the secretion in the clitellar mucous cells of their study. The large granular type of mucous cell equates morphologically, but not histochemically, to the granular phases in the integument of Megascolex mauritii (Krishnan & Rajulu, 1969), but Varute & Nalavade (1970) did not include any detailed description of the secretion in Pheretima elongata.

The histological appearance of the metachromatic cells also varied dependent on fixation procedures. In PTSA/f, globules of material were occasionally seen, sometimes coalescing into strands. In Helly material the secretion was generally in the form of strands, as it was after Bouin and formalin fixation. Such pronounced strands were rarely seen in Zenker material, the commonest state being the appearance of the mucus as a fine reticulum within the secretory cavity (Plate 1b; 2b). Fine focussing suggested that this network existed as a lining to the secretory cavity and so these cells were regarded as being nearly empty, the delicate strands representing the remnant of the extruded mucus. As the majority of the metachromatic cells assumed this appearance in Zenker material, and it is unlikely that all such cells would normally be fully discharged at any one time, the effect of Zenker fixation on the two mucous types is apparently very different, the metachromatic secretion being readily discharged during fixation and the orthochromatic (large

granular) type being little affected. The possibility of significant viscosity differences existing and being responsible for this is discussed in the Sections 6iii and 7iii.

The histological appearance of the metachromatic cells equates to the descriptions of reticulate, network, honeycombed or alveolar cells by Schneider (1902; 1908), Feldkamp (1924) and, at clitellar sites, Grove (1925), Grove & Cowley (1927), Lutfy (1965a) in region A, and the "spumeuse" (frothy) stage of Herlant-Meewis (1958/59). Cerfontaine (1890) did not describe a reticulate type and as his figure of the lumbricid epidermis has formed the basis for the descriptions by Stephenson (1930) and Laverack (1963), these texts make no reference to this cell type. Reticulate cells are not described in the megascolecid study of Krishnan & Rajulu (1969).

The small granular cells presented a much more uniform appearance with the various fixatives. The histochemical results (Section 8ii) reveal a higher protein content in these cells and, as most fixatives are designed to fix protein, it would appear that the process has been more effective with respect to this cell type. The histological representation of the small granular cells equates well to the Eiweisszellen of Schneider (1902; 1908), the fine granular cells of Feldkamp (1924), possibly the small granular type of Thomas (1932) as the description is minimal, and the albuminous cells of Herlant-Meewis (1958/59). The small granular cells of this study do not equate to the fine granular cells of Cerfontaine (1890), copied by Stephenson (1930) and Laverack (1963) and termed albumen cells, and the details and assessment of this cell type is given in Section 8iv. Small granular cells of similar appearance to those in lumbricids are recorded by Krishnan & Rajulu (1969) in the megascolecid Megascolex mauritii.

In some Zenker material, however, what appeared to be a fourth histological cell type was observed, in which the globules were distinctly demarcated by membranes but in which the contents were considerably condensed to about the size of a typical small granular inclusion. From the histochemical studies it was concluded that this type of cell was, in fact, an undischarged metachromatic cell, again evidencing the effect of Zenker on this type of secretory product.

The secretory cells extend the full depth of the epidermis, but since this is not constant throughout one segment, being shallower in the intersegmental regions and reaching its maximum depth mid-segmentally, the height of the secretory cells, irrespective of fixation, varied within any one segment of an individual. Wherever the epidermis narrowed to less than about half to a third of the maximum depth, no secretory cells were found, therefore secretory cells were absent from the intersegmental regions including the dorsal pore areas. They were also absent from the immediate vicinity of the chaetal areas, the epidermis narrowing as it approached these regions. These findings are in complete agreement with the detailed description of secretory cell distribution given by Cerfontaine (1890) for L. terrestris (agricola). No satisfactory explanation can be offered for this rather strict relationship between the height of the epidermis and the presence or absence of secretory cells.

Both the orthochromatic (large granular) and the metachromatic mucous cells show a random and frequent distribution within the secretory cell bearing regions. The small granular cells, however, are considerably less numerous and very irregularly dispersed.

The two types of mucous cell, orthochromatic and metachromatic, are of the 'goblet' type with a basal nucleus in which the nucleolus is less prominent than that found in the nuclei of the supporting

cells of the epidermis. The nucleus is often compressed and is surrounded by cytoplasm a thin continuation of which lines the secretory cavity. The small granular secretory cells do not contain an obvious goblet cavity. The nucleus is again placed at the base of the cell, the remainder of the cell being packed with small, discrete granules.

The histochemical results for the three types of secretory cell are given separately in Sections 6, 7 and 8, where the results are discussed and compared with the findings of other oligochaete workers. From these results it will be established that the three types are distinct entities and this is confirmed by the electron microscope observations. In this respect, the present work is in conflict with a suggestion of Schneider (1902; 1908) that possibly the cells are part of a secretory sequence, an idea which was firmly advanced by Thomas (1932), on no good evidence, and was supported by the histochemical findings of Herlant-Meewis (1958/59). She used Hollande Bouin's fixative, and the present study has shown that the granular type tends to show coalescence into strands with this fixative and that the metachromatic material is also strand-like. As she did not use other fixatives her interpretation of the results can be understood.

5 LUMBRICID CUTICLE HISTOCHEMISTRY

i) Introduction

Comparatively little detailed histochemical work has been done on lumbricid epidermal cuticle. However, Van Gansen (1960), on a study of gizzard cuticle in E. foetida, stated that the ectodermal cuticle and basement membrane showed the same affinities as the collagens but that the gizzard cuticle did not, basing this on several tests including WRF where she obtained a violet reaction at gizzard sites but a more typically collagenous reaction at epidermal sites. Her conclusion that a non-fibrillar elastin was present in the gizzard cuticle, but not in the epidermal cuticle, was challenged by Izard & Broussy (1964) working on L. terrestris. In an extensive histochemical study they found the gizzard cuticle to be Ald.F positive but susceptible to methylation and attributed this result to the presence of sulphate ester groups and not to elastin. The gizzard cuticle was strongly PAS positive, regaining the colouration after the acetylation-saponification sequence, metachromatic with TB, and alcianophilic. However, hyaluronidase did not abolish the acid mucopolysaccharide reactions, and they claimed that hyaluronic acid was not present at the gizzard site. All their protein tests were negative as were their lipid results.

Coggeshall (1966), on 0.5 µm resin sections of L. terrestris, found the outer edge of the body cuticle to be intensely PAS positive, as did Krall (1968) on the oligochaete Dero obtusa, the inner bulk of the cuticle staining less intensely. Burke (1974) recorded AB positivity at the cuticle outer edge and a general uptake of Ruthenium red by the cuticle of E. foetida processed for electron

microscopy. These results were similar to the findings of Potswald (1971) on Aeolosoma bengalense where the presence of acid mucopolysaccharides in the cuticle of this oligochaete was established.

At clitellar sites in A. caliginosa Lutfy (1965a) studied the cuticle mainly using trichrome and triple stains. However, he recorded a positive PAS reaction but got negative results with TB.

Apart from the Izard & Broussy (1964) study on the gizzard cuticle of L. terrestris, the only other extensive histochemical study of oligochaete cuticle has been by Rajulu & Krishnan (1967) on the megascolecid Megascolex mauritii. The cuticle was found to give a PAS positive reaction which was diastase-resistant and present after lipid extraction. Acid mucopolysaccharides were detected by TB metachromasia and the pH extinction point, using methylene blue, was between pH 4.9 and 5.4, showing the substrate to be a weakly acidic one. From this they deduced the presence of hyaluronic acid and checked their histochemical results against vertebrate reference material using domestic cock's comb. Their nitrogen estimations and chromatographic analysis supported their assumption that hyaluronic acid was present in the cuticle of this megascolecid. They did not carry out enzymatic studies or protein tests.

Biochemical and biophysical studies had revealed that the lumbricid cuticle had both a carbohydrate moiety (first demonstrated by Singleton (1957) on A. longa and extensively analysed by Watson (1958) on L. terrestris) and a protein component in the form of collagen (Watson & Smith, 1956). However, the earthworm cuticular collagen is known to be similar but not identical to vertebrate collagen. It differs from vertebrate collagens in certain amino acid proportions (Watson, 1958; Maser & Rice, 1962; Gross, 1963; Josse & Harrington, 1964; Fujimoto & Adams, 1965), in the location of given

amino acids in tripeptides (Goldstein & Adams, 1968; 1970) and in the type, amount and binding of carbohydrates (Lee & Lang, 1968; Muir & Lee, 1969; 1970). Furthermore, ultrastructural studies had indicated that the collagen fibres were housed in an appreciable amount of matrix material and that they were atypical in being unbanded (Reed & Rudall, 1948; Watson & Silvester, 1959). In these two respects they differed from the collagen fibres of the basement membrane which Ruska & Ruska (1961), Coggeshall (1966), Baccetti (1967) and Burke (1974) have demonstrated as being typically banded, though Baccetti (1967) detected certain topochemical differences between lumbricid banded collagen and mammalian collagen. Comparatively little ground substance surrounds these sub-epidermal collagen fibres in lumbricids. Biochemically the basement membrane and connective tissue collagen differs from the cuticle collagen (Fujimoto & Adams, 1964; Spiro, 1970).

The introduction of limited ultrastructural information in this section has been found necessary in order to allow full discussion of the histochemical findings.

The present study was directed towards an exposition of both the neutral and acid polysaccharide components of the cuticle and to an investigation of the protein component in the light of the known biochemistry and biophysics. Particular attention was directed towards the reactions of the outer cuticle edge, termed the epicuticle by Ruska & Ruska (1961), and to those of the basement membrane in view of its known, but different, collagenous nature.

ii) Results

The histochemical results for the lumbricid cuticle are presented in Tables I (Mucopolysaccharide results) and II (Protein results). No differences were detected between the species.

Mucopolysaccharides

The PAS results were strongly positive for the cuticle (Plate 1b) and also for the basement membrane subtending the epidermis. When the timing of the technique was carefully controlled so as not to give overstaining, the outer edge of the cuticle coloured a deeper magenta than the rest of the cuticle. The Schiff reagent alone did not produce colouration, therefore groups prerequiring periodic acid oxidation to aldehydes before visualisation by fuchsin were present.

The PAS reaction is given by a range of carbohydrates, some proteins and some lipids. The cuticle of material which had undergone lipid extraction was positive and deamination did not affect the reaction, thus suggesting that the periodate-reactive material was a type of carbohydrate. Diastase and salivary amylase did not reduce the PAS staining which was therefore presumed not to be due to glycogen. Acetylation destroyed the reaction, but colour was resumed after the acetylation-saponification - PAS sequence, indicating that the groups blocked by acetylation were OH groups, any other type of acetylated group being unable to respond to saponification so as to give OH groups which are again available for periodate oxidation to aldehydes. It was therefore presumed that vic (1,2) glycols were present and that the substrate responsible for the PAS reaction was a neutral polysaccharide.

Acid mucopolysaccharides are detected histochemically by a range of techniques including metachromasia with thiazin dyes, and staining by Alcian blue, and are further delineated into carboxylated or sulphated acid mucopolysaccharides by manipulation of these tests.

Thiazin dyes produced metachromasia in the cuticle, but if overstained with TB the bulk of the cuticle was orthochromatic, with the outer free edge still showing metachromasia. The cuticular metachromasia was not alcohol resistant and was considerably reduced

after acetone dehydration. The Az.A pH extinction point was around pH 4.0. These results indicated the presence of some acid mucopolysaccharide material in the cuticle, probably of a carboxylated nature, as sulphated mucins are more alcohol resistant and have a lower pH extinction point.

With AB at pH 2.5 the cuticle was positive, but not strongly so, and in sequences combined with PAS the cuticle was always strongly PAS positive (Plate 1b). Acetylation abolished the alcianophilia, as did methylation, but the methylation - saponification - AB sequence restored the staining and pointed to the presence of reactive carboxyl groups, as only these can be regained in the sequence and result in alcianophilia. However, AB at pH 1.0, a test for sulphomucins, gave weakly positive results, less strong than the basement membrane, which suggested that some sulphated acid mucus might be present. But the possibility of a weakly sulphated mucin is not entirely consistent with the AB at pH 2.5 results which were not intense, because weakly sulphated mucins stain, paradoxically, strongly at pH 2.5. Nor were the deamination - AB results suggestive of a weakly sulphated mucosubstance, for at mammalian sites such substances are associated with amine groups (Spicer, Horn & Leppi, 1967) and lose alcianophilia after deamination. It therefore seems unlikely that a weakly sulphated mucin is present.

The enzyme studies, used to categorise carboxymucins, failed to reveal glucuronic acid as the reactant acid residue, also sialic acid residues, the latter being confirmed by the acid hydrolysis results. However, TB metachromasia was abolished in the cuticle after hyaluronidase treatment, and therefore suggested that hyaluronic acid, by virtue of its carboxyl groups on D-glucuronic acid, was responsible for the acid mucopolysaccharide reactions of the cuticle. This agrees with

the AB at pH 2.5 results where the staining intensity was not strong, since hyaluronic acid is the only acid mucopolysaccharide giving weak acid reactions (Pearse, 1968).

The CEC.AB technique is used to distinguish between sulpho- and carboxymucins and the results support the evidence that carboxyls were the reactive group, the absence of alcianophilia at high molarities confirming that sulphomucins were not present. Sulphate groups were not revealed by the Tetrazonium technique, nor was there S^{35} uptake by the cuticle in E. foetida. The HID and LID-AB sequences of Spicer (1965) also failed to reveal sulphated mucins, the cuticle displaying alcianophilia consistent with the other results indicating carboxylated mucus. However, the parallel technique of Ald.F-AB (Spicer & Meyer, 1960) for delineating sulpho- and carboxymucins produced conflicting results.

The cuticle was strongly Ald.F positive, especially the outer edge, as was the basement membrane, and suggested, as the technique was performed as the Ald.F-AB sequence, the presence of sulphomucins. However, in mammals, the Ald.F positive reaction in this sequence is also given by elastica, but the two substrates react differently to methylation, sulphomucins being susceptible and elastins not. Drastic methylation at 60°C for 4 hours abolished the Ald.F staining in the basement membrane and the bulk of the cuticle, the free outer edge, however, remaining positive. The methylation susceptibility suggestive of the presence of sulphomucins is in conflict with the other acid mucopolysaccharide tests which had contraindicated their presence. These results, at the same time, indicated that elastin was not present in the bulk of the cuticle, and this was supported by the negation of the Ald.F reaction after trypsin pretreatment, trypsin being known to have no elastolytic activity (Adams & Bayliss, 1962).

The outer edge of the cuticle, however, remained positive, though not intensely, after trypsin digestion. After deamination the cuticle staining by Ald.F was either markedly reduced or abolished, unlike the basement membrane which became intensely positive. These results suggested two things: that the Ald.F reaction was due to the protein moiety of the cuticle; and that the Ald.F reactive material was different at the two sites. Fullmer & Lillie (1957) state that collagen is made more reactive to Ald.F after deamination, so it would appear that the technique was displaying the presence of the collagen known to be present in the basement membrane (Baccetti, 1967), whereas the collagen known to occur in the cuticle (Reed & Rudall, 1948; Watson, 1958) was responding differently.

Proteins and Lipids

The protein results were either negative (Ninhydrin, BS), extremely weak (Millon's - showing low levels of tyrosine-containing protein to be present, a result consistent with the biochemical findings of Josse & Harrington (1964)), or weakly positive (BPB, Amido black, FALG). The only test giving strong reactions was WRF, an elastin stain, though the colour was not that of mammalian elastin.

The BPB was consistently weaker at cuticular sites than at basement membrane sites and was abolished after deamination, permanganate oxidation and proteases. Amido black failed to give the dark green typical of collagen, the cuticle stained pale green in contrast to the strong green/blue of the basement membrane. As Amido black is a technique for demonstrating collagen and earthworm cuticle is collagenous, this result was unexpected. A similar failure was recorded with van Gieson, where the red colouration typical of collagen was observed in the basement membrane, but the cuticle, at best, assumed a pale pink tinge. The faint green result with FALG was uninformative in that both mucin and collagen react with Light green,

and, as many mucins have protein components, the negative results after deamination, permanganate oxidation and proteases did not really clarify the basis for this staining. However, as the PAS results indicated a neutral polysaccharide and since acid mucins do not generally have histochemically detectable protein moieties, it would seem that the FALG reaction was responding to the protein in the cuticle.

WRF gave a red/brown colour to the cuticle, especially intense at the outer edge, and a similar colouration to all the secretory cells and the basement membrane, perhaps indicating that a common constituent was present at all these sites. The violet/black colour given by vertebrate elastin with WRF was not recorded, the colour being closer to that given by vertebrate collagen after this stain.

The range of lipid tests were all negative at cuticular sites.

iii) Discussion

The histochemical results have shown that the cuticle has many characteristics which are shared with the epidermal basement membrane, that it has a carbohydrate moiety which was more easily visualised than its protein moiety, and that the outer free edge of the cuticle displayed properties which indicated that it was different, perhaps both quantitatively and qualitatively, from the rest of the cuticle.

The cuticle and the basement membrane were PAS positive, and basement membranes in general in the animal kingdom react in this way. The lumbricid epidermal basement membrane is known to be collagenous (Coggeshall, 1966; Baccetti, 1967; Burke, 1974) but to be different from the cuticle collagen, and this would explain some of the differences recorded between the basement membrane and the cuticle. The increased staining with Ald.F after deamination, and the stronger Amido black

and van Gieson results can be interpreted directly in terms of the presence of conventional collagen at the basement membrane sites. As there is comparatively little ground substance surrounding these subepidermal collagen fibres and an appreciable amount of matrix material surrounding the fibres in the cuticle (Ruska & Ruska, 1961; Coggeshall, 1966; Burke, 1974) this might offer an explanation for the situations where a marginally more positive reaction was recorded for the basement membrane, as in the BPB technique.

The carbohydrate moiety of the cuticle is shown to consist of a diastase-resistant, vic (1,2) glycol-containing fraction easily demonstrated by the PAS technique. The outer edge of the cuticle responded more strongly. An acid mucopolysaccharide component is also present and was demonstrated, by many techniques, to be of the carboxylated variety. Enzyme blocking revealed that hyaluronic acid was present. Hyaluronic acid-containing mucopolysaccharides are common in mammals where they are restricted to connective tissue sites, and were stated as being exclusive to vertebrates by Kent & Whitehouse (1955), though Defretin (1951) had cited the presence of hyaluronic acid in the mucus of certain polychaetes. Hyaluronic acid appears to be physiologically bound up with the viscosity of the mucosubstance (Defretin, 1951), and Holt, How, Long & Hawkins (1968) have shown that its presence is necessary for normal synovial fluid function in man, the decreased levels in rheumatoid arthritis resulting in a less viscous synovial fluid. The significance of a hyaluronic type mucin in earthworm cuticle can be speculated upon in terms of providing a sufficiently viscous matrix for the functioning of the collagen fibres.

The interrelationships of the acid and neutral mucopolysaccharide moieties have not been revealed by this study. However, from parallels

in mammalian studies, it is not thought that the acid residues can be attached to the neutral macromolecules, because hyaluronic acid mucins are usually PAS negative. It is therefore presumed that two distinct carbohydrate macromolecule types are present in the earthworm cuticle.

The failure to display strong protein, especially collagen, reactions is difficult to explain. However, the biochemical and biophysical identification of collagen in the earthworm cuticle does not necessarily mean that in situ the molecules are free to react with histochemical reagents. Failure to give staining reactions may be due to the masking by the matrix material in which the fibres are embedded or may be due to inherent molecular arrangements. The fact that the cuticle collagen is ultrastructurally distinguishable from normal collagen in being unbanded is indication that the molecules are aligned differently, so failing to produce the characteristic banding. This difference might be sufficient to prevent histochemical visualisation of the material, groups which are free to give reactions in normal collagen may not here be in a position so to do.

However, protein material has been shown to exist, though the results were always weak with the exception of the WRF technique. It has not been possible to indicate whether the feeble staining is due to carbohydrate masking or to an intrinsic molecular configuration within the protein yielding insufficient groups for dye attachment.

The WRF results pointed more towards a collagenous reactive substrate than an elastin one, as recorded by Van Gansen (1960), since no violet/black colouration was produced. However, all the secretory cell types also coloured and although not giving results as for mammalian elastin, an elastin-like protein is to be described at these sites (Sections 6, 7 and 8). The Ald.F-AB results did not indicate elastin in the bulk of the cuticle, the reaction being

susceptible to methylation and trypsin digestion, as was that of Izard & Broussy (1964) for lumbricid gizzard cuticle. However, the outer edge of the cuticle remained positive after drastic methylation for 4 hours, and was denser in the WRF technique. The Ald.F results, in terms of mammalian histochemical evidence, would point to an elastin-like protein present at the free edge of the cuticle where it is known, from ultrastructural studies (Ruska & Ruska, 1961; Coggeshall, 1966) that only matrix material is present. The stronger PAS staining of this zone presumably reflects the absence of collagen fibres and the concentration of matrix material. Although collagen is known to be PAS positive and to have, itself, a carbohydrate moiety (Josse & Harrington, 1964) which may well be responsible for the basement membrane reactions, it is not thought to be responsible for the reaction in the cuticle because of the deamination results and the denser staining of the epicuticle where collagen fibres are not present.

In the light of the ultrastructural detail available in the literature it perhaps suggests that the WRF staining of the cuticle is in fact due to matrix rather than fibrous material, but it does not explain the methylation-resistant Ald.F staining of the epicuticle, which suggested that elastin was present at this site. If the deductions made from the results are correct, then interpretation of these results becomes complex. However, two series of comments in the literature give rise to interesting speculations regarding the epicuticle.

Firstly, Krall (1968) working on the ultrastructure of the oligochaete Dero obtusa (Naididae) suggested that the cuticle might be in a state of constant renewal from the base, and this was also hinted at by Potswald (1971) for Aeolosoma bengalense (Aeolosomatidae). Coggeshall (1966) on L. terrestris had earlier reported that the

collagen fibres were thickest at the centre of the cuticle, with the outermost and innermost ones being considerably thinner. One could perhaps imagine a formation-maturation-degeneration sequence occurring with regard to the fibres. Secondly, Burton, Hall, Keech, Reed, Saxl, Tunbridge & Wood (1955) and Hall, Keech, Reed, Saxl, Tunbridge & Wood (1955) have reported the transformation of human collagen into elastin.

If the earthworm cuticle could be shown to be in a state of constant renewal and if the collagen material underwent such a transformation, the elastin-like reactions of the outer free edge might be explained. However, at the moment, no satisfactory explanation can be advanced concerning the Ald.F reaction at the epicuticle, and such speculations as the above highlight the difficulties, and perhaps dangers, of applying mammal-oriented techniques and relying on deductions based on them when working with invertebrate material.

The current results show, with the exception of the hyaluronic acid component, that many similarities exist between the body cuticle and gizzard cuticle (Izard & Broussy, 1964) of lumbricids. They also reveal, in terms of the carbohydrate components, strong parallels between the megascolecid (Rajulu & Krishnan, 1967) and lumbricid epidermal cuticle. The more limited results of the workers on microdrile oligochaetes (Krall, 1968; Potswald, 1971) are not in conflict with the present findings and suggest that in many respects the histochemistry of the body cuticle of oligochaetes shows uniformity.

6 LUMBRICID LARGE GRANULAR, ORTHOCHROMATIC MUCOUS CELL HISTOCHEMISTRY

i) Introduction

The granular mucous cells in the unmodified skin of E. foetida have been shown to contain neutral mucopolysaccharide material exhibiting PAS positivity (Herlant-Meewis, 1958/59). It was suggested that these cells represented the early stage of the secretory cycle and that this neutral mucus was later transformed into strongly acid mucus no longer susceptible to periodic oxidation, and the granulation disappearing. Lutfy (1965a) also demonstrated PAS positivity in the ordinary mucous cells at clitellar sites of A. caliginosa (not to be confused with the "large granular" clitellar cells of his description), as did Defretin & Demailly (1953) in similar cells in the clitellum of L. terrestris.

No direct record of a histochemically demonstrable protein moiety within the mucus of lumbricids appears in the literature, though Needham (1957) claimed that 50% of excreted nitrogen in L. terrestris and E. foetida was lost in the form of mucus protein, and Bahl (1947) suggested that the protein found in collected urine was not a component of normal urine but had come from the mucus collected simultaneously. Herlant-Meewis (1958/59) recorded a paraldehyde fuchsin positive reaction and an AB positivity after oxidation in the granular stage of the mucous cells of E. foetida but did not comment on these results in terms of a protein component. The workers on clitellar sites (Defretin & Demailly, 1953; Lutfy, 1965a) did not record protein in the ordinary mucous cells of these modified areas.

Investigators this century have not carried out lipid histo-

chemistry on the granular mucous cells, though Cerfontaine (1890) recorded that the secretion of the granular gland cells coloured black with osmic acid and, even earlier, Horst (1878) had described the refringent corpuscles as globules of fat.

With the advent of more numerous and more strictly controlled histochemical techniques, it seemed appropriate to investigate, in some detail, the nature of the secretory cells of the unmodified epidermis of lumbricids, particularly since the literature assumed that only one type of mucous cell was present. Section 4 has established that this is not so, and this present section deals with the large granular, orthochromatic mucous cells. However, certain techniques used for investigating the metachromatic mucous cells (Section 7) produced unexpected reactions within the orthochromatic mucous cells, and these are reported and discussed in this present section.

The pattern of the current investigation was based on the available histochemical techniques formulated for mammalian studies. It is accepted procedure to carry out more than one type of test to establish any single feature of the complex. This has arisen because certain mammalian mucins reveal histochemical contradictions and very few workers now base conclusions on a single technique.

ii) Results

The secretory product of the large granular, orthochromatic mucous cells is a complex in which carbohydrate, protein and lipid moieties are detectable. The results of these three components are presented separately and discussion pertinent to a technique and its results is included at the appropriate stage.

Mucopolysaccharides

The significant mucopolysaccharide histochemical results are given in Table III and no differences were recorded between the species.

The large granules are PAS positive (Plate 1b). A wide spectrum of tissue substances react positively to PAS including glycogen, neutral polysaccharide, glycoprotein, phospholipid, glycolipid and some unsaturated lipid, though most Schiff-reactive lipids generally require an organic acid oxidation in the form of performic or peracetic acid rather than a periodic one. When Schiff reagent was used alone, the result was negative, indicating that a periodate-reactive material was present in the large granules.

Free hydroxyl groups appear to be a prerequisite for periodic acid oxidation which is known to affect 1,2-glycol (vicinal glycol groups), 1-hydroxy 2 amino (primary amino groups), 1-hydroxy 2 alkyl-amino (secondary amino groups) and 1-hydroxy 2 keto groups, forming aldehydes which are then visualised by the Schiff reagent.

In order to define the tissue component or components producing the observed PAS result and to confirm the presence and participation of hydroxyl groups in the reaction, several techniques are available including sulphation, acetylation, the acetylation-saponification sequence and the use of enzymes.

Sulphation esterifies hydroxyls to sulphuric esters and causes them to be no longer available for periodic acid oxidation. After the sulphation-PAS sequence the result was negative. The ease with which this blocking was achieved - 5 minutes in sulphuric ether reagent - suggested that the tissue periodate-reactive material was not glycogen which is known to be difficult to esterify with this technique, taking up to 24 hours (Casselman, 1959). Diastase and

salivary amylase failed to block the PAS reaction, confirming that glycogen was not present in the large granules.

Acetylation also esterifies hydroxyls, forming O-acetyl groups, and can also affect amino groups converting them to N-acetyl groups, both of which are no longer capable of responding to periodic acid to yield Schiff-reactive aldehydes. The acetylation-PAS sequence produced negative results and, by itself, contributes little to a further understanding of the PAS positive substrate. However, the negation was achieved after 45 minutes at room temperature in the 2:3 solution of acetic anhydride and anhydrous pyridine, and the speed and ease of this supports that the carbohydrate is not glycogen, since this and some mammalian epithelial mucins require 18-24 hours to complete esterification. Furthermore, Casselman (1959) claims that the PAS reaction is not blocked by acetylation if primary amino groups are responsible for it, blocking only occurring if reactive secondary amino groups are present. It would therefore appear that primary amino groups are not involved in the normal PAS reaction and the failure of deamination to affect the PAS result would seem to confirm this.

The more conventional way of determining whether amino groups are involved or not is the use of the acetylation-saponification sequence. This is based on the easy reversal of O-acetyl groups by saponification (deacetylation) and the extreme difficulty with which N-acetyl groups can be deacetylated. Such a failure to re-establish hydroxyl groups results in the PAS colour not being restored. Consequently, PAS positive substrates containing 1,2 glycols show full resumption of colour after the acetylation-saponification-PAS sequence, but substrates with adjacent hydroxyl and amino groups give only partial resumption.

The large granules showed full restoration of the PAS reaction

(assessed subjectively) when KOH was used as the saponifying agent. The colouration was not so intense when ammonia in ethyl alcohol was employed, probably due to the less drastic nature of this solution which therefore requires longer timings. The same was true for the saponification at low temperature as suggested by Stoward (1967a). It therefore seems that the PAS reaction is due to the presence of vicinal (vic) (1,2) glycols in the carbohydrate moiety.

The possibility that the pyridine of the acetylating mixture removed periodate-reactive lipid has to be considered, for once removed, such lipid would not be available for participation in the saponification-PAS part of the sequence. If such lipid levels were very low, their failure to be restored might affect the PAS colouration so marginally as to be undetected in the subjective assessment of 'full resumption of colour'. The lipid extraction-PAS sequence, performed on specially fixed tissue, was visually indistinguishable from the control material. Such a result pointed to the PAS reaction not being due to periodate reactive lipid and as such diminishes the concern regarding the acetylation mixture, but the assessment process, in both cases, is less than satisfactory should very low lipid levels be present. However, most Schiff-reactive lipids are not periodate-reactive and so would not have contributed to the control colouration anyway.

The large granules are strongly orthochromatic when the thiazin dyes Toluidine blue (Plate 2a) and Azure A are employed. TB basophilia is often associated with the presence of RNA, but RNase failed to decrease or abolish the reaction and showed that RNA was not present in the granules. The absence of metachromasia indicated that no acid mucus was present and this was confirmed by the pH controlled AB which was negative. In the AB-PAS sequence, the granules stained

pink (Plate 1b) as was expected.

Many orthochromatic TB substrates, including some mammalian PAS positive, vic glycol-containing epithelial mucin sites, can be manipulated so as to display metachromasia by sulphation. This technique provides terminal groups which permit TB dye molecule stacking in such a way as to cause the observable spectral shift characteristic of metachromasia. Sulphation failed to induce TB metachromasia in the vic glycol-containing large granules. The literature reveals, however, that notall orthochromatic substrates, even ones displaying PAS positivity, become metachromatic after sulphation (Kramer & Windrum, 1954), collagen being one, as are certain mammalian serous secretions (Spicer, 1963). These two examples suggest that protein interference might be the cause of the absence of induced metachromasia. Protein is also present in the large granules, and the presence of protein is known to mask the expected (but non-induced) azurophilia at some mammalian sites (Spicer, Horn & Leppi, 1967). Pearse (1968) suggests that the success of induced metachromasia after sulphation depends on the correct relationship existing between the time, dye concentration and tissue component. The result at the large granule sites could be due to the failure to achieve these relationships, the protein present affecting the tissue component.

The Ald.F technique, employed as Ald.F-AB, coloured the large granules pink (Plate 2b) and, in the absence of blocking and enzyme studies, would appear to indicate the presence of a sulphated acid mucus, for the detection of which the method was designed. The parallel diamine technique (not tabled) coloured the granules grey/black and gave rise to similar indications. However, both techniques also colour elastica in mammalian tissue (Spicer & Meyer, 1960;

Mowry, 1963; Spicer, 1965), but elastica responds to manipulation differently from sulphomucins. The Ald.F staining of the large granules was unimpaired after trypsin digestion and was resistant to drastic methylation. Since sulphomucins are susceptible to both these treatments and elastica is not, these results contraindicated the presence of sulphomucins, and in so doing supported the earlier results establishing that acid mucins were not present, and, at the same time, indicated an elastica-like substrate. This will be discussed in the protein section.

During the study on the acid mucus-containing cells (Section 7), the use of the CEC.AB technique, designed to delineate carboxy- and sulphomucins (Scott & Dorling, 1965; Quintarelli & Dellovo, 1965), resulted in AB positive staining of the large granules at low molarity $MgCl_2$ (0.1M, 0.2M) with a sharp drop in staining intensity above 0.2M, and negative results at high molarities (1.0M and above). This result was unexpected. The pH controlled AB (pH 2.5) had given negative results and had indicated against the presence of acid mucins. The CEC.AB results, though showing that sulphomucins (which alone stain at high molarities) were not present, suggested that carboxymucins (which, together with sulphomucins stain at low molarities) were. The failure of both pH controlled AB and thiazin dyes to demonstrate acid mucus therefore demands some explanation of these CEC.AB results.

A possibility might be that the low molarity dye binding of AB was to carboxyl groups in substances other than mucopolysaccharides within the secretion complex. Other tissue polyanions such as acidic proteins have the capacity to bind cationic dyes (Barrett, 1971), but such staining is generally suppressed by pH and salt concentration control, which was not the case in the lumbricid material.

A further unexpected result was the substrate reaction to the

combined CEC.AB-PAS technique. In the more usual AB (pH 2.5)-PAS sequence the granules revealed their high level of neutral mucopolysaccharide material and were PAS positive. In the CEC.AB-PAS at 0.2M the AB dye binding was dominant and the granules coloured blue (Plate 3b) and failed to respond to the subsequent PAS part of the sequence though vic glycol sites were known to exist in the granules. This suggested that the AB binding was, in some way, interfering with the PA oxidation to aldehydes or was preventing the colouring of these aldehydes by the Schiff reagent. At 0.5M, some granules coloured blue (AB+ve) and some pink (PAS+ve) (Plate 3a), indicating that less interference was occurring, fewer molecules of AB being bound, as is the case in the CEC.AB technique at this molarity. Such an argument might hold if the technique involved two direct dyeing processes competing for binding sites, but this is not strictly the case, the PAS reaction being based on chemical conversion to aldehydes which are then visualised, and not on primary dye attachment.

If the AB binding was at sites other than mucopolysaccharides within the secretory complex, it is not easy to visualise a molecular configuration which would allow dye binding at sites on one component and at the same time interfere with the PAS reaction at the 1,2 glycol sites on the carbohydrate moiety. However, carbohydrate-protein complexes are thought to consist of protein cores or chains to which the carbohydrate units are attached terminally or to side chains. If the firm AB binding were to be at COOH sites of the protein moiety, the size of the AB molecule (a phthalocyanine nucleus with 4 immonium basic side chains) might be such that although the PA oxidation would produce aldehydes at the 1,2 glycol sites the AB interference would preclude the attachment of the fuchsin of the Schiff reagent to such aldehydes. At the higher molarity (0.5M) fewer AB molecules bind and

therefore more fuchsin molecules could become attached. A similar AB interference with the PAS reaction in the large granules will be noted in the protein section.

The mucopolysaccharide results therefore indicate the presence of a diastase-resistant neutral mucopolysaccharide though fail, by the nature of the techniques, to distinguish the different hexoses in the polysaccharide. The mucopolysaccharide moiety of the large granules contains no acidic residues.

Proteins

The protein moiety of the large granules is evidenced by a wide range of techniques, the results of the major tests being presented in Table IV. Again, no differences were detected between the species.

The negative results of the ninhydrin reaction were unexpected as this test is stated as revealing any alpha amino acids and is given by all materials containing them (McManus & Mowry, 1964) and by some primary aliphatic amines (Glenner, 1963). It is possible that not sufficient terminal amino acids with available NH_2 groups were present (insulin has only one alpha amino acid group free for the reaction and is ninhydrin negative), or that the amino acids present had blocked carboxyl or amino groups (Puchtler & Sweat, 1962), or that the protein configuration was folded such that reactive groups were unavailable. All such conditions would lead to negative results (Kasten, 1962). Alternatively, the carbohydrate material within the granule may be interfering with the reaction, as it is known to do at many mammalian sites where protein results are weak or negative (Lev & Gerard, 1967). It is, however, known that not all protein is oxidised during the ninhydrin technique (Serra, 1946) and that proline, hydroxyproline and glycine respond negatively (Puchtler & Sweat, 1962), but Kasten (1962) has pointed out that proline and hydroxyproline are alpha imino acids and would not be expected to respond.

The Millon's reaction, though weak, indicated the presence of some tyrosine-containing protein and the DMAB-nitrite technique visualised tryptophan, both of which have been recorded in mammalian salivary mucin (Quintarelli, 1963). The PFA-AB sequence, with appropriate control, is used histochemically to detect S-S groups and was positive, probably indicating cystine revealed after oxidation. This result prompted the use of the PFA-AB-PAS sequence to investigate the relative dominance of the two techniques. The granules stained blue (PFA-AB+ve) and no PAS positivity was observed. This would appear to be similar to the CEC.AB-PAS sequence discussed earlier, with the AB binding at protein sites interfering with subsequent PAS reactivity.

The Bromphenol blue and mercuric BPB results were positive and were blocked by the proteases papain, pepsin and trypsin, the latter two being more effective. The BPB reaction was completely negated by permanganate oxidation and deamination. Runham (1961) considers, because of the complete BPB blocking after deamination, that the technique only demonstrates amino groups and that it does not enable predictions about the basic or acidic nature of the protein to be made. Wholly basic proteins are thought to react similarly with or without mercury (Lillie, 1965) and this was not conclusively the case in the lumbricid large granules. This would suggest that the protein is not wholly basic. Prior methylation (3 hours at 60^{o}C) was used to determine whether anion groups in the complex were interfering with the protein reaction (Lev & Gerard, 1967), but only marginal improvement was recorded in the large granules, whereas blood and muscle tissue showed a marked increase in the HgBPB staining intensity after methylation.

The Biebrich scarlet technique, used at high pH (Spicer & Lillie,

1961; Spicer, 1962a) is a test for basic proteins. The BS results were negative at high pH. Staining started around pH 4.5 and continued with increasing intensity in the lower pHs, thus the protein is stained by this acid dye only at low pH. The presence of formalin in the fixative, or as a pretreatment, is known to affect the pH at which staining occurs, and this was depressed in the granules. In mammalian epithelial mucins two distinct pH staining levels have been recorded by Lev & Gerard (1967) who suggested that both intrinsic and extrinsic proteins may exist within mucus. This was not the case in the lumbricid large granules and the results equate more to the intrinsic type described by these workers.

Basic protein (histone) was not displayed by the naphthol yellow S technique.

The FALG stain of Gurr & MacConaill (1961), when used sequentially with phosphomolybdic acid between the two dyes and when used as one solution with a 10 minute 1% acetic acid pretreatment, coloured the granules green, a result suggestive of collagen or mucin (Plate 4e). The reaction was negated by pepsin, permanganate oxidation and deamination, but not wholly by trypsin. Collagen is rarely susceptible to trypsin (Baccetti, 1967).

The Amido black technique for collagen was positive, but this and the FALG reaction were consistently weaker than the reactions given by the basement membrane.

Freshly prepared Weigert's resorcin fuchsin stained all the secretory cell types and the cuticle highly selectively, failing to colour the epidermal supporting cells or any other tissue in the section. These results suggest a common constituent. The colouration was red/brown, and not the metachromatic purple recorded by Ewer & Hanson (1945) for some polychaete mucoproteins, nor the typical

violet/black associated with mammalian elastica. The present results indicated a more collagenous-like protein.

It has been recorded earlier that the large granules stained pink in the Ald.F-AB sequence and that such results, with substrate manipulation treatments, might indicate an elastin-like protein. The FALG, Amido black and WRF results, however, suggested a collagen-like protein. Deamination did not affect the Ald.F staining intensity and it is stated (Fullmer & Lillie, 1957) that collagen only reacts strongly with Ald.F after deamination. So, if present in the complex and contributing to the control reaction, some increase would be expected after deamination, and this was not so. It would therefore appear, from this manipulation, that collagen was not present and this questions the validity and specificity of the Amido black result as being indicative of collagen. The unimpairment of the Ald.F reaction after trypsin was suggestive of elastin, as trypsin is known to have no elastolytic activity, and the resistance of the reaction to drastic methylation (4 hours at 60°C, and even when continued for 24 hours) strongly indicated an elastin-like material.

The protein results therefore indicate a not wholly basic protein exhibiting weak collagenous and strong elastin-like properties.

Lipids

The lipid results (Table V) show that no free lipid was present. The existence of bound lipid was evidenced by the Sudan black technique. This reaction was negated after pyridine extraction, weakened after permanganate oxidation and deamination, and negated after protease pretreatments. Such results point to the lipid being associated with the protein moiety. The Luxol Fast blue techniques (MBS and ARN) were negative but the pale reaction with Lillie's Nile blue and the

stronger reaction with Menschik's Nile blue sulphate pointed to the presence of phospholipid. Since none of the positive lipid reactions was ever strong in the large granules, this raised the question of solution strengths and timings. It was found helpful to have whole transverse sections of worm for many of these tests so that the techniques could be judged against sites known to contain lipid. In such tissue, the results for the large granules were consistently paler than those given by blood and chloragogen tissue, and the enzyme and chemical manipulation results at these sites would suggest that the lipid (SB+ve) material is different, possibly both quantitatively and qualitatively, and that in the granules the lipid component is perhaps more intimately bound to the protein.

Mammalian elastica is known to have a phospholipid component, but Adams & Bayliss (1962) point out that "elastic lipids cannot be completely extracted by organic solvents unless the bonds binding lipid to protein are first disrupted by acid". Pyridine extraction completely removed the sudanophilic lipid moiety in the lumbricid large granular cells and so it appears that although a lipid-protein complex occurs which gives strong indications of being elastin-like, there are no grounds for suggesting a complex such as is found in mammalian elastica.

iii) Discussion

Although the present investigation has allowed limited definition of the secretory complex and has identified a diastase-resistant neutral polysaccharide component, a protein moiety with detectable tyrosine, tryptophan and cystine which exhibits strong elastin-like and weak and debatable collagen-like properties, and a protein-bound lipid fraction which is probably a phospholipid,

the results have revealed only slender information regarding the molecular interrelationships of these moieties within the complex. That the lipid is intimately bound to the protein component has been demonstrated, but the exact association of the carbohydrate and protein moieties is not clear.

Barrett (1971) states that in mucosubstances the protein and polysaccharide components are linked together by strong, that is covalent, bonds. The protein component is always a single chain with polysaccharide side chains attached to the protein 'backbone', and it is the number and nature of these that go to determine the properties of the complex. This type of molecular configuration has not been the opinion of all biochemists and Bernardi (1957) suggested, from cartilage studies, that the polysaccharide units were linked by protein bridges to form a chain. By applying only histochemical techniques in this lumbricid study it has not been possible to decide which of the alternatives obtains in the large granular mucous cells. The idea of a polypeptide chain is attractive in that the elastin-like properties of the protein moiety point to a fibrous rather than a globular type of protein. The failure of deamination to suppress the PAS reaction merely indicated that the polysaccharide and protein moieties were not linked through the NH_2 groups, but does not contribute further information regarding the overall molecular configuration.

In the discussion of the AB interference in the CEC.AB-PAS at low molarity and the PFA.AB-PAS sequences, it was suggested that the AB binding on the protein (COOH or other groups) might be spatially such that the fuchsin visualisation of aldehydes produced by subsequent PA oxidation was precluded, but this, if correct, again does not fully resolve the question of molecular arrangement within the complex. Equally, the failure of methylation to produce marked increases in

protein reactions suggested that the carbohydrate interference was not great within the mucosubstance, but yielded no information regarding the size, number or arrangement of the polysaccharide units. Without biochemical delineation, the exact design of the secretion cannot be elucidated.

It is customary in mucus studies to attempt to categorise the complex in general terms, but the terminology of mucosubstances is complex (Stacey & Barker, 1962; Spicer, Leppi & Stoward, 1965; Barrett, 1971; Cook, 1972) and the literature abounds with synonyms, terms of ill-precision and conflicting definitions. Carbohydrate-protein complexes, as detailed here for the large granular cells, were, in the older terminology, called mucoproteins (Stacey & Barker, 1962; Cook, 1972) but the more recent terminology distinguishes between glycoproteins (Spicer, Leppi & Stoward, 1965; Barrett, 1971) and proteoglycans (Barrett, 1971).

The lumbricid complex is not a proteoglycan for these substances, although having a polypeptide backbone with several covalently linked polysaccharide chains, are characterised by the predominance of the polysaccharide component and are always acidic due to uronic acid units or ester sulphate groups or both.

Glycoproteins, on the other hand, are compounds which, according to Barrett (1971), often show the polypeptide to be the major component, though Spicer, Leppi & Stoward (1965) do not state this. A large and important group of glycoproteins have residues of the strongly acidic sugar, sialic acid, so that the molecules as a whole acquire a strong negative charge and stain as acid mucosubstances. This is not the case in the lumbricid large granular cells. However, Spicer, Leppi & Stoward (1965) describe a group of histochemically distinct substances in mammalian connective tissue and epithelia

which lack demonstrable acid properties but are rich in vic (1,2) glycols presumed to originate largely from sugar residues. Although technically neutral glycoproteins, the protein component is not detectable histochemically and so these authors prefer the use of the term neutral mucosubstance for this type of secretion. Again, the lumbricid secretion does not conform to this group, as the protein moiety is clearly demonstrable.

It would therefore appear that the mucus of the lumbricid large granular cells is unlike any that has been charted in mammalian studies, even the exceptions within the dichotomy of Barrett (1971). The use of such a term as 'non-acidic glycoprotein' seems appropriate at the moment, for it underlines most of the histochemical properties which this present study has elucidated. However, it fails to indicate the presence of the bound lipid moiety, a component unrecorded in mammalian mucosubstances other than in the specialised category of cerebrosides and gangliosides which are collectively termed mucolipids and have a restricted distribution.

Claims have been made in the literature that various fractions within mucosubstances could be responsible for the viscosity of the secretion. Campion (1961) stated that the presence of protein affected the mucus viscosity in Helix, and Hashimoto, Tsuiki, Nisizawa & Pigman (1963) more precisely demonstrated that the protein moiety contributed to the viscosity by showing that the proteolytic enzymes pepsin, chymotrypsin, papain and trypsin reduced the viscosity of mammalian submaxillary mucin increasingly in that order.

More recently, Lewis (1970) has shown that in fish mucus, using three species for detailed analysis, the phospholipid level varied in proportion to the viscosity of the mucus, a result which was in accord with the work of Breckenridge & Pommerenke (1951) on human

cervical mucus. These workers had shown that the documented viscosity changes in cervical mucus during the menstrual cycle coincided with increased phospholipid and cholesterol levels during the pre- and post-ovulatory phases. Since the cholesterol levels in fish mucus were the same for all species Lewis (1970) concluded that in fish the major factor in determining the viscosity was the phospholipid.

The present results are in agreement with earlier lumbricid workers (Defretin & Demailly, 1953; Herlant-Meewis, 1958/59; Lutfy, 1965a) with respect to the existence of a neutral polysaccharide moiety in the large granular mucous cells, but these workers either did not record protein (Defretin & Demailly, 1953; Lutfy, 1965a) or failed to reason its presence from certain of the results (Herlant-Meewis, 1958/59).

Bound lipid has not previously been recorded in lumbricid mucus, though phospholipid in association with mucous/slime secretions has been documented in schistosome cercariae (Stirewalt & Evans, 1960), Fasciola hepatica (Clegg, 1965) and certain fish (Lewis, 1970).

Investigations have been carried out on the epidermal mucous cells of certain megascolecid earthworms: Megascolex mauritii (Krishnan & Rajulu, 1969); Pheretima elongata (Varute & Nalavade, 1970); Eutyphoeus incommodus and E. nicholsoni (Menon & Singal, 1969). The Eutyphoeus studies did not contribute to the histochemical knowledge, only basophilia being recorded for the granular mucous cells. More extensive studies were performed on the other megascolecids.

The present lumbricid results, although agreeing with a protein moiety (though undefined) present in the granular cells of M. mauritii (Krishnan & Rajulu, 1969), are in marked contrast when the mucopolysaccharide component is compared. In both M. mauritii (Krishnan &

Rajulu, 1969) and Pheretima elongata (Varute & Nalavade, 1970) the nature of the large granular cells has been recorded as PAS negative, but strongly positive for acid mucins. These findings also conflict with those of Lutfy (1965b) who, parallel to his clitellar studies on A. caliginosa (Lutfy, 1965a), examined the clitellum of Pheretima hawayana and showed that, with respect to the ordinary mucous cells present in the clitellum, it was identical to the lumbricid in being PAS positive and acid mucus negative.

The megascolecid results would seem to suggest that either the ordinary mucous cells of the clitellum are unlike those of the normal integument or that there are major differences in the chemical nature of the mucus within the genus Pheretima. Further comment on these results cannot be made other than to state that in a brief survey of the clitellar epithelium of the lumbricid species in this present survey there was nothing to indicate differences between the ordinary mucous cells of the clitellum and those of the normal epidermis in the Lumbricidae, and the present histochemical survey has not revealed generic or specific differences in the mucus.

7 LUMBRICID METACHROMATIC MUCOUS CELL HISTOCHEMISTRY

i) Introduction

Most of the accounts of the metachromatic mucous cells of normal lumbricid skin date from work carried out before the existence of the present-day histochemical techniques and consequently merely record the presence of acid mucus without attempting to define its nature.

Schneider (1902; 1908) described the reticulate cells of A. (E.) rosea as staining red with German Toluidine blue (Thionin). The acid nature of certain epithelial cells of E. foetida was demonstrated by Herlant-Meewis (1958/59) using Alcian blue, and she suggested that such cells were the final stages in a secretory sequence. Negus (1963) also reported Alcian blue positive reactions for the epidermal goblet cells of L. terrestris.

The glandular clitellum of lumbricids contains, in addition to the specialised elements, mucous cells which are similar to those of the unmodified epidermis. In such cells, acid mucus has been recorded by Grove (1925) on L. terrestris, Grove & Cowley (1927) on E. foetida, Defretin & Demailly (1953) on L. terrestris and Lutfy (1965a) on A. caliginosa. With the exception of Defretin & Demailly (1953) no qualification is made. They interpreted their uncontrolled Alcian blue results as being indicative of sulphated acid mucus and established that hyaluronic acid was not present by enzyme study.

The present study attempts to define more accurately the nature of the secretion in lumbricid metachromatic mucous cells.

ii) Results

Neutral mucopolysaccharides

These cells gave a weak PAS reaction, in contrast to the granular, orthochromatic mucous cells, but this could be due to the fact that after fixation the majority of the cells are almost empty and therefore there was less substrate for oxidation. But such an argument would have to hold equally for the acid mucus components and their reactions were generally strong. It therefore would seem that the periodate reactive moiety in the complex is small. That the PAS reaction arose from a protein fraction must be dismissed as the results were similar whether a pretreatment deamination was applied or not. The PAS reactions after diastase digestion, acetylation and saponification were the same as for the orthochromatic cells and indicated the presence of vic glycols. Sulphation to induce metachromasia at neutral mucopolysaccharide sites could not be used because of the normal metachromasia shown by the acid mucous cells with thiazin dyes. When the PAS reaction was used in conjunction with pH controlled AB (pH 2.5) as AB-PAS (Plate 1b), the cells exhibited alcianophilia, indicating their acid mucus nature, and did not respond to the subsequent PAS, thus contrasting with the PAS positivity obtained after this sequence in the orthochromatic cells.

Acid mucopolysaccharides

The acidic nature of acid mucins is known to be due to carboxyl groups or sulphate ester groups or to a combination of both, and these groups can be distinguished by a variety of histochemical methods: metachromasia (degree of, alcohol resistance, pH extinction point, restoration of by saponification after methylation); AB (pH controlled, CEC controlled); Ald.F-AB sequence; diamine-AB sequence; fluorescence techniques; enzyme techniques; acid hydrolysis; autoradiographic

visualisation of S^{35} uptake. The acknowledged (mammalian) results of these techniques together with the present lumbricid findings are presented in Table VI (techniques excluding enzyme studies) and the enzyme and related results are presented in Table VII.

The γ metachromasia obtained with the thiazin dyes TB (Plate 2a) and Az.A indicated that the acidic substrate material present influenced these dyes to form polymers of sufficient complexity to result in a spectral shift from the orthochromatic blue to red. Such metachromasia is only obtained if negative charges on the substrate offer a minimal periodic distance of about 0.5 nm. As the strength of thiazin dye metachromasia is difficult to quantify it was not possible to determine whether the phenomenon was due to carboxyl or sulphate groups. However, the stacking of the dye molecules on to sulphated groups is known to be at an intercharge distance of less than 0.4 nm (Pearse, 1968) and this confers on the metachromasia a greater stability than does the stacking with respect to carboxylated mucins where the intercharge distance ranges from 0.6 - 1.0 nm.

The stability of the metachromasia is generally expressed in terms of alcohol resistance and the γ metachromasia of the lumbricid acid mucous cells tended to be alcohol labile and not resistant to normal dehydration procedures. These results are suggestive of the presence of carboxylated mucus, though few histochemists would rely solely on alcohol resistance to delineate the two forms. For comparative studies on the species, it was therefore necessary to make all observations at the aqueous stage. However, it was found that the mucous cells, both orthochromatic and metachromatic, would stain with TB in wax sections. After staining, the wax was removed in xylol and the sections mounted in the usual way. Although such preparations began to fade after a few days, the procedure allowed comparisons to

be made with more ease than trying to maintain numerous sections in the aqueous state. Dehydration in acetone preserved some of the metachromasia.

The Az.A pH extinction point of the metachromasia in lumbricid material was around pH 4, very pale or negative results being obtained below this figure, and so indicated the presence of carboxylated or weakly sulphated mucins. The failure to stain at low pHs (pH 1.5, 1.0) indicated that strongly sulphated mucosubstances were absent as they alone stain at these pH levels because the pH is below the pK value of carboxyls. At pHs 2.5 - 4.0 both groups may be contributing, and the very pale results at pH 3.0 may be due to the presence of some sulphated mucin in addition to carboxylated mucins. However, the pH extinction results alone cannot be taken as conclusively diagnostic of the nature of acid mucins as the problem of masked azurophilia has been raised in mammal tissue where some sulphated mucopolysaccharides, detected by other techniques, stained only at pH 3.5 - 4.5 (Spicer, Horn & Leppi, 1967). Such masked metachromasia is often due to protein inhibition, but this is not the case in the lumbricid material since both pepsin and deamination did not alter the pH controlled alcianophilia of the metachromatic cells.

The restoration of TB metachromasia after the methylation-saponification sequence pointed strongly to the presence of a carboxymucin in the lumbricid acid mucous cells. The technique is valued by mammalian histochemists (Fisher & Lillie, 1954; Spicer & Lillie, 1959; Spicer, 1962b; Mowry, 1963) and involves the esterification of carboxyl groups to $COOCH_3$ and the desulphation of sulphated esters by methanol with hydrochloric acid as catalyst. Both modified groups then fail to exhibit metachromasia as was the case in the lumbricid material, the TB metachromasia being abolished after methylation for 2 hrs at $58^{o}C$.

Subsequent saponification restored TB metachromasia to the acid cells and this is thought only to be possible if the original substrate contained carboxyl polysaccharides (Fisher & Lillie, 1954), the desulphation by methylation having produced OH groups in sulphated mucins, and such OH groups cannot be saponified to groups which will show metachromasia. However, if small quantities of weakly sulphated mucin were present as well as carboxylated mucin, the question of subjectivity in assessing the restoration is raised. Visually the metachromasia appeared as strong as in the material not subjected to the sequence, but without microspectrophotometric methods the degree of metachromasia is difficult to quantify.

Because the technique of methylation involves the use of an acid catalyst, it raised the question of possible acid hydrolysis of material within the cells, and so the reaction was also carried out using the thionyl chloride methylation technique of Stoward (1967a) which, on mammalian substrates, effectively blocks alcianophilia. This it failed to do on the lumbricid material and for which no explanation can be advanced. A similar failure has been recorded by Lawrence (1972) on the mollusc Arion hortensis.

The strong alcianophilia shown by the metachromatic cells at pH 2.5 indicated the presence of carboxylated or weakly sulphated acidic mucopolysaccharide material, since strongly sulphated mucopolysaccharides paradoxically stain only weakly at this pH. At pH 0.5 and 1.0, however, sulphated acid mucins are said to stain well (Lev & Spicer, 1964) and carboxymucins not at all because of their pK value. The technique involves the dry-blotting of the sections, otherwise hydration brings the tissue within the pK value of carboxyls and causes them to stain also. The lumbricid results were variable at these low pHs. Very faint alcianophilia was obtained in Zenker

fixed material and a slightly stronger reaction occurred in formalin fixed material. These results again raised the possibility of there being some sulphated mucus present in the complex which showed, overall, strong carboxyl properties.

Low sulphate mucosubstances in mammals often contain galactosamine as their amino sugar (Spicer, Horn & Leppi, 1967) possibly linked to the sulphate groups, in which case deamination would effectively cause loss of staining. An alternative possibility may be the attachment of the sulphated polysaccharide groups to a protein core. In both these configurational possibilities deamination and pepsin digestion should negate AB staining. This was not the case in the lumbricid material, strong alcianophilia at pH 2.5 being retained after both these pretreatments and so indicating that it was due to carboxymucins which were present and not intimately linked with a protein moiety. However, if low levels of weakly sulphated acid mucin were present and destroyed by deamination and pepsin, the degree of loss of alcianophilia might be so slight as to be undetectable visually.

The results of the CEC.AB technique of Scott & Dorling (1965) were not overwhelmingly satisfactory in determining the exact nature of the acid moiety. Strong, vivid turquoise staining occurred at low molarities of $MgCl_2$ with a sharp drop above 0.2M $MgCl_2$ and a further drop at 0.6M $MgCl_2$, at which molarity carboxymucins rarely stain. At higher molarities, theoretically, only sulphomucins should react. In the lumbricid material some very faint staining was recorded at 1.0M $MgCl_2$ and above, and might suggest, again, the presence of small quantities of weakly sulphated acid mucus.

With the Ald.F-AB technique (Spicer & Meyer, 1960) (Plate 2b) and the diamine (HID and LID)-AB sequences (Spicer, 1965) the metachromatic cells showed strong alcianophilia indicative of carboxymucins,

sulphomucins reacting to the Ald.F and diamine reagents. The failure of Geyer's Tetrazonium method to evidence the presence of sulphated groups is taken to be supportive of the carboxylated nature of the acid mucus.

Fluorescence techniques are widely used to detect and distinguish tissue acid mucopolysaccharides, and Stoward (1967b) regards Coriphosphine as one of the best fluorescent indicators of sulphated mucus. This technique failed to show the presence of any sulphated mucus in the lumbricid material. The preliminary autoradiographic study using S^{35} as sodium sulphate also failed to indicate sulphomucins.

The acidic nature of the secretion would therefore appear to be conferred by carboxylated groups.

Carboxymucins

The commonly occurring carboxylated mucosubstances in mammals are hyaluronic or sialic acid-containing compounds or, less frequently, glucuronic acid-containing ones. All three types, with some exceptions respecting sialomucins, are digestable by the appropriate enzymes and such techniques afford definition of the groups conferring the carboxy staining of carboxylated mucins. The lumbricid enzyme results are presented in Table VII.

Testicular hyaluronidase (6 hours) did not abolish the TB metachromasia of the lumbricid metachromatic cells. It would therefore seem that hyaluronic acid, chondroitin 4-sulphate (old terminology chondroitin sulphate A (Spicer, Horn & Leppi, 1967)) and chondroitin 6-sulphate (chondroitin sulphate C) are not present, since testicular hyaluronidase splits the endo-β-N-acetyl-D-glucosaminidic residues (Leppi & Stoward, 1965) present in these substances and removes their ability to stain with TB. The present results, in fact, showed

enhanced TB metachromasia in wet mounts, as noted by Fullmer (1960) on avian and various mammal tissue. The absence of hyaluronic acid is also supported by the negative results after the CTAC-AC.O fluorescence technique.

Neuraminidase did not block the alcianophilia in the lumbricid acid mucous cells and therefore suggests that sialomucins are not present. However, not all sialomucins appear to be sensitive to sialidase or neuraminidase (Quintarelli & Dellovo, 1965; Warren & Spicer, 1961), but all sialic acid residues, whether sensitive or resistant to enzyme, can be removed by acid hydrolysis. On the lumbricid tissue, acid hydrolysis, using both N/10 HCl and N/10 H_2SO_4 at temperatures in excess of $55^{o}C$, failed to abolish the alcianophilia of the metachromatic cells. The secretion, therefore, does not contain sialic acid residues.

The possible occurrence of glucuronic acid residues within the secretory complex was considered from both the standpoint of defining the carboxy nature of the mucus and that of attempting to reconcile the alcianophilia at low pH and at high molarity CEC (both admittedly weak), both sets of results being suggestive of low quantities of weakly sulphated material being present. In mammals a mucosubstance exists described as a low sulphate chondroitin (Spicer, Horn & Leppi, 1967) containing galactosamine, glucuronic acid residues and some sulphate groups, such a substance presumably showing weak reactions for sulphates as well as those for carboxylates. The action of β-glucuronidase is to affect the terminal non-reducing glucuronic residues (Curran, 1964) so as to abolish alcianophilia or TB metachromasia. On the lumbricid mucus the use of β-glucuronidase, with or without peracetic acid (Fullmer, 1960), did not negate the AB staining or suppress the TB metachromasia, thus indicating that

the acidic properties were not due to the presence of glucuronic residues.

The acid moiety, which is predominant in the complex, is shown to be a carboxymucin but not of the hyaluronic, sialic or glucuronic type.

Proteins & Lipids

The range of lipid and protein tests applied to determine the nature of the secretion of the orthochromatic cells (Section 6) yielded either negative or extremely pale reactions in the metachromatic cells. The cells were weakly positive with SB, BS, HgBPB and WRF, but the Ald.F reaction (as Ald.F-AB) (which, with the chemical manipulations employed, gave information on the elastin-like nature of the protein moiety of the orthchromatic cells) was not open to manipulation in the metachromatic cells due to the dominance of the acid mucus which stained with the AB part of the sequence (Plate 2b).

It would therefore seem that the lipid and protein moieties, although possibly qualitatively similar to those of the orthochromatic cells, are quantitatively very different, forming, together with the neutral mucopolysaccharide component, but a small part of the mucosubstance in the metachromatic secretion as visualised histochemically.

iii) Discussion

Further definition of the metachromatic secretion has not been possible, other than to point out that it is unlike most forms of charted mammalian acid mucus. The limitations of the existing, mammal-oriented, histochemical techniques become apparent when attempting to define invertebrate mucus with the same precision as has been applied to mammalian mucus. But even in mammalian histochemistry there are limitations and reservations in that the techniques

are not always specific and often only indicate the types of substances and groupings present, not the actual secretion as a whole. Nor can they show the detailed ordering of linkages within the substance.

The lumbricid acid mucus could be classified as a non-(or very weakly-) sulphated, sialidase-, hyaluronidase-, β-glucuronidase-resistant mucin in which the reactive group is a carboxyl. However, the possibility of a uronic type mucin resistant to hyaluronidase and β-glucuronidase, and thus paralleling the sialidase-resistant sialomucins becomes a real one when trying to define, in detail, lumbricid acid mucus. Such a compound is unlikely to appear among the charted epithelial mucins of mammals, where uronic acid mucins are generally confined to connective tissue, but Cook (1968) reported the presence of a carboxylated, sialidase-, hyaluronidase- resistant mucin in human pyloric glands.

A further possibility must be considered, namely that of the presence of sulphate groups that do not stain as sulphated mucin. Such a situation has been described by Lamb & Reid (1969) in human tracheo-bronchial glands where a secretion failed to reveal sulphate groups histochemically, and although suspected of being carboxylated failed to respond to enzyme and acid hydrolysis treatments, yet displayed S^{35} uptake. In the lumbricid material no S^{35} uptake was recorded so the tracheo-bronchial situation is not paralleled here.

However, these negative S^{35} results, using both high dosage levels (for parallel electron microscope study) and low levels, raise two points: the advisability of using inorganic sulphur, and the mode of introduction into the animals.

The incorporation of labelled inorganic sulphur into the sulphated mucus of mammalian duodenum is well documented (Kent,

Whitehouse, Jenning & Florey, 1956), and so the use of labelled sodium sulphate would seem to have been appropriate.

There is the possibility, in earthworms, that materials introduced into the coelom by injection are not retained but are immediately expelled through the dorsal pores, as reported by Keng (1895). Irritants, such as fixatives, and handling readily cause coelomic fluid expulsion (Plate 5a,b). However, Nakahara & Bevelander (1969), Valembois & Cazaux (1970) and Valembois (1971a), using small quantities similar to those employed here, successfully introduced labelled materials by coelomic injection into lumbricids. The target sites in these instances were not epidermal cells, and Macha & Ditadi (1972), who reported S^{35} uptake (as sulphate) in the epidermal mucus of the echiuran worm Lissomyema, did not use injection techniques but immersion ones. Although the present negative results might be indicating a metabolic inability to incorporate the label in the form administered, the other histochemical observations would point to the acceptance of the S^{35} results as indicative of the absence of sulphomucins in earthworm acid mucus.

The present study has revealed no information as to the relationships between the moieties of the secretion complex. How the neutral polysaccharide fraction would fit into the molecule can only be guessed. Possibly the vic glycol bearing units are attached to the protein-lipid chain, with the acidic mucin residues occurring as terminal or side groups on the neutral carbohydrate substance. Such a configuration would, in broad terms, resemble the arrangement in many mammalian glycoproteins (Barrett, 1971) but for the lipid component and the fact that most documented (acid) glycoproteins have sialic acid residues, which is not the case in the lumbricids. The possibility of a simple protein-lipid-acid mucin configuration,

without a neutral mucosubstance moiety, the protein displaying the periodate reactive groups, is not tenable in the light of the PAS manipulations that were carried out.

The recording, in the lumbricids, of a hitherto uncharted type of acid mucus is not altogether surprising, since the lumbricids are a group known to possessspecial, and unexpected, biochemical pathways as evidenced by their body wall muscle phosphagen - lombricine (Thoai & Robin, 1954), which is in contrast to the arginine phosphate typical of so many invertebrates and creatine phosphate which characterises vertebrates. Jakowska (1965b) predicted that mucous secretions, as they became studied in a wider variety of animals, would not conform in physico-chemical make up to the patterns established for the better known mammalian mucins.

The very low levels of detectable protein and phospholipid and the absence of hyaluronic acid in the secretion of the lumbricid metachromatic cells lead to speculation concerning its viscosity and function. All three components have been described, in the literature, as contributing to a viscous mucosubstance, and the presence of protein and phospholipid moieties in mucus has been discussed earlier (Section 6iii).

Defretin (1951) claimed that the presence of hyaluronic acid in the mucus of some tubicolous polychaetes accounted for the increased mucus viscosity compared with that of non-tube building polychaetes and Holt, How et al. (1968) have shown that diseased human joints have a less viscous fluid and contain appreciably lower hyaluronic acid levels than normal fluid.

The histochemical differences between the metachromatic and orthochromatic mucus of lumbricids lead to a suggestion that the

former has a lower viscosity than the latter, and the difference in the effect of light microscope fixatives upon the two types has been mentioned earlier (Section 4). Being less viscous, the metachromatic mucus could form, more easily, the permanent 'respiratory' film, the as yet unidentified residue that confers the carboxylated properties imparting a strong negative charge and so attracting water molecules. Further evidence supporting this idea that the metachromatic mucus serves a general, all purpose role such as the provision of a respiratory surface will be advanced after the ultrastructural study of this cell type (Section 12). In the context of a 'respiratory' mucus, it has not been possible to relate these present lumbricid findings to those of many other invertebrates which make respiratory provision by means of a mucous covering. This is primarily because of the paucity of detailed, controlled histochemical studies in other groups, and where such studies exist the workers often draw histochemical conclusions about the nature of the acid mucus from slender and unconvincing evidence.

The present work confirms the presence of acid mucous cells, noted by Schneider (1902; 1908), Herlant-Meewis (1958/59) and Negus (1963) in the lumbricid epidermis. It also supports the statement by Defretin & Demailly (1953) that hyaluronic acid is absent from the acid mucous cells, but has shown that their interpretation of their AB results, leading to the conclusion that sulphated mucus was present, was incorrect.

Sialomucins and glucuronic acid residues have not been investigated by previous lumbricid workers and are shown not to be present, though Dhainhaut (1970), on polychaete eggs, established by enzyme blocking that glucuronic acid was responsible for the acid mucus staining of these eggs.

Workers on megascolecid earthworms have reported the occurrence of acid mucous cells in the skin and Lutfy (1965b) recorded cells in the clitellum region of Pheretima hawayana similar to the acid mucous cells found at that site in the lumbricid A. caliginosa. In the normal skin of Megascolex mauritii Krishnan & Rajulu (1969) described two types of acid cell, one which exhibited alcohol-resistant metachromasia and was presumed to be sulphated from the large mucous glands, and one from the small mucous glands in which hyaluronic acid was identified. In Pheretima elongata Varute & Nalavade (1970) also reported sulphated mucus, based on low pH AB results, and a hyaluronidase-susceptible mucin.

The present lumbricid results differ widely from those reported for the megascolecids, the absence of hyaluronic residues in earthworms contrasting with their presence in the megascolecids and in some polychaetes (Defretin, 1951). Furthermore, the histology of the megascolecid cells is different, in that in M. mauritii the acid cells are distinctly granular and, from published micrographs (Krishnan & Rajulu, 1969) more nearly resemble the granular, orthochromatic cells of lumbricids than the metachromatic ones.

Neutral polysaccharide (vic glycol-containing), bound lipid and protein moieties have not previously been reported in association with acid mucus by lumbricid workers, but Krishnan & Rajulu (1969) detailed positive protein reactions in the hyaluronic acid type mucous cells of M. mauritii, using BPB, but this reagent gave negative results in the supposedly sulphated cells where a weak Millon's was recorded. No PAS positive material was detected by them or by Varute & Nalavade (1970) on P. elongata, but it is known from mammalian studies that hyaluronic acid-containing mucins are rarely if ever PAS positive. Lipid techniques were not employed by these megascolecid workers.

It would therefore seem that lumbricid acid mucus is markedly different from that recorded for megascolecids.

8 LUMBRICID SMALL GRANULAR, PROTEINACEOUS CELL HISTOCHEMISTRY

i) Introduction

The diversity of terminology in the literature relating to this cell type has been documented in Section 4.

The Eiweisszellen of Schneider (1902; 1908), on A. (E.) rosea, were described as showing an atypical green colour with German Toluidine blue and as exhibiting strong eosinophilia, and although not directly relating these facts to the presence of protein within the secretory granules, Schneider intimated this by his choice of the term to describe this cell type.

Ewer & Hanson (1945), on an unnamed lumbricid, failed to reveal the proteinaceous character of the "albumen" cells, using techniques including WRF where a purple metachromatic effect was apparently expected if protein were present. The cells were stated as not containing acid mucus.

On E. foetida Herlant-Meewis (1958/59) obtained a weak PAS reaction in the "albumineuse" cells and recorded the presence of protein based on disulphide detecting techniques, but she did not consider her paraldehyde fuchsin and Gomori positive results as indicative of it. In fact, she interpreted these results as displaying the acid mucus nature of the albuminous cells.

Workers on lumbricid clitellum have described "albumen" cells (Grove & Cowley (1927) on E. foetida) or fine granular cells (Lutfy (1965a) on A. caliginosa; Defretin & Demailly (1953) on L. terrestris) but from the histological accounts of these elements it would appear that such cells are modified components of this specialised area, whereas the mucous cells, also present there, conform exactly to the

equivalent cells in the normal skin. Because the histochemical techniques used by these workers were limited, it has not been possible to decide if the clitellar "albumen" cell type is histochemically the same as the type found and described by other workers for the unmodified epidermis.

The present histochemical investigation was directed towards a fuller characterisation of the secretion of the small granular cells of the normal skin, and the final part (iv) of this Section reassesses the so-called "albumen" cells of the literature.

ii) Results

General histological techniques showed the small granules to be eosinophilic and to stain with the Acid fuchsin component of Mallory's Triple stain. The histochemical results which readily distinguish the small granular from the large granular orthochromatic cells are presented in Table VIII.

Mucopolysaccharides

The PAS results were positive and with appropriate chemical manipulation and enzyme treatment, as applied to the orthochromatic cells, indicated the presence of a neutral, diastase-resistant mucosubstance containing vic glycols. However, the results were often paler than those given by the large granular mucous cells, and some cells in the sections failed to give reactions, but were recognisable by the shape and refractive index of the granules. The cells did not exhibit metachromasia with the thiazin dyes and did not stain with AB at pH 2.5, and indicated the absence of acid mucopolysaccharides.

The Ald.F and diamine-AB sequences for sulphomucins were, when used without chemical or enzyme treatments, Ald.F and diamine positive, but the Ald.F reaction was unimpaired by trypsin and resistant to

drastic methylation and so contraindicated sulphomucins, indicating a protein of the elastin type. In some Zenker fixed material the small granular cells were purplish rather than deep pink, perhaps suggesting some AB binding in addition to the Ald.F binding. The ferric alum-Coriphosphine fluorescent technique produced a yellow/orange result, in contrast to the background green of the orthochromatic and metachromatic cells. The CEC.AB at low molarity $MgCl_2$ was positive, the small granules exhibiting an intense turquoise colouration, but as no other test for carboxylated acid mucus was positive, this result must be attributed to reactive groups present in other than acid mucus.

The small granular cells therefore contain no acid mucus but possess a diastase-resistant, vic glycol-containing neutral mucopolysaccharide.

Proteins

The ninhydrin test was negative, but not all protein is oxidised this way, and Millon's produced only a weak reaction in the small granules and so indicated that some tyrosine-containing protein was present. The DMAB-nitrite technique for tryptophan gave a strong reaction and the PFA-AB sequence for S-S groups was positive.

The BPB and HgBPB techniques were positive and prior methylation to remove anionic groups which might be interfering with the reaction induced only marginal improvement. Deamination and permanganate oxidation negated the BPB staining and the proteases papain and trypsin reduced the staining but did not abolish it, whereas pepsin left the small granules stained. It is possible that the timings were not sufficient in the enzyme tests, but some specimens were left until almost total digestion of the section had taken place, and still the small granules showed staining. Although BPB is used as a protein-

indicator, the technique is thought only to demonstrate the presence of amino groups and not to be of significance in determining the nature of the protein. The lumbricid results support this assessment of the technique.

The Biebrich scarlet on non-formalin fixed material, carried out over a wide pH range, only produced positive results at low pH (4.5 or lower) and failed to reveal any basic protein stainable at high pH. The positive results were negative after deamination. In tissue fixed in formalin, and in formalinised non-formalin material, there was no impairment of the staining within the positive pH range and the small granules, in fact, stained more intensely (Table VIII). In mammalian tissue such a formalin unimpairment is indicative of either elastica or the eosinophilic leucocyte granules (Lillie, 1965). Although many of histochemical tests on lumbricid material point to an elastin-like protein being present in the small granules, it must be stated that mammalian elastica exhibits BS staining at much higher pHs than recorded here in the lumbricid small granular cells.

The WRF gave a red/brown colour, unlike the violet/black of mammalian elastica, but the Ald.F (as Ald.F-AB), with appropriate controls, indicated an elastin-like material. The reaction was resistant to trypsin, which is known to have no elastolytic activity, and also to drastic methylation (4 hrs at 60^{o}C) which abolishes the reaction if sulphomucins are responsible for the aldehyde fuchsin staining (Spicer & Meyer, 1960).

The Amido black technique for collagen was negative, the granules assumed a yellow/brown colour in contrast to the green staining of the large granular, orthochromatic cells (Table VIII) and the positive reaction of the basement membrane collagen site. The proteinaceous moiety of the small granular cells would therefore

appear not to be collagenous.

The FALG sequence resulted in strong Acid fuchsin uptake by the granules (Plate 4e) and clearly differentiated them from the other secretory types (Table VIII). The staining was negated by proteases, deamination and permanganate oxidation. Human erythrocytes also exhibit fuchsinophilia with this technique, the extent to which they display it depending on the acidity of the solution (Gurr & McConaill, 1961). In the present lumbricid studies the acidity was achieved through an acid bath between the dyes when used sequentially, or by acid pretreatment when using the FALG mixture. Although polychrome staining was observed in lumbricid muscle tissue, the secretory cells stained monochromatically, with the small granular cells showing strong fuchsinophilia and the large granular, orthochromatic cells displaying affinity for the Light green component in the technique.

Similar acidophilia was exhibited by the small granules in the Mallory technique where the result might be interpreted as being indicative of elastin-like material, elastica displaying such fuchsinophilia with Mallory. In the van Gieson technique, even in the presence of concentrated Acid fuchsin, the small granules failed to exhibit fuchsinophilia and coloured yellow, as does mammalian elastin after this technique.

With TB the small granules showed atypical orthochromasia (Table VIII) and were stained green-blue in preparations that were not overstained. This was in contrast to the large granular cells which exhibited typical orthochromasia (blue). A similar atypical orthochromasia has been recorded in a variety of tissue: Lillie (1965) comments on the green colouration of erythrocytes with TB, the azures and thionin; Terner & Magidson (1962) on kidney cell inclusions of Chediak-Higashi syndrome suggested a pH effect since

they were green at pH 4.5, blue-green at 5.5 and blue at 6.5; Spicer, Staley, Wetzel & Wetzel (1967), on mouse Paneth cell granules, recorded a green-blue colouration with Az.A at pH.4.0 in the protein rich granule core and suggested that "the green-blue orthochromasia of this type is more typical of protein-rich sites than sites containing mucosaccharides". It would therefore seem that atypical orthochromasia can be regarded as indicative of protein material in the lumbricid small granular cells, and it adds to the staining similarities shared by these cells and mammalian erythrocytes which are also eosinophilic and fuchsinophilic.

The intense turquoise staining of the small granular cells (Plate 3a, b) in the CEC.AB technique at low molarities, with or without subsequent PAS, can be interpreted, in the absence of any other positive acid mucus results, as the binding of the dye to the COOH groups of the protein moiety. Fisher & Lillie (1954) have suggested that certain staining reactions may be due to carboxyl acid groups in protein components and Barrett (1971) has commented on the possibility of tissue polyanions such as acidic proteins binding with cationic dyes. Such an interpretation confirms that the protein is not wholly basic.

The intense pyroninophilia in the MG-PY sequence, which is abolished by RNase, suggested high levels of RNA, though the function of RNA in the fully formed granules is difficult to imagine. One would imagine RNase to be specific and that failure to stain after its use would unquestionably indicate the presence of RNA. However, Scott (1967) has shown that in the absence of electrolytes many non-nucleotide polyanions exhibit pyroninophilia, but that in the presence of electrolytes the staining is suppressed in all but RNA. He further points out that as RNase is made up in a buffer, the electrolyte

in this could be sufficient to prevent the dye from binding at the non-nucleotide polyanion sites and so give the appearance of a valid test for RNA and thus lead to dubious conclusions. On the lumbricid material, the presence of 2M $MgCl_2$ prevented the uptake of pyronin in the small granular inclusions and the buffer control in the RNase test also suppressed the pyroninophilia, there being sufficient salt ions present to prevent pyronin uptake by the granules. Therefore, the present results must be regarded merely as indicative of the presence of pyroninophilic non-nucleotide polyanions in the granules and not of the presence of RNA. The technique did not indicate the donor of such polyanions.

Lipids

No free lipid was detected, but weak reactions indicative of bound lipid, possibly a phospholipid, closely associated with the protein occurred. The weak SB staining was negative in pyridine-extracted material and also after protease treatments and deamination in non-extracted material. Luxol fast blue (both MBS and ARN) were negative, but Lillie's Nile blue was positive in the small granules and was more intense than in the large granular, orthochromatic cells. Menschik's Nile blue sulphate, also a phospholipid indicator, was positive.

Non-lumbricid material

Because the small granular cells have been described as "albumen" cells in the literature, the techniques which visualised them as distinct from the mucous cells in lumbricids (CEC.AB at low molarity, FALG, TB) were applied to sections of pigeon and frog oviduct at the level where the albumen is known to be secreted. The cells at these sites did not parallel the lumbricid results and so indicated that

the secretion of the small granular cells of earthworms is different from that found at the albumen-secreting regions in these two vertebrates, nor is it similar to that of the albumen gland of the garden slug Arion ater (Smith, 1965).

iii) Discussion

The lumbricid results have shown the granules of the small granular cells to contain no acid mucus, to possess a diastase-resistant, vic glycol-containing neutral polysaccharide, to be low in phospholipid, but high in a protein which is not wholly basic, is not of the collagenous type but is strongly elastin-like.

These results are, in broad histochemical terms, very similar to those given by the large granular mucous cells and the differences can, in many cases, be explained on the basis of quantity of protein present. The considerably higher levels of carbohydrate in the large granular cells would have a masking effect on many of the protein tests at those sites, though methylation failed to increase appreciably the protein staining in those cells, as it does in many mammalian carbohydrate-protein complexes (Lev & Gerard, 1967). In the Mallory and FALG techniques, the presence of large quantities of polysaccharide material could be responsible for the visual differences recorded for the large and small granular cells, the latter being acidophilic (fuchsinophilic) and the former not offering sufficient sites for this, but presenting numerous, and successfully competing, sites for the aniline or Light green dyes.

As with the orthochromatic and metachromatic cells studied, the present results do not allow proposals to be made concerning the molecular construction of the secretion. Within the terminology of mucosubstances (Spicer, Leppi & Stoward, 1965; Barrett, 1971) the

secretion, apart from its lipid component, might be classified as a glycoprotein, or, in older terms, a mucoprotein (Stacey & Barker, 1962). The latter term suggests mucoid properties, but in the lumbricid small granular cell material these are not abundantly evident, the variable PAS being singular in this respect.

The term glycoprotein is applied to macromolecules containing more than 0.5% hexosamine firmly bound to protein, but has now come to include substances containing oligosaccharides glycosidally bound to hydroxyl or to carboxyls of amino acids, as side groups on a polypeptide chain (Spicer, Leppi & Stoward; 1965). The majority of mammalian glycoproteins are acidic, due to sialic acid residues, and this is not the case in the small granular cells. However, some mammalian glycoproteins lack demonstrable acid groups and are rich in vic glycols and the term neutral glycoprotein has been applied to them, though in mammalian tissue the protein component is not revealed histochemically. For this reason Spicer, Leppi & Stoward (1965) prefer the less committal term "neutral mucosubstance". The secretion of the small granular cells may technically be a neutral glycoprotein, but it is one in which the protein moiety is high, elastin-like, bound to phospholipid and easily demonstrable, and the vic glycol carbohydrate moiety is comparatively low. In these respects it conforms, because of its low carbohydrate and high protein, more to the definition of a glycoprotein given by Barrett (1971) than to that of Spicer, Leppi & Stoward (1965), but, nevertheless, differs from any charted category of mucosubstances.

The detailed histochemical studies and classification of mucosubstances have, to date, been restricted to mammalian tissue and it is therefore not surprising that invertebrate secretions fail to fit in to the recognised categories. Ewer & Hanson (1945), using limited

techniques and surveying species ranging from coelenterates to mammals, found a variety of combinations of staining reactions indicating that in the animal kingdom there is a wide diversity of mucoproteins.

In order to avoid mammalian comparisons, it is suggested that the secretion of the small granular cells be referred to as proteinaceous rather than the more technically correct term neutral glycoprotein (with bound phospholipid), thus emphasising the high protein component which can be variously demonstrated, and avoiding the histochemical parallels that the strict use of this term implies.

The present results do not support the continued usage of the word "albumen" to describe these cells. Apart from the application of the term "albumen" by Grove & Cowley (1927) to describe the small granular cells in the clitellum of E. foetida (these cells are greatly elongated elements in the clitellum and it is questionable if they are homologous to the small granular cells of the normal epidermis), in the belief that they were responsible for the "albuminous" secretion found in the deposited cocoon, the use of the term "albumen" in connection with cells in normal skin seems to have come about through a tortuous mis-reading of the early literature. A discussion and assessment of this problem is given in the final part (iv) of the Section, but most workers since 1930 have employed the term "albumen" whether in lumbricid or megascolecid studies (Ewer & Hanson, 1945; Herlant-Meewis, 1958/59; Krishnan & Rajulu, 1969; Menon & Singhal, 1969; Varute & Nalavade, 1970; Valembois, 1971b).

Although the phrase "albumen cell" carries with it proteinaceous implications, the term from which it was translated - Eiweisszellen - does not carry reciprocal albumen connotations (Wondrak (1969) on molluscs) or when translated into English (Pedersen (1963) when reviewing German turbellarian literature). It is simply used to

denote a proteinaceous cell.

The function of the secretion of the small granular cells of the normal epidermis remains obscure and no suggestion has been advanced in the literature. The proteinaceous nature of the secretion could well affect the characteristics of the secreted products of the mucous cell types, altering the viscosity and perhaps increasing the water holding properties, particularly of the mucus of the metachromatic cell type which is thought to form the respiratory film (Sections 7, 10, 12).

When comparing the present results with those of earlier workers the findings appertaining to the clitellar sites will be included, but because of the reservations stated in the introduction (8i) such results will be asterisked (*).

The occurrence of vic glycols indicating a neutral polysaccharide component in the small granular cells is in agreement with the results of Herlant-Meewis (1958/59) who recorded weak PAS reactions by these cells in the normal skin of E. foetida and with Lutfy * (1965a) on the clitellum cells of A. caliginosa. Defretin & Demailly * (1953), on clitellar sites of L. terrestris, recorded negative reactions with PAS. However, this present study has shown that frequently there are cells which, although recognisable by the refractive index of the granular inclusions, fail to exhibit a given staining reaction.

The absence of acid mucus accords with the findings of previous workers on lumbricids (Ewer & Hanson, 1945; Lutfy * 1965a) but not with Herlant-Meewis (1958/59) who claimed its presence on her interpretation of certain techniques. The present author contends that these results were indicative of the protein moiety of the secretion.

There has been no previous record of the occurrence of bound

lipid of the phospholipid type in the small granular cells of the lumbricid epidermis. Pedersen (1963), however, has recorded phospholipid in the acidophilic, eosinophilic, proteinaceous gland cells (Eiweisszellen) of two fresh water triclads, much of the histochemistry of these cells paralleling that of the lumbricid small granular cells revealed by this study.

The presence of a protein moiety detected by Herlant-Meewis (1958/59) is sustained by the present results but the evidence of protein at clitellar sites by Defretin & Demailly * (1953) has to be challenged. They recorded RNase sensitive pyroninophilia and the presence of alkaline phosphatase (often associated with protein synthesis) and deduced that the RNA-rich cells indicated the proteinaceous character of the secretion. Although in this they might have been correct, their histochemical basis for this assumption (RNase) has proved to be unfounded (Scott, 1967). Lutfy * (1965a), also on clitellar sites (A. caliginosa), failed to demonstrate protein with either Millon's technique or a SH detecting technique, and concluded that the secretion was not albumen, so questioning the suggestion of Grove & Cowley * (1927) that these cells gave rise to the albuminous material found in the cocoon. Lutfy * (1965a) described the secretion as being a mucoprotein, neutral mucopolysaccharide or glycoprotein.

The present results are therefore in conflict with those of Ewer & Hanson (1945) and Lutfy * (1965a) who failed to record protein, but are in agreement with those of Herlant-Meewis (1958/59) who described a protein moiety but wished to reserve the term "albuminous" for these cells in E. foetida.

The megascolecid epidermis also contains "albumen" cells which exhibit PAS positivity and lack metachromasia and alcianophilia

(Krishnan & Rajulu, 1969; Varute & Nalavade, 1970). Menon & Singhal (1969) did not undertake any histochemical tests on the two species of Eutyphoeus they studied, merely recording the presence of "albumen" cells. Varute & Nalavade (1970) did not carry out any protein tests on the megascolecid Pheretima elongata, but Krishnan & Rajulu (1969) on Megascolex mauritii, using Millon's, BPB and diazotization tests, recorded positive protein reactions in the "albumen" cells.

It would therefore seem that with respect to the small granular ("albumen") cells a greater similarity exists between the megascolecids and the lumbricids than occurred in the mucous cell types (Section 6 and 7).

iv) Reassessment of the so-called "albumen" cells

The origin of the use of the term "albumen" to describe a secretory cell type of the lumbricid epidermis dates from 1930 when Stephenson chose to reproduce Cerfontaine's figure (1890) of a fine granular cell but, at the same time, elected to use Schneider's terminology Eiweisszellen (1902; 1908) translated as "albumen" in preference to the "granules fines" of Cerfontaine. Furthermore, Stephenson chose to accompany the Cerfontaine figure and the Schneider label with a text description translated from Schneider (1902; 1908) where the Eiweisszellen were described as having the shape of a wine glass with a thick stem ("die Form eines Weinglases mit dickem Stiel besitzen") which had little in common with the Cerfontaine figure of a pear-shaped cell with a slender neck opening to the outside. The histological appearance and the text descriptions of these cells given by the two earlier authors are very different and tracings of their figures are given in Plate 4a and b.

The amalgam of this earlier information so produced by Stephenson

(1930) has formed the basis of descriptions of a secretory cell type in an elementary text by Grove & Newell (1946) and in a more advanced work by Laverack (1963) who does not detail the cell types but merely reproduces Stephenson's figure. More recently Brinkhurst & Jamieson (1971) refer to the albuminous cells of the glossoscolecid Callidrilus and state that, as in Lumbricus, they have amorphous vacuolated contents as described by Cerfontaine (1890).

Cells similar in shape and histology to the fine granular cells of Cerfontaine (1890) (Plate 4b - tracing) have not been observed in this study, nor was such a cell type described by Herlant-Meewis (1958/59), but her study and the present one both reveal cells similar to the Eiweisszellen of Schneider (1902; 1908).

If the fine granular cells of Cerfontaine (1890) are not equivalent to the Eiweisszellen and to the small granular cells of the present study, and they would appear not to be, two questions arise: to what are they equivalent in the lumbricid epidermis; and did Cerfontaine fail, in his detailed study in 1890, to observe cells comparable to the small granular, Eiweisszellen type.

As the histological techniques used by Cerfontaine (1890) (chromic acid maceration, Fleming's fixation) were different from those used by later workers, and as it is known that different fixatives can produce differing cell shapes and visualisation of cell contents, particularly mucosubstances, it is possible that his fine granular cells equate to the metachromatic mucous cells.

This suggestion arises from two observations. Firstly, Cerfontaine (1890) did not describe any cell equatable to the metachromatic type, yet this type is plentiful in lumbricid skin (Plates 1b, 2a, 2b). Secondly, most light microscope fixatives cause considerable loss of substance from the metachromatic cells,

many of which appear empty. It is therefore conceivable that the fine granular cells of Cerfontaine (1890) were, in fact, more or less empty, apically contracted, metachromatic cells.

Cerfontaine himself (1890) made two comments which are pertinent to this suggestion. Firstly, he raised (p 355) the question as to whether his very finely granulated cells may in fact be empty, but he discussed this in relation to the empty stages of the large granular cells. He dismissed the idea and favoured the interpretation that they were a distinct cell type, found constantly in the normal epidermis and also in the clitellum region, and suggested that they produced mucus. However, in his figure - to be copied by Stephenson (1930) and repeated by Laverack (1963) - he showed only one such cell together with several large granular ones. From this alone, the present suggestion that this cell type was in fact an empty metachromatic cell is difficult to sustain since metachromatic cells occur frequently in the epidermis. But, secondly, on p 359 Cerfontaine (1890) noted that these cells were more abundant in preparations other than the one he chose to figure. It would therefore seem that the fine granular cells of his study were the reticulate or "spumeuse" cells of other workers and the metachromatic cells of this study.

Regarding the absence of a description of a secretory cell type equatable to the small granular type in the Cerfontaine account (1890), it must be remembered that the bulk of his description of the glandular elements of the epidermis was based on macerated cell preparation, supported by sectioned material. The small granular cells occur randomly and are often infrequent in any particular section. They are also small cells and tend, in sections thicker than 5 µm, to be obscured by the other secretory elements unless stained with one of the techniques which clearly differentiates them (Table VIII). It is

therefore possible that Cerfontaine (1890) using Mayer's haematoxylin, aniline, safranin, fuchsin and Lyon blue failed to detect the small granular cells in sectioned epidermis.

However, in his account of the non-secretory epidermal cells (supporting cells) he described and figured four types. One of these - type c (Plate 4c - tracing) - has numerous, discrete, small granules larger than those figured in the other supporting cell types, and a more basally placed nucleus. This cell type was only found in the anterior part of the lumbricid. Ignoring the basal projections, which represent the interdigitation processes of the cells in the intact epidermis and which Cerfontaine (1890) described for all four types of supporting cell and for the mucous cells, this cell type c more nearly equates to Schneider's Eiweisszellen (1902; 1908), to the "albuminous" cells of Herlant-Meewis (1958/59) and to the small granular cells of the present study. The erratic distribution of the small granular cells in the lumbricid epidermis could account for the fact that Cerfontaine (1890) recorded his type c only from certain areas of the lumbricid body.

It would therefore appear that Stephenson (1930) confused the accounts of two quite different secretory cell types, making an amalgamation of what is to be considered as a metachromatic mucous type and labelling it an "albumen" cell. This present work, together with the ultrastructural study (Section 13) shows that the small granular, proteinaceous cells are equivalent to the Eiweisszellen of Schneider (1902; 1908) and are totally unlike the "albumen" cells cited in the English literature since 1930.

9 LUMBRICID HISTORICAL BACKGROUND (ELECTRON MICROSCOPY)

The lumbricid cuticle has received considerable attention from electron microscopists and their work will be summarised here.

In 1948 Reed & Rudall, using layer stripping and layer digestion techniques, demonstrated that the cuticle of A. longa was made up of a number of thin layers lying parallel to the surface, each layer being composed of fine parallel fibrils, with the fibril directions in alternate layers approximately at right angles to one another. They established that the fibrils ran at 45° to the long axis of the worm. The fibril arrangement was later to be described as orthogonal, cross-lamella patterning. It is of interest that Cerfontaine (1890) had also established that fibres ran at right angles to one another and at an angle of 45° to the long axis, though he believed there to be just two layers of fibres, one in each direction. Reed & Rudall (1948) also noted that the lowermost 2 or 3 fibres were smaller than those situated in the bulk of the cuticle and speculated that they might represent growth stages. They established, as had Picken, Pryor & Swann (1947), using X-ray diffraction techniques, that the fibres were collagenous and documented the absence of cross-banding typical of mammalian collagen. They suggested that the cuticular layers were formed by secretion from subtending epidermal cells and that the cell surface might, due to "intrinsic orientation", control the pattern seen in the cuticle. At the base of the cuticle they described a "granular" layer.

Reed & Rudall (1948) were also the first to describe the "superficial layer of corpuscles", the particles being 600Å in diameter. These particles were evenly distributed and generally

covered the surface continuously, but where absent, they claimed that the main cuticle layers were at the surface. Between the corpuscles they described a continuous membrane and suggested that by means of a series of "tails" or "leaflets" the corpuscles were attached to the fibril layer, such tails resembling, in their estimation, tailed bacteriophages. The corpuscles were recorded as being present in cuticle regenerated for a period of 30 days.

This paper of Reed & Rudall (1948) laid the foundation for further studies by other workers and these later studies will be reviewed in terms of the additional information revealed or the challenging of the original study.

Randall (1957), on a species of Lumbricus, noted that the epidermal cells were highly vesiculated and that the cuticle edge of these cells was thrown into many evenly spaced folds that appeared to taper into fine cytoplasmic processes that penetrated the cuticle and connected with the "exterior membrane" of the worm. This was the first ultrastructural observation of connections running from the cells through the cuticle to the exterior, though Cerfontaine (1890) had observed extremely fine vertical striations in the cuticle and had suggested such a connection. Randall (1957) described the bases of these cytoplasmic processes as oval, 700-1000Å in diameter and 2000Å apart and equated these with the "granules" of Reed & Rudall (1948).

He agreed with Reed & Rudall (1948) in that the lowermost layers (about 4) contained fibres of smaller diameter than those forming the bulk of the cuticle, but suggested that the outermost fibres were also smaller, and difficult to distinguish. The fibres were described as forming a basket weave with respect to the perpendicular cytoplasmic processes. Randall (1957) described the fibres as being embedded in

an amorphous material which extended beyond the fibril zone so forming a thin amorphous stratum. External to this he described a layer of double membraned "microvilli" 1500Å long and 500Å wide, corresponding to the "corpuscles" of Reed & Rudall (1948).

Randall (1957) made two speculations about the cuticle: that the cytoplasmic processes might take part in the orientation of the unbanded fibrils which themselves are probably secreted from precursors in the epidermal cells; and that there might be a connection between the presence of the cytoplasmic processes and that of the "microvilli" on the exterior of the worm.

Millard & Rudall (1960) on A. longa confirmed the size differences of fibres within the cuticle and put forward a hypothesis of constant renewal of the cuticle from below, with growth occurring within the 6 lowermost layers and resorption in the outermost layers. They noted papilla-like projections arising from the epidermal surface and suggested that these might influence fibre direction within the cuticle. They also recorded "protoplasmic continuations" running upwards through the cuticle.

In 1961 Ruska & Ruska, on L. terrestris, schematised the fibre orientation and vertical cytoplasmic processes and showed these to be regular and occurring in the spaces between the fibre-crossings. They also established the outermost zone - free of fibres - as the epicuticle and demonstrated both the unbanded cuticle collagen and banded collagen from other lumbricid sites. This more typical, banded collagen was commented on by Coggeshall (1966) and studied more extensively by Baccetti (1967) and Valembois (1971a) and shown to possess a periodicity of 560 Å, the figure quoted by Burke (1974) for E. foetida.

Coggeshall (1966), on L. terrestris, supported many of these earlier observations, but distinguished the cytoplasmic extensions

through the cuticle as being of two types - the "long" and "short" microvilli. He claimed that the "long" microvilli were so irregularly arranged as to be discounted as orientation factors with respect to the fibres. A similar categorisation into "long" and "short" was referred to by Knapp & Mill (1971). Coggeshall (1966) described the small ellipsoidal projections on the outer surface as being homogenous and electron opaque, and 400Å wide and 800Å long. They were shown to possess a unit membrane and to have, from their tips, fine filaments making up a layer 1 μm thick. As this layer was not PAS positive, he dismissed the possibility of its being mucus. He also dismissed Randall's suggestion (1957) of a possible connection between the projections and the microvilli purely on numerical grounds, the "long" microvilli being few in number, although their tips "rest among them", and the projections being numerous. He did not suggest either the origin or the function of the projections.

The works of Chapron (1970), Valembois (1971b) and Burke (1974) on E. foetida all comment on the similarities in cuticle ultrastructure between this species and the description of Coggeshall (1966) on L. terrestris. Burke (1974) describes the microvilli that pass through the cuticle to the free edge as being regularly arranged but does not discuss their role. She details hemidesmosomes arising from the epithelial surface (= "short" microvilli of other workers) and regards them as distinct from the microvilli. Ellipsoidal bodies occurring on the free edge of the cuticle are described, as is the layer of material trapped by them. Although this gave positive reactions with AB and Ruthenium red, Burke (1974) did not associate this with mucus material. The origin of the ellipsoidal bodies was not considered.

With the use of fixation techniques other than glutaraldehyde and osmium tetroxide, Djaczenko & Cimmino (1973) have revealed further ultrastructural details concerning the fibrous components of the

oligochaete cuticle. By means of the tris 1-aziridinyl phosphine oxide method (TAPO) combined with pre-treatment acrolein and post-treatment osmium tetroxide they have visualised the polysaccharide component of the collagen (established by Singleton, 1957). Their results show, with silver staining, the deposition of silver proteinate exclusively at the peripheries of the fibres, the central core remaining unstained. They guardedly suggest that the collagen component of the fibres is located centrally and the carbohydrate peripherally, though admit of the possibility that lack of penetration of the TAPO mixtures into the central core might be responsible for this effect. Earlier, Maser & Rice (1963) had suggested that the carbohydrate component of the fibres might be bound to the tropocollagen dimers in such a way as to obscure any ultrastructural patterning in the collagen due to the unavailability of the protein side groups to react with heavy metals.

The studies of Djaczenko & Cimmino (1973), carried out on four lumbricids, Tubifex tubifex and the enchytraeid Enchytraeus albidus further revealed a cross-banding periodicity in the peripheral zone of the cuticular fibres of 185Å and a regular spacing of the filaments within the zone of 62.5 Å. Such a periodicity is, as they point out, one third of that of lumbricid non-cuticular collagen and their evidence supports the idea of different alignment of the molecules within the cuticular fibre from that at other sites.

With respect to the epidermis of lumbricids, much work has been done on the sensory cells (Aros, Röhlich & Vigh, 1971a; 1971b; Röhlich, Aros & Viragh, 1970; Knapp & Mill, 1971), and the localisation of monoamines (Myhrberg, 1971). Coggeshall's paper (1966) on the fine structural analysis of the epidermis of L. terrestris dealt fully with the supporting cells and intercellular junctions but gave little

detail respecting the secretory cells. He described vacuoles filled with mucus in some cells whilst others possessed a single mucous cavity, and concluded that these were stages in a cycle of mucus production and release. Valembois (1971b), on regeneration after body wall grafting on A. caliginosa and E. foetida, distinguished mucous and albumen cells in the normal epidermis, both rich in ergastoplasm and dictyosomes and containing secretory masses, but did not give any further ultrastructural details of the secretion itself. Burke (1974) on E. foetida gave some ultrastructural detail concerning the epidermal secretory cells, but unfortunately the depth and clarity of her descriptions of the gland cells fell short of her observations on other aspects of the epidermis. She recorded a "mucous-type", a "speckled type" the nature of which was not investigated, and also described and figured a further type showing electron density variability and suggested that this might be a less than fully mature stage. As the details of her findings are pertinent to the present study, these will be given in the appropriate section (Sections 11, 12, 13).

10 LUMBRICID CUTICLE ULTRASTRUCTURE

i) Observations

The depth of the cuticle of the eleven lumbricids studied is given in Table IX and is seen to range from 1.1 - 3.2 μm depending on the species. The cuticle region can be conveniently divided into (a) the collagenous fibres, (b) the microfibrillar matrix, (c) the microvillar extensions from the epithelial cells, (d) the epicuticular projections and the supracuticular mucoid coat, and (e) the interruptions caused by the mucous cell pores and the ciliated sensory cells where they occur.

The collagenous fibres

In all the eleven species the fibres are revealed, by the techniques employed, as being unbanded and show the typical orthogonal cross-lamella pattern. The fibres vary in thickness depending on their position in the cuticle. Nearest the epithelial surface they are small, there being a gradual increase in thickness towards the mid-region where they attain their maximum width, beyond this they decrease again, eventually becoming difficult to distinguish clearly (Plate 6).

The number of fibre layers varies in the species studied (Table IX), the bulky worms A. longa and L. terrestris having 20 - 24, the medium sized worms D. zebra, E. foetida, L. rubellus and O. lacteum in the 15 - 18 range and the smaller or more slender worms with 10 - 14.

The mean fibre diameter, taken at the mid-cuticular level, is also given in Table IX and ranges from 200 nm in A. longa to 86 nm in D. hibernica.

Consistently throughout the study irregularities in the heavy metal staining of the fibres were observed within a single section.

Usually they stained only at their surfaces, thus appearing pale or white in the section (Plate 8a, b), rarely appearing as dense fibres as in Plate 6.

The cuticle fibres of lumbricids were very susceptible to pronase digestion (0.25% for 30 min) and were completely removed during this treatment (Plate 7), whereas the banded collagen fibres of the layer below the basal lamina (basement membrane of light microscope studies) were scarcely affected, their outline remaining distinct and their banding discernable.

The microfibrillar matrix

The microfibrillar matrix can be divided into four zones (Plate 8b). Nearest the epithelial surface is a narrow basal zone lying below the collagenous fibres. Next is the deep zone surrounding the fibres and above this is a fairly loosely arranged layer of microfibrils of low electron density, the pale zone. The extreme outer zone is of electron dense microfibrils, the surface zone, closely packed together and occasionally showing some degree of parallel alignment at right angles to the surface (Plate 14a). This surface zone has no limiting membrane, though the epicuticular projections which rest on it are membrane-bound. In thick resin sections the surface zone (and possibly the pale, fibre-free zone) and the epicuticular projections stain intensely with PAS and TB and correspond to the epicuticle.

These zones of the matrix are common to all the species in this survey except E. foetida. The only variables are the depth of the deep zone consequent upon the number of collagenous fibre layers, and the depth of the surface zone and its ratio to the fairly consistent pale zone which subtends it (Table IX). In A. chlorotica, D. zebra and L. terrestris the surface zone is comparatively shallow (65-72 nm)

and has an approximate 1:3 ratio to the pale zone, in A. longa it is about 120 nm deep and has a 1:1.5 ratio, and in the other species, except E. foetida, the surface zone is 85-95 nm in depth and a roughly 1:2 ratio exists.

In E. foetida the outermost region can often be distinguished as two layers of approximately equal thickness (40-45 nm), a very dense layer lying above a less dense layer (Plate 8b). Because the latter sometimes shows a parallel alignment of microfibrils it is suggested that the two layers together are equivalent to the surface zone of the other species, giving thus a 1:2 ratio to the pale zone.

In the region of the microvillar extensions, the matrix microfibrils form a loose but distinct sheath around them, and in sections to one side of a microvillus the sheath appears as a band running vertically through the cuticle (Plate 8a) and, in serial sections, this can be followed through to a microvillus.

The microfibril size is of the order of 2 nm thick and often the microfibrils lie closely, but irregularly, applied to the collagenous fibres.

The microvillar extensions from the epithelial surface

The cytoplasm of the distal areas of the epithelial cells is characterised by small membrane-bound vesicles (52 nm) of varying electron density, often lying close to or in contact with the distal plasmalemma (Plate 11c), larger vesicles (95 nm) of low density or showing a heterogeneous matrix, multivesicular bodies and bundles of tonofilaments (Plate 8b). The cells are also characterised by the regular extensions into the cuticle.

In favourable L.S. (Plate 8b) these microvillar extensions are seen to be very regularly arranged, running right through the cuticle and occupying the majority of the channels between the fibres.

They terminate among the epicuticular projections. Most of the microvilli follow a more or less straight path but, occasionally, they are seen passing obliquely or almost horizontally through the upper zones of the cuticle (Plate 14a). The microvilli are not digested during pronase treatment (Plate 7).

In horizontal section of the cuticle (Plate 9) the microvilli are seen to be arranged in regular staggered rows along the longitudinal axis of the worm. The distances between the individual microvilli of a row vary between 200 and 400 nm, the most frequent spacing being 300 nm. The distance between rows is more constant, between 70 nm and 100 nm.

Each microvillus arises from its own oval base protruding from the cell surface. It is itself ovoid above the base, becoming more or less circular in cross section higher up. Its diameter decreases from 90 - 95 nm above the base to 50 - 55 nm at the distal end. These measurements are marginally smaller than those of the microvilli surrounding the mucous cell pore and considerably smaller than the microvilli of the uniciliate sensory cells (120-130 nm). The surface membrane of the microvillus is continuous with that of its base and the subtending epithelial cell.

The base is roughly twice as long as wide (195 x 100 nm) with the two poles forming low shoulders on either side of the microvillus (Plates 10a, 11c). Closely applied to the inner surface of each shoulder is a dense basal body, and from each of these a prominent bundle of tonofilaments, the microfilaments of Goodman & Parrish (1971), extends a considerable distance, often traceable within one section for at least 1 μm, into the cell. Other tonofilaments from the cell pass on up the centre of the microvillus, but are not detectable towards the distal end. Because of the slightly sinuous path of the microvilli through the cuticle, these latter filaments

are rarely seen clearly (Plate 11a, b, c).

From the outer surface of each shoulder, adjacent to the basal body, arises a regular array of microfibrils, in an arc of 130^{o} - 180^{o}, 30 nm long and terminating among the microfibrils of the basal zone of the cuticle (Plate 10d).

The microvillar bases are uniformly oriented at right angles to the longitudinal axis of the worm and, depending on the plane of sectioning, their complete structure is not always apparent. Plate 10a shows both shoulders whereas Plate 10b is in a plane whereby neither of the shoulders is apparent. Plate 10c shows one shoulder and the microvillus and Plate 10d reveals only the shoulder and gives the appearance of a "short" microvillus.

The epicuticular projections and the supracuticular mucoid coat

Lying above the surface matrix zone is the region of the regularly arranged, membrane-bound epicuticular projections (the "corpuscles" of Reed & Rudall (1948)). In all the species other than E. foetida they have a peanut shell shape with a 'waist', 20 - 25% smaller, occurring at the halfway level (Plate 12a). In E. foetida they are wider at the base than at the apex and the waist is about one-third of the distance from the top (Plate 13a, b, c). The dimensions of the projections for the eleven species are presented in Table X.

The projections in all the species show a definite substructure, having an electron dense lining of 8 nm, surrounding, in the basal portion, a pale core. Above the waist two parallel electron dense discs, each 8 nm deep, occur, the upper one being about 8 nm below the apical lining (Plates 12a, 13 a, b, c). Plate 12b shows the projections in cross section, one of them being cut so as to show the dense disc.

Fine parallel microfilaments can be discerned extending from the base of the epicuticular projections into the surface matrix zone where they lose their identity among the parallel alignment of the microfibrils described earlier. No branching of the epicuticular extensions has been observed. These connections with the rest of the cuticle form a vague pedestal but do not show up clearly enough to allow enumeration or positional definition.

The epicuticular projections arise from the exposed tips of the microvilli and Plate 13a-c shows the series of events starting with the pinching-off of the microvillus tip at the appropriate level. The substructural details of the projections become established before they are finally separated from the microvillus. Both the projections and the 2 parallel electron dense discs are retained after pronase treatment of fixed material (Plate 7).

There is evidence that the microvillus tip may branch and give rise to more than one epicuticular projection (Plate 14b). The number of projections is greatly in excess of the number of microvilli and the bending of the latter (Plate 14a) suggests that one microvillus may be the source of a group of epicuticular projections.

Surrounding the epicuticular projections and extending well beyond them are fine strands of mucoid material forming the supracuticular coat (Plate 8a, b). Some of the strands lie very close to the projections especially in their apical regions and in transverse section (Plate 12b) the threads may appear to radiate from them, but no consistent pattern exists and no clear connection has been found between the mucoid strands and the projections. The filamentous nature of the mucoid material is ultrastructurally similar to the contents of the metachromatic mucous cells of the epithelium, suggesting that this cell type is the origin of the mucoid coat.

The cuticular interruptions

The cilia of the sensory cells cause little disruption to the cuticular arrangement, passing up through the microfibrillar matrix and leaving the collagenous fibre pattern undisturbed (Plate 9). The openings of the mucous cells are, by comparison, large (diameter 1.5 μm) and result in definite cuticular interruptions (Plates 15, 16).

Mucous cells are abundant in the epidermis and both the metachromatic and the orthochromatic cells have a cytoplasmic lining to the goblet cavity that is uniformly thin (about 0.16 μm) to within about 2 μm of the cell apex. At this point the lining gradually increases threefold to give a wedge-shaped area which, however, does not contain any specialised occlusor organelles. Arising from the outer surface of the cytoplasm is a circlet of 13 - 15 short microvilli, 400 nm long and 85 nm wide at their mid-point, which are prominently supplied with parallel tonofilaments.

Surrounding the mucous pore microvilli are numerous membrane-bound mucous pore particles which line the edge of the pore nearest the cuticle for one-quarter to one-third of its length (Plates 15, 16). They have an average diameter of 36 nm and an unstructured core. Close physical proximity to the mucous pore microvilli often exists but no clear evidence of their origin can be given.

The pore leading through the cuticle is straight and is lined by a microfibrillar zone 0.3 - 0.4 μm wide (Plates 15, 16). In sections where extruding mucus can be seen in the pore cavity (diameter 0.75 μm) the pale microfibrils show an oblique and parallel orientation (Plates 15, 16). At the level of the mucous pore particles a close association exists between these and the microfibrils as though the latter may be derived from the particles. At the distal end of the mucous pore the pale fibril lining merges with the pale matrix

zone of the cuticle. The surface zone of the matrix and the epicuticular projections are not involved in the pore lining.

ii) Discussion

The cuticle depth measurements given in Table IX show a broad correlation with the size of the species, the large A. longa and L. terrestris having a cuticle depth twice that of the small B. muldali and D. hibernica. Measurements were made only on micrographs where the cuticle was in good section, but it is recognised that the depth of the cuticle may depend on the contraction state of the worm during fixation. This accounts for the smaller figure of 1.6 µm for L. terrestris calculated from the micrographs of Coggeshall (1966) who used relaxants before fixation. However, in the text of Edwards & Lofty (1972) a figure of 7 µm is quoted for the cuticle of L. terrestris but the region of the body examined is not given nor is the type of fixation. It has been noted (Section 4) that fixatives with a high proportion of swelling agent can affect the resultant thickness of the fixed cuticle (Plate 1a) and this might account for the discrepancy. The cuticle depth figure of 1.5 µm given by Burke (1974) for E. foetida accords well with the present results.

The mean diameter figures for the cuticle fibres, taken at the mid-cuticular level, agree, in the two instances where the same species have been studied, with the recorded measurements of Djaczenko & Cimmino (1973).

The irregularities in the heavy metal staining of the fibres reported here would appear contrary to the claims of uniform density with conventional fixation techniques made by Djaczenko & Cimmino (1973). The published micrographs of other workers display similar irregularities, and this non-staining may account for Kryvi's description (1972a) of the cuticle of Sabella where he detailed the absence of dense fibres

but recorded "light tubules" of varying diameters, lacking a membrane but revealing a border "wall" slightly denser than the matrix. From his micrographs it would appear that his "light tubules" were, in fact, unstained collagen fibres showing the graded size pattern described above for the lumbricids.

The susceptibility of the cuticle fibres to pronase treatment contrasts with the findings of Burke (1974) on E. foetida where, after 20 minutes immersion of whole worms in 0.1% pronase prior to fixation, the enzyme failed to digest the fibres. In view of the fact that the enzyme strength, exposure time and tissue state were all different from those undertaken in this study, the precise reason for the contrasting results cannot be ascertained.

The arrangement of the collagenous fibres of the cuticle is common throughout the species in this survey and agrees with the findings of previous workers on lumbricid cuticle (see Section 9). The pattern is also similar to that recorded for polychaetes belonging to diverse families by Storch & Welsch (1970), and such an arrangement is also apparent in the cuticle of the microdrile Mesenchytraeus solifugus (Goodman & Parrish, 1971).

What is unclear, and will remain so until developmental studies are performed, is whether the successive parallel layers are in fact formed sequentially, increasing in number corresponding to the enlargement of the cuticle with the growth of the worm, or whether the number of layers is laid down at an early stage and increased girth is accommodated by the intercalary accretion of unit filaments to individual fibres.

In either case, it seems likely that the source of the unit filaments is the microfibrils of the matrix, since these are of the same order of thickness as those isolated from lumbricid native cuticular collagen by Maser & Rice (1962) and they are present

throughout the cuticle.

Krall (1968) reported similar microfibrils in the less organised cuticle of Dero and he postulated that these were formed by polymerisation on the cuticle side of the epithelial cell membrane from subunits secreted from apical vesicles within the cell. In the lumbricids examined here, the numerous small vesicles of the epithelial cells, many appearing to coalesce with the apical cell membrane, point to a similar origin for the microfibril subunits. Krall's further suggestion that perhaps tonofilaments (also present in Dero) were involved in the dual role of microfibril formation in addition to their cytoskeletal supporting function would appear to be unnecessarily complicated in view of what is known of the formation of connective tissue collagen in other oligochaetes and in the leeches. In E. foetida and A. caliginosa (Valembois, 1971a) and Hirudo medicinalis (Bradbury & Meek, 1958) the collagen of the connective tissue has been shown to form its unit filaments outside the cell, as in vertebrates.

The factors determining the orthogonal, cross-lamella arrangement of the successive fibre layers remain to be determined. Earlier workers (Randall, 1957; Millard & Rudall, 1960) have suggested that the "cytoplasmic processes" are partially responsible for the fibre pattern, but this idea was dismissed by Coggeshall (1966). He, together with Knapp & Mill (1971), distinguished between "long" and "short" microvilli and Coggeshall claimed that the former were too irregularly spaced to be fibre orientation factors. The present study indicates that such a distinction is not valid and that the "disparate phenomena" of Goodman & Parrish (1971) (long and short microvilli, epicuticular projections and cuticular fibre orientation) are in fact interrelated. There are only "long" microvilli, the so-called "short" ones being the shoulders of the microvillus bases.

These long microvilli are very regularly arranged in relation to the fibres (Plate 9), but it is not suggested that Cerfontaine (1890) was in fact seeing them when he reported extremely fine striations running perpendicular to the cuticle surface and connecting the hypodermis (epidermis) to the exterior. Since the microvilli run in the spaces between the cuticular fibres it is however possible that he was observing, if the angle was correct, the result of the differing densities of the fibres and the matrix housing the microvilli.

Although developmental studies have not been undertaken, the regeneration studies of Potswald (1971) on Aeolosoma cuticle showed that the "filamentous layers" (= fibre layers of lumbricids) did not appear before the elaboration of the microvilli. Thus the microvilli would in fact appear to be suitable candidates for influencing fibre orientation.

Since there is no apparent alignment of microfibrils within the matrix, fibre orientation must presumably occur during unit filament aggregation and be controlled by some 'intrinsic' factor. The precise nature of this factor remains unclear. One possibility could involve the interaction of the unit filaments with the stresses set up in the cuticle by the normal movement of the worm, modified by the arrangement of the microvilli.

Although the microfibrils of the matrix appear variously bent and twisted and of indeterminate length in fixed, sectioned material, this may be partly artifactual. Maser & Rice (1962; 1963) present convincing physico-chemical evidence that the unit filaments of native lumbricid collagen consist of dimers of tropocollagen molecules having a relatively stiff rod-like structure about 540 nm long. Josse & Harrington (1964) present a calculated figure of 940 nm.

If the molecules have a fairly stiff structure, then the waves

of continually varying longitudinal and circumferential stresses, induced throughout the depth of the cuticle and parallel to its surface by the worm's movement, could perhaps tend to align the unit filaments parallel to the cuticle surface. It is also possible that other longitudinal stresses caused by local annular flexing of the cuticle, acting differentially throughout its depth, could reinforce this tendency.

The arrangement of the microvilli, as seen in surface view, is of more or less regular staggered rows along the longitudinal axis. The distance between the microvilli in each row is most frequently 300 nm and the distance between the rows is between 70 nm and 100 nm. Thus, as the 540 nm long filaments react to the various stresses within the cuticle, they would tend to take up the preferred orthogonal orientation presented by the spaces between the microvilli.

Once small groups of filaments are roughly aligned, they would become tightly bound together by intermolecular forces, and such protofibres would thus increase in length, diameter and stiffness. This increasing stiffness might tend to prevent them from bending at those points where they cross each other, so that layers of fibres would develop with all the fibres of each layer parallel. Since this layering would develop solely as the result of their crossing, successive layers would be oriented more or less at right angles to each other.

Most probably the main function of the microvilli is to act as pivotal anchorage points for the flexible cuticle, as evidenced by their core of tonofilaments and the firm connections of their basal bodies to both the cuticle matrix and the epithelial cell cytoplasm. The tonofilaments would provide longitudinal stiffness, while the polarity and firm attachments of the bases would provide torsional stability.

An interesting speculation is that information about local stresses set up in the cuticle by the movement of the worm could be transmitted via the tonofilaments of the microvilli, and thence by the bundles of tonofilaments that have been observed in the epithelial cells extending down to the cell bases where there is a network of nerve fibres. Thus the microvilli could function as the proprioceptors whose apparent absence was commented on by Knapp & Mill (1971).

The present study establishes the microvillar origin of the epicuticular projections in the Lumbricidae, showing them to be pinched-off from the ascending microvilli. A similar origin has been recorded in other oligochaetes (Hess & Menzel, 1967 - Enchytraeidae; Krall, 1968 - Naididae; Potswald, 1971 - Aeolosomatidae; Goodman & Parrish, 1971 - Enchytraeidae) and also in some polychaetes (listed by Michel, 1972) suggesting that these structures, also present in leeches (Damas, 1969), and their mode of formation are characteristic of annelid cuticles. Structures resembling these projections have also been recorded in the egg envelope of the polychaete Hydroides (Colwin & Colwin, 1961) and in the primary envelope of Sabellaria (Franklin, 1966). The similarity between the epicuticular projections and the mammalian glycocalyx has been commented upon by Potswald (1971) and Michel (1972).

The number of projections, in all cases, greatly exceeds the number of microvilli and the branching and tortuous pathway of the distal end of the microvillus suggests how this could arise. In other oligochaetes it has been suggested that after epicuticular projection formation the microvillus either withdraws or degenerates. Potswald (1971), from his regeneration experiments, presumed withdrawal as the number of microvilli decreased after the establishment of the full complement of projections. A regeneration sequence is not

necessarily the same as a developmental sequence. In the present lumbricid studies there is nothing to suggest withdrawal or degeneration, all the microvilli that were seen or followed through serially emerged into the epicuticular projection zone, and the occasional 'missing' microvillus is not regarded as supportive evidence of a normal and regular loss of microvilli.

The substructural detail observed in the lumbricid epicuticular projections is unlike any recorded in other annelids studied where, if mentioned, the ground substance of the projection is described as uniform, and Coggeshall (1966) recorded the particles as homogeneous for L. terrestris. However, the micrographs of Burke (1974), although the projections were not in very good section plane, showed evidence of the substructure described here, but she did not comment on this detail. In the oligochaete Dero, Krall (1968) described density changes in the microvillus tip during the formation of the projections.

The epicuticular projections were resistant to pronase in fixed material. This contrasts with the findings of Potswald (1971) on Aeolosoma where pronase treatment on living worms caused detachment of the epicuticular projections after 20 minutes in 0.1% pronase, at which time the cuticular filaments showed erosion, the filaments not being effectively removed until treated with pronase for 30 minutes. It is not possible to assess whether these differences are fundamental or merely reflecting the fact that the lumbricid material was fixed and the aeolosomatid material living. However, the collagen fibres responded to the enzyme in the same way and with the same speed in both, contrasting with the findings of Burke (1974) on E. foetida.

The attachment of the projections appears as a vague pedestal composed of fine parallel microfilaments which merge into the surface matrix zone and lose their identity in the lumbricids studied here.

No organised attachments as described for Enchytraeus fragmentosus by Hess & Menzel (1967) were observed.

Intermingled with the epicuticular projections are the fibril strands of the supracuticular mucoid coat. In microdrile studies an orderly arrangement of such fibrils has been described (Hess & Menzel, 1967), and a suggestion put forward that they are derived from the surface corpuscles by Krall (1968). No pattern was seen in lumbricids and in this present study the origin of the strands cannot be traced to the projections. It is unlikely that the secretion of mucus would be located at this point since, in lumbricids, large mucous cells are plentiful within the epidermis, and the ultrastructural nature of the mucoid coat fibrils is similar to the mucus of the metachromatic mucous cells (Section 12). Burke (1974) using AB and Ruthenium red has shown this superficial material to take up these dyes selectively in E. foetida, but did not define the material as being mucus on the grounds of the questionable specificity of these dyes for demonstrating acid mucopolysaccharides in electron microscope studies.

The close proximity of the strands to the projections supports the idea that the epicuticular projections function to trap and mechanically stabilise the acid mucopolysaccharide coat which lies as a fine fibrous blanket around the worm. The rather complex substructure of the projections is difficult to explain; it is perhaps involved in the maintenance of the physical integrity of the projections as stabilisers of the mucoid coat, but seems rather elaborate for this function. The arrangement of the two parallel electron dense discs in the tip suggest that it is not entirely impossible that they are sensitive to external pressure variations, e.g. physical vibrations of the soil, or even, perhaps variations in the osmotic pressure of

the mucoid coat associated with different degrees of hydration.

The structure of the mucous cell pore indicates that there is little in the way of control mechanism guarding against excessive loss of mucus apart from the narrowness of the neck compared to the width of the goblet and the circlet of microvilli with their tonofilaments. The cytoskeletal function of these might well be important in forming a physical barrier at the base of the pore. Pore microvilli have been recorded in polychaetes by Dorsett & Hyde (1970a, b) on Nereis, Kryvi (1972b) on Sabella and Michel (1972) on Notomastus, but membrane-bound mucous pore particles located in the lower regions of the pore have not been documented. That these might be the origin of the pale fibril lining is a possibility but as yet their own origin is uncertain. The orientation of the pale fibrils lining the pore of discharging mucous cells may merely reflect the drag effect of the mucus on the lining as it passes through the pore.

11 LUMBRICID ORTHOCHROMATIC MUCOUS CELL ULTRASTRUCTURE

i) Observations

The orthochromatic mucous cells are characterised by large, distinctive mucous globules lying in the very large secretory cavity, the lateral cytoplasmic lining of which is extremely thin (about 0.16 μm). Leading through the cuticle are the mucous pores, the fine structure of which has been dealt with in Section 10. Basally, the cytoplasmic lining is thicker and it is here that the nucleus, numerous mitochondria, extensive granular endoplasmic reticulum and the Golgi complex are located. The relationship of the rough endoplasmic reticulum to the polarised Golgi apparatus is that typical of secretory goblet cells, and from this basal region arise the membraned secretory sacs, with their contained globule material, that come to lie in the goblet cavity. These globules are the granules of the histochemical study.

The standard fixation schedule, while adequate for general epidermal fixation and for the metachromatic mucous cells (Plate 17 and Section 12), proved species variable for the orthochromatic cells. In many the globule membrane always appeared fragmented; in others the globule area was definable by reasonably intact membranes but the globule material occupied only a portion of this and gave the appearance of considerable condensation having taken place; in still others, both fragmentation and condensation occurred. For any given species the appearance was, in most cases, consistent. Trials with varying strengths of fixative, timings, buffer molarities and pHs, also the use of Durcupan A to eliminate dehydration, on E. foetida,

failed to alter the membrane rupture and appearance of condensation in this species.

Secretory globule ultrastructure

The mature globule lying in the goblet sac consists of a limiting membrane inside which lies the mucous material. This assumes the appearance of a two-phase system with the inner core substance surrounded by rim material, varying, with the species, in compactness, quantity and degree of delimitation from the core. In some species the core material of the globule is consistently and characteristically patterned, heterogeneous globules, in one there is a faint substructure which is occasionally indiscernable, and in four there is no observable pattern, the globules being homogeneous, even though vague areas of marginally denser core material might occur.

Heterogeneous globule types

The electron dense patterning within the globule core takes a variety of forms depending on the species, ranging from an ill-defined mottling (O type as in Octolasion), through a more pronounced but irregular mottled effect (L type as in Lumbricus), a more or less regular spotted pattern (A type as in Allolobophora) to a mixed arrangement of solid and hollow spheroids (E type as in Eisenia).

L type

Lumbricus rubellus (Plate 18a). The core of each globule is characterised by electron dense areas giving an irregular mottled effect, the remainder of the core material forming a matrix of medium electron density. The rim is distinct and delimited from the core, and the globule material fills the membraned secretory sacs. A small number of cells showed a more regular patterning.

Lumbricus terrestris (Plate 18b). The mottling is less pronounced

than in L. rubellus and is more irregular, sometimes the electron dense areas being large enough to be observed in the 0.5 μm resin sections stained with Toluidine blue. The rim of the globule is less clearly defined than in L. rubellus and some wrinkling and fragmentation of the sac membranes occurs, but the contents fill the area.

A type

Allolobophora longa (Plate 19a). The globule cores of this species are characterised by numerous, discrete, spherical, electron dense bodies. Each dense body has a distinct, clear, peripheral area. Occasionally a globule may have one or more very large dense bodies, again with clear peripheries. Rim material lacks compactness, does not touch the sac membrane and merges with the core matrix. The globule membranes are often wrinkled and fragmented.

Allolobophora caliginosa (Plate 19b). In general the globule cores are similar to those of A. longa, but the spheres are more numerous, less electron dense and have a characteristic thin, distinct, denser outer limit surrounded by a less conspicuous clear periphery. Loose rim material is present around the condensed core.

Allolobophora chlorotica (Plate 20a). The core patterns are variable, often with ill-defined spherical areas only slightly denser than the matrix, but sometimes without any discernable pattern. There is greater condensation of the core material than in the other species of this genus studied and the area between the membrane limit and the core is filled with diffuse rim material.

E type

Eisenia foetida (Plates 17, 20b, 27). Numerous very electron dense solid and hollow spheroid formations characterise the globules of this species. The smaller, less clearly defined, dense areas are

surface cuts of the larger formations, both solid and hollow, and the formations are not always discrete, occasionally joining up with one another. There is no rim material and the membranes are frequently ruptured. The core material always appears condensed, even when a wide range of technical variations is employed. In a very small number of cells the globule core patterning was lacking in density and gave a 'ghost' effect. In material fixed in glutaraldehyde alone, the patterns are discernable but are marginally less dense than in double fixed material. After fixation in osmium tetroxide alone, the patterns are also slightly less intense.

O type

Octolasion lacteum. The globule cores show some variation in their substructure. At low magnification, they are either so finely patterned as to appear almost structureless or show a type of mottling (Plate 21a). At higher magnification, the fine patterning consists of very regular dense dots (Plate 21b) which in some section planes show parallel lines suggesting an orderly, perhaps rod-like substructure not unlike the paracrystalline arrangements described in the developing spermatocyte granules of the turbellarian Anaperus gardineri (Silveira, 1967). A thin rim surrounds the cores showing mottling, the finely patterned cores are rimless and the membranes in both types show wrinkling and fragmentation.

Homogeneous globule types

Dendrobaena subrubicunda (Plate 22a). The globule material is unpatterned and condensed, with loose material occupying the rim area and the membranes showing fragmentation. One cell in one block showed a mottled core and rim structure similar to that found in L. rubellus.

Dendrobaena veneta var. hibernica. The globule is, at low magnifications, homogeneous and condensed, with very diffuse rim material between it and the much fragmented membrane system. At higher magnifications (Plate 22b) the core reveals small, ill-defined, irregular areas only slightly denser than the rest of the material.

Dendrobaena veneta var. zebra (Plate 23a). The homogeneous cores are condensed and diffuse rim material occupies the spaces between the cores and the fragmented membranes.

Bimastos muldali (Plate 23b). Condensed homogeneous cores occur within the fragmented membrane area. The rim material is very diffuse.

The secretory process

There is a close relationship between the granular endoplasmic reticulum and the formation face of the Golgi complex (Plate 24), and the smooth membraned transition vesicles arise from the ribosome deprived areas of the reticulum. The contents of these transition vesicles have a low electron density as have the contents of the endoplasmic reticulum cisternae. Towards the mature face of the Golgi complex the typically closely applied cisternae (commonly six) with dilated margins occur and the contents are frequently more electron dense than in the formative regions of the Golgi (Plate 25a). Small secretory vesicles arise from the cisternae and these enlarge and fuse (Plate 24) to give the secretory sacs described earlier.

In the species with unpatterned, homogeneous, secretory globules, the medium electron density of the contents of the small secretory vesicles is similar to that of the fully formed globule. In those species with patterned, heterogeneous, secretory globules, the heterogeneity is first apparent within the small Golgi vesicles of the mature face (Plate 25a, b), there being one dense region per

vesicle, and in E. foetida both the solid and the hollow spheroids are evident at this level in the Golgi complex. Fusion of such vesicles gives rise to the mature globule with its numerous dense areas.

Action of proteolytic enzymes

After pronase treatment of E. foetida ultrasections the characteristic dense heterogeneity was no longer discernable, the patterning of the spheroid formations now appearing as areas of low electron density (Plate 26a), much lower than the surrounding globule matrix material which was seemingly unaffected by the treatment. This gave a 'ghost' patterning to the globules. Similar 'ghosting', but less intense, occurred after the block treatment with pepsin.

The orthochromatic mucous globules of A. longa, after pronase treatment, also showed loss of the electron dense patterning (Plate 26b), the spheres having a low electron density except at their extreme peripheries. The patterning in L. terrestris was also pronase-susceptible.

Mucus extrusion

There is some evidence of coalescence of the globules at the goblet pore prior to extrusion and in species with heterogeneous globules, the patterning is retained as the globule material is extruded (Plate 27).

This present study has not revealed any ultrastructural detail directly referable to a mucus extrusion control mechanism in the orthochromatic mucous cells. Portions of the epidermal nerve plexus are frequently seen in close proximity to the mucous cell base,

similar to those reported by Burke (1974) in E. foetida, but no synaptoid connections have been observed. The thin lateral lining to the goblet cavity is devoid of contractile organelles and no sphincter organelles have been observed in the lumbricid pore areas, though mucous pore microvilli are present (Plates 22a, 27) and have been described earlier (Section 10). However, tonofilaments running vertically through the neighbouring supporting cells are frequently numerous and often lie close to the intercell boundary (Plate 28a). Portions of the epidermal nerve plexus are to be seen at the bases of such cells. The bundles of filaments are occasionally detectable in light microscope, paraffin, sections, staining with Acid fuchsin (in FALG) and also Heidenhain's haematoxylin.

Beneath the epidermis is a thin basal lamina, below which is the layer of banded collagen fibres. Prominent hemidesmosomes characterise the bases of the epidermal supporting cells, but such features appear to be lacking at the bases of the orthochromatic mucous cells in this study, though Burke (1974) described basally situated hemidesmosomes in the secretory cells of E. foetida. The cells of the present study, for the most part, do not make contact with the basal lamina, the area between their base and the lamina being occupied by baso-lateral extensions of neighbouring supporting cells. The orthochromatic mucous cells would therefore appear to be serous fluid sacs suspended from the cuticle and surrounded by supporting cells rich in tonofilaments.

ii) Discussion

The only published work pertinent to the detail given in this present study is that of Burke (1974) on E. foetida. The speckled type of gland cell described, though not considered as a type of

mucous cell by her, appears to be identical to the orthochromatic type dealt with in this section. Her single micrograph shows all the globule characteristics described here, including membrane fragmentation, but her account and figure did not include details of the secretory areas of this cell type and she did not comment on the point within the cell at which the heterogeneity was established. Burke (1974) claims that "intermediaries can be seen between the homogeneously dense granules and the speckled granules", but does not figure these or state whether such intermediaries occur within a single cell, or clearly define the dense granular type. However, she speculates that the denser class of granules may represent a gland cell series whose terminal secretion is the large speckled granules. The results of the present lumbricid study do not provide evidence to support these ideas, and the only figure given of dense (but variably so), homogeneous granules by Burke (1974) equates closely with the small granular, proteinaceous cell type to be described in Section 13.

The lumbricid orthochromatic cells under review here are characterised by their prominent secretory globules which show species variation with respect to globule substructure, globule condensation and secretory sac membrane fragmentation. In these respects they differ from the metachromatic mucous cells (see Section 12). Condensation of the secretory product and failure of the sac membranes to remain intact has been encountered by other workers on invertebrate epidermal secretion (Krall, 1968; Chassard-Bouchard & Hubert, 1973) and by Silveira (1967) in the secretory granules in developing turbellarian spermatocytes.

In this present study the species variation in core condensation, rim density and membrane fragmentation might well be an osmotic

fixation effect (unresolved in E. foetida) reflecting differences in the naturally occurring hydration state or viscosity of the mucus. Those species showing core condensation also showed diffuse or no rim material and invariably displayed membrane rupture in the fixed state. If different species are producing mucus of differing hydration states then a standard fixation schedule, applied to all the species, might reflect any such specific differences in the mature globules. The more or less 2-phase system which has been described in the mature globule is not apparent in the small Golgi vesicles, so presumably changes occur as the globules coalesce and mature in the goblet sac. Changes in mucous globules during maturation have been recorded in polychaetes (Dorsett & Hyde, 1970a, b). If the core, rim and membrane variations in the fixed state are indicative of physical differences in the mucus of the species, it has not been possible to relate such variations to any system of species grouping based on the eco-physiological factors of Piearce (1972) (Table XI).

The pronounced heterogeneity of the cores of five species, the variable or slight heterogeneity of two and the homogeneity of the remaining four species is not absolutely coincident with current lumbricid taxonomy, but it approximates more to this than to other groupings. Plate 28b may be found helpful during the following taxonomic discussion.

At the generic level Lumbricus, Allolobophora, Eisenia and Octolasion show distinctive substructural patterns, and Bimastos and Dendrobaena have plain, unstructured cores. Where more than one species was studied in a heterogeneous genus, specific differences were apparent.

The two species of Lumbricus have a mottled pattern to the core

and a rim to each globule, but are distinguishable from each other on the basis of intensity and size of the pattern components. Allolobophora longa and A. caliginosa possess numerous dense spheres with clear peripheries, unlike the substructure of the previous genus, and the two species are ultrastructurally distinct, caliginosa having more numerous, smaller spheres with denser limits to them. A. chlorotica, however, does not show the 'generic' substructure as clearly, having either a similar but considerably less dense patterning or no discernable heterogeneity. Only one species was studied from the genera Octolasion and Eisenia. Eisenia foetida characteristically shows solid and hollow spheroids and samples of this species from Vancouver Island, B.C., Canada showed exactly the same patterning as the British ones, as did the material from Massachusetts, U.S.A. of Burke (1974). Bimastos muldali and the three members of the genus Dendrobaena all have homogeneous globules, though a single heterogeneous cell was found in D. subrubicunda.

The ultrastructural detail of the globules in the species studied here lines up well with modern lumbricid taxonomy and in many cases supports the recent changes that have been made. In the extensive revision made by Omodeo (1956), and not yet accepted by all taxonomists, veneta var. hibernica and veneta var. zebra were transferred from the genus Eisenia to that of Dendrobaena and they, together with D. subrubicunda, all show homogeneous globules. In terms of British lumbricids the genus Eisenia is now monospecific, containing only foetida, the globules of which are very distinct. This study shows that, at high magnification, veneta var. hibernica has a vague pattern of ill-defined areas only marginally denser than the rest of the globule material and in this respect differs from the other homogeneous types and is, perhaps, more similar to the

situation in A. chlorotica than to veneta var. zebra. Morphologically the worm is unlike zebra, being much smaller and lacking dark pigmentation, and in many ways resembles the grey/pink form of A. chlorotica. For more substantial reasons Gates (1956) split the veneta forms and suggested that hibernica be placed in the genus Allolobophora as A. hibernica. Bimastos muldali was originally classified as Allolobophora minima and was renamed by Omodeo (1956), and its globules are shown to be unlike those which characterise the genus Allolobophora. However, A. chlorotica does not show the typical 'generic' substructure, only a vague representation of it. Omodeo (1956), in his revision, erected the subgenus Allolobophora (genus Allolobophora) and within this he established three groups, placing chlorotica in the chlorotica-group and so separating it from both longa and caliginosa which he designated to the caliginosa-group.

When groupings other than taxonomic are made, e.g. size, pigmentation, eco-physiological (Table XI), the substructural patterns do not satisfactorily align themselves with any of these and this present study therefore indicates that a wider and more comprehensive survey may be of some taxonomic interest.

The histochemistry of the orthochromatic mucous cells (Section 6) of seven of the species under review here showed that the secretion contained protein. The enzyme part of this ultrastructural study indicates pronase-susceptible protein to be located at the points of electron density within the globules of E. foetida, A. longa and L. terrestris, thereby suggesting that the heterogeneous patterning is a reflection of certain protein sites. That these sites may also be the location of the bound lipid, shown histochemically to be intimately associated with the protein moiety of the mucus, is a possibility, as suggested by the density differences when single

fixation techniques were employed.

In cells secreting both protein and carbohydrate it is now widely accepted (Bloom & Fawcett, 1968; Neutra & Leblond, 1966a, b) that the rough endoplasmic reticulum is the site of the protein synthesis and the Golgi complex the location of the carbohydrate synthesis. In this study, the electron dense bodies (protein sites in E. foetida, A. longa and L. terrestris) were not observable, in any of the heterogeneous species, in the endoplasmic reticulum, the formative Golgi face or the Golgi stacks. Within the secretory complex they are first detectable at the mature face of the Golgi. This suggests that the protein complex susceptible to fixation so as to give electron dense areas is produced as a result of elaborations within the polarised Golgi. Similar positional visualisations of structured protein components of secretions have been recorded in the epidermis of the crustacean Palaemon serratus (Chassard-Bouchaud & Hubert, 1973) where the protein constituent of the intermoult secretory cells appears at the Golgi vesicle level, as do the structured granules of the developing spermatocytes in the acoelous turbellarian Anaperus gardineri studied by Silveira (1967).

The failure of the very occasional cell in normal E. foetida material to show the typical dense substructural pattern, so resulting in an appearance resembling the pronase treated material, may be regarded as an indication of irregularity in protein synthesis. A similar explanation could be advanced for the aberrant cell observed in D. subrubicunda and for the occasional variation in some of the other species.

In demonstrating that the heterogeneity of the globules reflects the site of the pronase-susceptible protein moiety of the mucus, it leaves unanswered the protein location in those species with homo-

geneous globules, three of which were studied histochemically and revealed no detectable differences in the nature of the mucus when compared with the other species studied. This is not to say that differences do not exist, and the occurrence of interspecific differences in mucus has been recorded by Reygrobellet (1963) in Deroceras (Agriolimax). Jakowska (1965b) put forward the speculation that with further knowledge of mucosubstances it might be possible eventually to relate taxonomic differences to definite fractions within the mucus. Although the present histochemical study failed to do this, the ultrastructural observations have revealed certain characteristics which align themselves more to the taxonomy of lumbricids than to any other grouping.

The mechanism of mucus extrusion still remains unclear. No synaptoid endings have been observed in this study, nor were found by Myhrberg (1971) in epidermal studies on L. terrestris, though Smallwood (1926) believed mucus secretion to be controlled by nerves in earthworms. In the medicinal leech Lent (1973) has demonstrated mucus secretion to be under the control of the Retzius cells, but microscopic details were not given as this was a physiological study. Earlier workers on earthworms had suggested a direct muscular control and Coonfield (1932), working on peripheral nerves in earthworms, supported a mechanism in which muscular contraction was the main influence. The present study shows that the goblet lining is extremely thin and devoid of contractile organelles, but that the surrounding supporting cells, which embrace the orthochromatic mucous cells, are rich in vertically oriented tonofilament bundles, also reported by Valembois (1971b). The mucous cell is, for the most part, not in contact with the basal lamina and acts as a serous fluid bag suspended in the epidermis from the cuticle, and is therefore

susceptible to any pressure changes that are directed towards the epidermis, below which is the layer of banded collagen fibres and the muscle coats of the body wall. Since there is no apparent special mechanism for mucus extrusion, it could conceivably happen as a result of locomotory activities, in which case the secretion of the orthochromatic mucous cells in lumbricids would automatically accompany, and at the same time assist, locomotion.

However, recent literature supports the idea that microfilaments, which have the same substructural appearance and approximately the same dimensions as described for tonofilaments, may be 'contractile' (Cloney, 1966; Schroeder, 1969; 1970; Selman & Perry, 1970; Huang & Pitelka, 1973; Singal & Sanders, 1974), actin-like proteins (Adelman, Borisy, Shelanski, Weisenberg & Taylor, 1968; Lyons, 1972; Helper & Palevitz, 1974) and if this were found to be universally so, then the tonofilaments of the supporting cells could possibly have other than a purely cytoskeletal function and provide a structural basis for contractility.

A contractile function and a cytoskeletal function are, of course, not mutually exclusive, the structures possibly acting in both capacities as has been suggested for microtubules (Anderson, Weissman & Ellis, 1966). The presence of tonofilaments near the mucous cell would more subtly influence the extrusion of mucus and it is noted that nerve fibrils occur regularly at the bases of these supporting cells. It has been stated that the tonofilament bundles are detectable in suitably stained paraffin material where they assume an appearance similar to the intracellular fibrillae demonstrated by Millott (1948) in the ciliated cells surrounding the intestinal gland cells of L. terrestris. He advanced a hypothesis whereby contraction of the fibrillae would effect discharge

of the gland cells, and although he did not demonstrate nerve endings within the surrounding cells he had previously shown that gland discharge occurred in response to the stimulation of the secretory nerves.

The exact function of the mucous pore microvilli (described in Section 10) is not clear. The mucous goblet cells appear to be in permanent connection with the outside, unlike the sequence of events proposed by Freeman (1966) for mammalian goblet cells which were shown as being devoid of mucous pore microvilli. The presence of mucous pore microvilli could form a resistance to mucus flow from the cells and their possession of tonofilaments, in the light of the suggestion that they might be contractile, would support this idea. Kryvi (1972b) speculated on the possible "spreading" effect of the mucous pore microvilli on the mucus of Sabella, though in this polychaete the microvilli would appear to be more numerous, longer and less endowed with tonofilaments than in the lumbricids of this study. Dorsett & Hyde (1970a, b) did not comment on the possible function of the mucous pore microvilli in Nereis.

The orthochromatic mucus would appear not to be involved in providing the thin film of mucus permanently covering the cuticle in fixed preparations, the film being trapped there by the epicuticular projections and so acting as 'respiratory' and 'dehydration prevention' mucus. This is derived from the metachromatic cells, and the secretion of the orthochromatic cells is not found clinging to the cuticle after fixation.

Nothing in the literature indicates any possible reason for the subtle species differences noted ultrastructurally in the orthochromatic mucus, but the obvious problem of species recognition

exists in cross-fertilising hermaphrodites.

The ultrastructural characteristics have been identified as protein sites in three species of differing genera. Such specificity suggests a chemical individuality the function of which might be an attraction pheromone when the mucus is extruded in normal quantities. This hypothesis could be advanced for those lumbricids having heterogeneous globules, but presents difficulties for those with homogeneous globules. However, the two genera in this study with homogeneous cores both contain species which are polyploid (Muldal, 1952) and are either facultatively parthenogenetic (D. subrubicunda, B. muldali) or obligatorily so, and thus would have no such recognition requirement. Parthenogenesis in the megadrile oligochaetes is much more widespread than generally realised (Reynolds, 1974). Furthermore, within the heterogeneous genera, Octolasion shows a less pronounced patterning than the other three genera, and O. lacteum is singular in the British lumbricid fauna in being an obligatory parthenogen which is apomictic and diploid.

It is therefore suggested that the specific differences observable at the ultrastructural level could possibly be a reflection of an intraspecific recognition mechanism associated with the locomotory mucus and serving a species recognition function. The idea that mucus from other than specialised areas might be associated with sexual behaviour has not arisen in the oligochaete literature, though sexual dimorphism reflecting itself in the nature of the mucus was shown to be the case in the hamster (Shackleford & Klapper, 1962) and the Amazonian "discus" fish is documented as producing copious integumentary mucus during the parental nurturing period following the hatching of the young (Hildeman, 1959). A similar situation occurs in the leech Glossosiphonia complanata where the

epidermal mucous cells empty during the cocoon nurturing period (Gondran, 1954). But none of these examples is directly comparable to the present suggestion.

The other function of mucus which has been put forward in the literature is that of an alarm pheromone. Krishnan & Rajulu (1969), working on a megascolecid earthworm, claim that the large gland cells discharge only after stimulation and support the alarm pheromone function described by Ressler, Cialdini et al. (1968) for the stimulated mucus of L. terrestris. Ratner & Boice (1971) have also demonstrated a pheromone in L. terrestris mucus and noted, in addition, a cross-species generality of the mucous secretions of earthworms, obtaining the same behavioural responses using L. terrestris and E. foetida, irrespective of which was the secreting and which the detecting worm.

However, the pheromonal effect was only produced after severe stimulation, and the violent electrical or mechanical stimulation, as used by these behaviourists on lumbricids, would certainly cause coelomic fluid discharge through the dorsal pores, a reaction well documented in the literature (see Section 3), seen in Plate 5a, b, and recorded in Appendix II. Consequently the secretion used for these pheromone experiments would have been a mixture of mucus types together with coelomic fluid. Ratner & Boice (1971) record the presence of "cells" (their inverted commas) in the secretion when studied microscopically and these would have come, presumably, from the coelom. The possibility that the pheromonal effects were due to extruded coelomic fluid is less acceptable in view of the report by Ressler, Cialdini et al. (1968) that the pheromone effect persisted at the same potency after the secretion had been dried for three months, such long effective durations perhaps suggest a

mucus rather than a coelomic siting of the pheromone.

The results of the workers on earthworm behaviour point to the existence of a type of mucus secreted in stress conditions and demonstrate that worms can recognise worm mucus, though the mechanism of recognition is unclear, as it is in the oligochaete Chaetogaster which has been shown to detect its mollusc host by means of the mucous trail the latter leaves (Buse, 1972).

It might not be too speculative to suggest that with the species differences evidenced by this present study, there might exist a species recognition component within the orthochromatic mucus. The cross-species generality findings of Ratner & Boice (1971) appear to conflict with this idea but their techniques were such that the large quantities of mucus produced, which always elicited an avoidance response, might have swamped any possible positive tactic response which lower, more normal, mucus levels might have produced.

In accepting a mucus siting of the alarm pheromone as documented by Ressler, Cialdini et al. (1968) and Ratner & Boice (1971) the present results lead to the suggestion that the orthochromatic mucus, with its primary locomotory function, also acts as a 2-level pheromone mechanism, whereby a single substance, in different amounts, elicits different responses, as with some plant auxins. Such a mechanism, located in the orthochromatic mucous cells, would produce, at normal low levels, a specific attraction pheromone for use in pairing, and at high levels, as under stress, act as a non-specific alarm pheromone.

At present such a complex hypothesis seems necessary in order to embrace the results of the behaviour studies, but future experiments might well show the cross-species alarm pheromone to be

located in the coelomic fluid, known to be ejected under stress. If this were to be so, then the 2-level pheromone explanation would be unnecessary and would enable a simple hypothesis to be advanced for the location of an attraction pheromone in the orthochromatic mucus of the heterogeneous, amphimictic species. Ultrastructural investigation of the orthochromatic mucous cells of such species before maturation might well be of interest.

12 LUMBRICID METACHROMATIC MUCOUS CELL ULTRASTRUCTURE

i) Observations

The metachromatic mucous cells, like the orthochromatic type, extend the full depth of the epidermis and are characterised by the membrane-enclosed secretory globules lying within the secretory area. The thin cytoplasmic lining of this is thicker basally where it houses the nucleus, mitochondria, granular endoplasmic reticulum and Golgi apparatus, the latter two bearing to each other a relationship typical of secretory cells. Apically each metachromatic cell opens to the exterior by means of a pore which perforates the cuticle. In all these architectural respects the metachromatic cells are similar to the orthochromatic mucous cells, yet are readily distinguishable from them by reason of the ultrastructural details of the secretory globules.

Whereas a standard fixation schedule applied to all the species in the survey proved species variable for the orthochromatic cells, a greater consistency occurred with respect to the metachromatic cells and it has not been possible, with one exception, to relate the ultrastructural representation of the secretory globules to a particular species. Nevertheless, the globule material did not always present a uniform appearance, variations occurring between cells within a section, between globules within a cell on occasions and within areas of an individual globule.

The secretory globules

Ultrastructurally the globule material is fibrous in nature and similar to the fibrous material forming the supracuticular mucoid

coat which lies external to the cuticular surface. The disposition of the fibrous material varies and presents itself in three main forms which will be referred to as finely fibrous, stranded, and densely whorled.

The finely fibrous representation is shown in Plate 29a and the fibrils are randomly arranged and give rise to no detectable pattern. The stranded form, on the other hand, has areas of greater electron density within the globule, these appearing as strands of the order of 25-30nm thick, frequently arranged in parallel to one another. The alignment of the strands is not always uniform within the globule and Plate 29b shows globules in which some strand groups have been cut along their length and others across their width within the same globule. The densely whorled form presents an exaggerated type of stranding, the strands being about 50nm across (Plate 30a).

Although within one cell the majority of the globules show the same ultrastructural form, gradation between the categories exists and two of the forms can exist within a cell or even within a single globule. Plate 30b shows the finely fibrous and stranded globules occurring together in the same cell and Plate 31a depicts globules in which the material is finely fibrous in some areas and stranded in others.

This occurrence of two distinct areas within a single globule is not uncommon and most frequently takes the form of the presence of a denser area, generally one per globule except in D. hibernica. The denser areas are either ultrastructurally uniform (Plate 30b) or show a whorled, stranded substructure (Plate 31b). In either case the denser zone is surrounded by an area of lesser electron density which is traversed by thin, radiating, fibrous strands, suggesting that the observed dense area is perhaps the product of

condensation of globular material at that point.

In D. hibernica the most commonly occurring metachromatic cell type was one where the globules were very large (in excess of 2 μm) and possessed several electron dense areas lying within a finely fibrous matrix (Plate 32a). This form was not observed in any of the other species studied. Also present in D. hibernica, but fewer in number, were metachromatic cells with globules filled by uniformly fibrous material and resembling cells found in the other species.

A broad correlation appears to exist between the size of the globules within a cell and the ultrastructural representation of the secretory material. Allowing for the fact that not all the globules of one cell will be cut across their maximum diameter, observation and measurement reveal that in most cells the majority of the globules are roughly the same size and present the same general ultrastructural picture. In cells with globules in excess of 1.5 μm and less than 2 μm the secretory material is uniformly distributed and of the finely fibrous type. Plate 32b shows two adjacent metachromatic cells, one containing large, finely fibrous globules and the other medium sized globules with whorled strands and denser areas. This association of pattern and size was observed in all the species under review, the stranded form being most generally found in globules with a size range of 0.9 - 1.3 μm and the finely fibrous type in globules larger than this. The densely whorled form is associated with globules smaller than 0.9 μm. However, cells with very large globules - in excess of 2 μm - were occasionally found in species other than D. hibernica and in these cases the material did not show uniform distribution within the membraned area but presented a presumed condensation eccentrically within the globule limit (Plate 33).

The other broad correlation noted in this study was that of the globule size and the state of the granular endoplasmic reticulum. In cells where the majority of globules were medium or small sized the basally situated secretory apparatus appeared active, with the endoplasmic reticulum cisternae filled with uniform, medium electron dense material (Plate 34). In those cells filled with large globules the rough endoplasmic reticulum was often bloated, distorted and invariably empty (Plate 35a).

A similar situation to this exists in Plate 33 where the secretory material is housed in large globules and the basal region of the cell contains bloated and almost empty endoplasmic reticulum and a disorganised Golgi system. In Plate 35b a metachromatic cell, identified from the portions of globular material, shows even greater indications of disorganisation of the secretory apparatus, together with a nucleus that lacks electron density and shows a distended perinuclear space. It is suggested that the cells in Plates 33, 35a, b represent stages in the senescence of the metachromatic cell type.

Within an actively secretory epithelium and one in which scenescing and effete cells are found it might be expected that young, replacement cells would also occur. Plate 36 shows what might be interpreted as such a cell. This contrasts with the normal metachromatic cell in two important respects: (a) the height of the nucleus within the cell is unusual for a secretory cell and approaches the mid-epithelial level of the nuclei of the supporting cells (see Plate 33) and (b) the granular endoplasmic reticulum and the Golgi apparatus are not restricted to the basal area of the cell, as they are in mature cells, but extend throughout the lateral cytoplasmic lining, and portions of the secretory complex are found amongst the globules in the more apical parts of the cell (Plate 37). The globules are mainly of small to medium size and contain whorled material.

Plate 33, apart from showing what is considered to be a senescing cell, also shows, to the right of the ageing cell, one which could possibly be interpreted as an even earlier stage in the formation of a metachromatic mucous cell than that shown in Plate 36. This is discussed later. The portion of tissue shown in Plate 33 also displays part of an epidermal blood capillary loop.

Action of proteolytic enzymes

In contrast to the action of pronase and pepsin on the ultrastructure of the orthochromatic globules, the fine structural appearance of the metachromatic cells remained unimpaired after proteolytic enzyme treatment.

The secretory process and extrusion

The globules of the metachromatic cells arise from the mature face of the polarised Golgi apparatus which lies distal to the granular endoplasmic reticulum in the basal regions of the cytoplasm of the cell (Plate 34). The endoplasmic reticulum cisternae contain uniformly amorphous material and the first indication of material reacting to fixation in a manner similar to that found in the fully formed globules occurs within the secretory vesicles of the mature face of the Golgi system.

The mature globules are extruded from the cell, generally with membrane breakdown before extrusion, through the mucous pore which perforates the cuticle. Plate 33 shows this and indicates the ultrastructural similarity between the mucus and that of the supracuticular mucoid coat trapped by the epicuticular projections of the cuticle. At the base of the mucous pore is a ring of microvilli, stouter than the cuticle microvilli, similar to those found in the orthochromatic cells and described earlier.

No occlusor organelles have been found in relation to the pores, and although portions of the epidermal nerve net are frequent in sections and often located close to the mucous cell bases, no synaptoid endings associated with the mucous cells have been observed.

ii) Discussion

Although three main forms of ultrastructural expression of the metachromatic secretory material have been described in this present study, there is sufficient evidence of overlap and coexistence within one cell to justify the interpretation that they are all images of the same type of material. Because such differences are evident in all the species it can be assumed that they are a reflection of the fixation on a similar secretory product in all the species studied. As the differences do not seem to be related to fixative penetration, there being no relationship between globule form and its position in the cell, the variations would therefore appear to be indicating some physical or physico-chemical difference within the secreted globules.

The hydration state of the secretion suggests itself as a likely cause. It is not possible, at present, to argue as to whether the observed differences are due to variations in the amount of water present in a globule at the time of fixation or to differences in the degree of binding of the water to the acid mucopolysaccharide complex. From the ultrastructural appearance of the globule material it would seem that more water has been removed from the stranded and whorled forms than from the finely fibrous expressions. Such differences would not be detected in the histochemical study where, in any case, the light microscope fixatives failed to retain fully the secretion within the cell.

The correlation between the size of the globule and the ultrastructural form of the secretion, and that existing between the globule size and the appearance of the secretory apparatus, which showed signs of less organised activity in cells secreting large globules, gives rise to the hypothesis that the hydration state of the mucus, and linked to this its viscosity, may change during the life-span of the cell.

With age, the ultrastructure of the secretory cell alters, the secretory apparatus giving rise to larger globules of material of a different hydration level and the granular endoplasmic reticulum and Golgi eventually showing indications of disorganisation. This alteration in the size of the secreted unit during the life of a cell is an acknowledged phenomenon in vertebrate endocrine cells where the production of small granules is associated with the fast secretion rate of vigorous cells and large globules with a slower secretion rate. Such a changing physiology during the activity-span of a lumbricid acid mucus cell could also affect the quantity or degree of chemical bonding of the water in the secretion sufficient to account for the observed ultrastructural variations after fixation.

Within an actively secretory epithelium containing ageing cells it might be expected that replacement cells would also be observed. The cell type interpreted as being a young stage shows several micro-anatomical features not observed in the mature cells. The position of the nucleus and the widely dispersed secretory apparatus are markedly different from the typical metachromatic cell. It is presumed that as the cell matures the nucleus, endoplasmic reticulum and Golgi apparatus become displaced into the usual basal position as the majority of the cell area fills with secretory product.

Although the present study has shown a number of stages within the hypothesised sequence of events during the life of a metachromatic

cell it has not revealed a complete enough series of the early stages to resolve the controversy concerning the origin of the secretory cells within the epidermis. The oligochaete literature presents two possible candidates for secretory cell replacement: by direct modification of existing supporting cells; or by growth and differentiation of basal cells. The literature is slightly weighted in favour of the basal cell origin, but no detailed, recent work exists on this point, the grafting and regeneration studies of Valembois (1971b) not revealing the precise origin of the mucous cells. However, in the polychaetes there is evidence that mucous cells differentiate directly from the supporting cells (Thouveny, 1967; Cornec, Solbani & Turki, 1970), and the same claim is made for the leech Glossosiphonia (Gondran, 1954).

Without true serial sections it has not been possible to interpret such a cell as the one adjacent to the ageing cell in Plate 33. The secretory material is ultrastructurally of the metachromatic mucus type and occurs basally and apically to the nucleus which is similar to that of the secretory cells and unlike that of supporting cells. If it could be shown with certainty that this was a cell confined to the basal regions of the epithelium, and not a slice of a cell not lying in the exact plane of the section but nevertheless extending the full depth of the epidermis, one could imagine a sequence of events involving the accumulation of secretory product both sides of the nucleus to such an extent initially that the nucleus would be pushed into a more mid-epithelial position and assume the form seen in Plate 36 before fully maturing into a typical metachromatic cell.

The described ultrastructural variations in the globule material were present in all the species studied, D. hibernica being singular in depicting features in the very large globules that were not found

in any other species, namely numerous electron dense areas within the large globules. No satisfactory explanation can be offered for this in taxonomic or eco-physiological terms and the histochemical study (Section 7) did not reveal any differences between the metachromatic mucus of this species and that of the other lumbricids studied.

If the hypothesis of the globule size being related to the secretory age of the cell holds, it would appear that the majority of the metachromatic cells in the processed tissue pieces of D. hibernica showed every appearance of being 'old', that is, producing very large globules. All the individuals of D. hibernica processed for this electron microscope study were, in fact, collected from one site at one time and fixed immediately. This raises the question of the environmental conditions operating on the individuals during the period before their selection for this study. This point has rarely been given consideration in the literature, but Wohlgrab (1971) has drawn attention to the need for standardisation of not only the genotype but also the environment of animals used in detailed investigations. However, other species, also collected from one site at one time for this study, did not exhibit a predominance of one phase. This does not necessarily eliminate the environmental influence which might have been operating on the specimens of D. hibernica, nor does it allow total disregard of the possibility that a real difference, undetected by histochemical techniques, exists which causes this particular ultrastructural pattern to occur when globules above a certain size are subjected to the fixation schedule.

The unimpairment of the ultrastructural pattern after proteolytic enzyme treatment contrasted with the effect produced by these enzymes on the orthochromatic globules (Section 11). The histo-

chemical survey showed extremely low levels of protein within the metachromatic secretion (Section 7) and failed to reveal qualitative differences in the protein fraction of the two mucous cell types. The enzyme treatments at the ultrastructural level indicate that the protein present in the metachromatic cells is not visualised in the same way as in the orthochromatic cells and is not susceptible to the enzymatic treatments undertaken.

The cell type described as the mucous type by Burke (1974) on E. foetida, and stated as resembling the mucous cells of L. terrestris reported by Coggeshall (1966), would appear, from the single published micrograph, to be equivalent to the metachromatic mucous cell type described in this section. Ultrastructural variation in the globule contents was noted by Burke (1974) ranging from "faintly granular to a more electron-opaque flocculence".

The fibrous ultrastructural nature of the metachromatic mucus in this present study accords with the published micrographs of cells producing acid mucus in many animal groups. In the polychaetes Nereis (Dorsett & Hyde, 1970) and Sabella (Kryvi, 1972) the ultrastructural nature of the acid mucus is one of fibrous material often showing a reticulate pattern similar to that described in some platyhelminthes (Pedersen, 1965; Best, Morita & Noel, 1968). However, a very striking similarity exists between the present lumbricid results, with their display of variations, and those presented by Pedersen (1965) for the predominantly carboxylated acid mucus of the posteroventral gland cells of Convoluta. He recorded densely whorled, stranded and finely fibrous expressions of the mucus, the latter occurring only in the large sized globules, and suggested that the variations might be reflecting differing states. A study of his published micrographs reveals a relationship between the

size of the globule and the fixed form of the secretion similar to that observed in the present study and it would therefore seem that the correlations described in this lumbricid study also occur in the histochemically similar acid mucus producing cells of a turbellarian platyhelminth.

The fine structural detail of the metachromatic mucus is similar to that found forming the supracuticular mucoid coat trapped by the epicuticular projections which occur on the free edge of the cuticle (Section 10). It has been suggested earlier (Section 7) that this ever-present mucous film plays an important part in the respiration of the animals, providing the medium through which the primary gaseous exchanges take place. The ultrastructural similarity of the metachromatic mucus throughout the species studied here, and when compared with that of other animal groups, would support the idea of a generalised function, contrasting with the lumbricid orthochromatic mucus which showed species variation (Section 11).

The involvement of the lumbricid integument in providing the respiratory exchange surface and the participation of mucus in forming the watery layer for dissolving the oxygen has been documented many times, though without reference to the nature of the mucus, most writings implying that there is only one type. Associated with the respiratory function of the epidermis in large oligochaetes is the presence of blood capillary loops within the epidermis, though Brinkhurst & Jamieson (1971) state that this is not the case in lumbricids. Plate 33 shows a cross-section of a blood capillary within 2.7 μm of the cuticle and frequently capillaries have been observed lying at a distance of 1 μm below the cuticle. In the truly aquatic microdrile oligochaetes there are no blood capillaries within the thin epidermis and comparatively few mucous cells, both

facts reflecting a relationship between the occurrence of mucous cells, vascularisation of the epidermis, body size and the environment.

During the ultrastructural study of the orthochromatic mucous cells (Section 11), carried out in parallel to this present investigation on the metachromatic cells, no cells identifiable as either effete or young stages of the orthochromatic type were observed. From this arises the suggestion that perhaps the metachromatic cell type has a shorter activity-span than the orthochromatic type, hence the occurrence of effete and young stages in the tissue at the moment of fixation. That this is a built-in character of this cell type is a possibility, but our present lack of knowledge concerning life-spans of cell types does not enable further serious comment on this at the moment. However, since the metachromatic mucus is providing what must be a constant respiratory surface, then this cell type is presumably permanently secretory during its life-span. This might well be expected, therefore, to be shorter than for a cell type which is less permanently active. The present results would, guardedly, seem to support such a hypothesis.

13 LUMBRICID SMALL GRANULAR, PROTEINACEOUS CELL ULTRASTRUCTURE

i) Observations

The small granular, proteinaceous cells extend the full depth of the epidermis and have their apical pore surrounded by pore microvilli of greater diameter than the supporting cell microvilli. The basally situated nucleus is often compressed. These microanatomical features they share with the large granular, orthochromatic mucous cells and the metachromatic mucous cells, but the small granular cells differ from them in the following respects: (1) the size and electron density of the secreted units; (2) the absence of a distinct secretory cavity housing these units; (3) the lack of restriction of the granular endoplasmic reticulum and Golgi systems to the basal regions of the mature cells; (4) the sparse and irregular distribution of this cell type within the epidermis; (5) the ultrastructural uniformity of the granules throughout the species studied, which contrasts with the condition found in the orthochromatic mucous cells (Section 11).

The secretory granules are electron dense (Plate 38) and even where variations occur (Plate 41) they are more dense than the globules of the mucous cells (Plate 39). The spherical granules are of the order of 0.6 - 0.7 μm in diameter, though in some tissue pieces fixation distortion has occurred and a more irregular outline results (Plate 40a). The granules are membrane-bound and arise from the polarised Golgi apparatus (Plate 40b). The contents of the granular endoplasmic reticulum cisternae are amorphous and of medium electron density whereas in the Golgi stacks those lamellae at the mature face are often of greater electron density than those at the

formative face (Plate 41).

The granular material appears ultrastructurally uniform at low and medium magnification (Plates 38, 41), but at high magnification the paler granules reveal, within the matrix, an extremely fine reticulum of tubules of the order of 16-18 nm wide the walls of which are approximately 4 nm wide (Plate 42a), such detail not being apparent at the levels of the granular endoplasmic reticulum cisternae or the Golgi stacks.

Plate 42b shows a young stage of a secretory cell type which cannot be related to either the orthochromatic or the metachromatic mucous cells and might therefore be interpreted as a developmental stage of the small granular type. The formed granules are mainly restricted to the apical third of the cell, the middle third being occupied by the extensive granular endoplasmic reticulum and Golgi and the remainder of the cell housing the large, uncompressed nucleus. Most of the granules near the secretory apparatus are less electron dense than those more apically situated and have an electron density similar to the contents of the secretory apparatus. The granules show the same fine structure at high magnification as that described earlier.

No control mechanisms in the form of occlusor organelles or nerve endings were observed in association with the small granular cells.

ii) Discussion

The secreted units of the small granular cells, whilst being considerably more electron dense than the secretory product of the mucous cells, showed some variation in electron density, the recently formed granules often having a lower density than those

in the more apical parts of the cell. This suggests that as the secretion matures there is a condensation of the osmiophilic material. A similar variation has been recorded by Dorsett & Hyde (1970a) for the type-1 cells of the spiral gland of two species of Nereis.

The granules arise from the mature face of the Golgi system which forms part of the polarised secretory apparatus of the cells. Such a topographical relationship suggests that both the endoplasmic reticulum and the Golgi are involved in the synthesis of the final secretory product. The participation of the endoplasmic reticulum in the secretory process is to be expected in a cell type producing highly proteinaceous granules (Section 8) for it is now acknowledged that this area of the cell is associated with protein synthesis and the Golgi system with carbohydrate synthesis. Although the electron density of a newly formed granule is often similar to that of the contents of the rough endoplasmic reticulum cisternae, the fine structural detail of the two sites if sufficiently different to give support to the idea that the effect of the passage of the material through the Golgi system is such as to produce a final complex which, on fixation, gives rise to the ultrastructural configurations within the granule which are not observable at the level of the endoplasmic reticulum.

The present study did not reveal developmental stages of the small granular cell type other than the stage presented in Plate 42b. It is not difficult to envisage how, with vigorous production of granules, the endoplasmic reticulum and Golgi would become more dispersed amongst the secreted units and eventually produce the mature form. Because of the absence of earlier stages in the examined pieces of tissue it is not possible to support

either the basal cell origin or the supporting cell origin of the secretory cells in the oligochaete epidermis.

In the recent study of Burke (1974) on E. foetida a cell type exhibiting variation in granule electron density is briefly reported and the basal region figured. This figure (Fig. 9, p.315) closely resembles the secretory areas of the small granular cells under review, particularly Plate 40b. It is unfortunate that Fig. 9 in the Burke paper does not carry any magnification information or scale from which the granule size could be calculated. Nevertheless, the structural similarities suggest to the present author that the cell illustrated is of the small granular type, a category not recorded by Burke (1974), and not a stage of the mature speckled type as intimated by her.

The membrane-bound granules of the present study are of the same order of size as those of the type-1 cells of the spiral gland of Nereis (Dorsett & Hyde, 1970a) which, at high magnification, also showed a reticulate substructure, but the components were 50 nm in diameter in Nereis and therefore of a much greater size than those described here for lumbricids. Furthermore, Dorsett & Hyde (1970a) questioned the involvement of the Golgi in the secretion of the granules of the type-1 cell in Nereis, the granules appearing to develop in the cisternae of the endoplasmic reticulum, and this is not the case in the lumbricid small granular cells. The histochemistry carried out on Nereis spiral gland cells was limited and revealed only neutral mucopolysaccharide material, protein tests not being undertaken. It is therefore not possible to relate this nereid cell type, found in the modified epidermal areas of the anterior portion of the body, with any certainty to the lumbricid cells under discussion.

In the less modified parapodial areas of Nereis no epithelial cells equivalent to the lumbricid small granular cells have been described (Dorsett & Hyde, 1970b) nor are any detailed in the extensive survey of the epidermis of polychaetes undertaken by Storch & Welsch (1970). Also, no cells comparable to the ones under present review have been reported in the light microscope study of leech epidermis (Gondran, 1954).

It would therefore appear from the literature that the small granular cells are a feature of the epidermis of the larger oligochaetes, their presence having also been recorded at the light microscope level in megascolecid earthworms (Menon & Singhal, 1969; Krishnan & Rajulu, 1969; Varute & Nalavade, 1970). Personal observations on microdrile oligochaetes have shown that cells equivalent to the lumbricid small granular type are not present in the fully aquatic families. However, in the Enchytraeidae a situation is found (described in greater detail in Section 26) in which littoral and sewage bed species of the genus Lumbricillus possess, in addition to mucous cells, a cell type with electron dense granules showing similar density variations and being of the same order of size as in the lumbricid skin, but terrestrial species of the genera Fridericia and Mesenchytraeus do not. It would therefore not appear possible to argue that the possession of a small granular type is the result of the adoption of the terrestrial habit in oligochaetes.

The lumbricid small granular cells show many similarities to the acidophilic cells (Eiweisszellen), the so-called albuminoid cells, of certain planarian platyhelminthes. Histochemically the lumbricid cells have many properties in common with those described by Pedersen (1963) for Planaria vitta and Dugesia tigrina, but comparisons do not hold at the ultrastructural level where striations at right angles to the long axis of the ovoid granules appear to be

characteristic for these platyhelminthes. However, in Dugesia dorotocephala Best, Morita & Noel (1968) described secretory cells which, like the Eiweissdrüsenzelle of Klug (1960), show spherical dense granules of the same order of size as those of the lumbricids of this present study arising from a polarised Golgi apparatus. Since no very high magnification micrographs of this cell type were presented in their paper, and as they did not do a histochemical study, further comparisons are not possible. It nevertheless remains of interest in view of the close histochemical and ultrastructural similarities that have been noted between the lumbricid metachromatic mucus and that of several turbellarian platyhelminthes (Section 12).

It has been suggested earlier (Section 8) that the proteinaceous secretion of the small granular cells of the lumbricid epidermis might well affect the viscosity of the secretion released from the mucous cell types and possibly increase the water retention properties of the metachromatic mucous film which maintains the body surface moist for gaseous exchange. The secretion of the acidophilic albuminoid cells of certain planaria, the histochemical and ultrastructural similarities of which have been already mentioned, is claimed to be sticky (Jennings, 1957; Pedersen, 1963). It is not, however, possible to make such direct claims for the product of the small granular cells of lumbricids. The random and infrequent distribution of this cell type and the absence of any detectable secretion-control mechanism make experimentation impossible, any type of generalised stimulation affecting not only the two mucous cell types but also, almost certainly, causing coelomic fluid discharge through the dorsal pores (see Plate 5b).

In advancing the suggestion that the secretion of the small granular, proteinaceous cells might play a part in determining the

overall viscosity of the slime it is recognised that the mechanisms whereby the secretory product of these cells could be extruded in response to environmental conditions demanding viscosity changes in the mucus still remain obscure.

14 LUMBRICID DISCUSSION

The present study, whilst delineating three types of secretory cell within the lumbricid epidermis and defining, by the use of histochemical techniques of varying specificity, the nature of the secretion more precisely than hitherto, has also revealed the limitations of this method of approach. These would appear to be twofold: the most specific and informative techniques have been established for essentially mammalian secretions or substances and when applied to invertebrate mucus often produce conflicting and unexpected results; histochemical methods can provide evidence for some of the types of units present within a secretion but give no indication of their arrangement.

Biochemical and electrophoretic methods have provided additional information in mammalian studies. They have allowed, together with supportive histochemical methods, for the confident use of such terms, deliberately avoided in this study, as glycosaminoglycan or glycosaminoglucuronoglycan in place of acid mucopolysaccharide in the literature (Spicer, Horn & Leppi, 1967). Nevertheless, the problem of linkages within the molecular complex remains unsolved.

At present, the analytical approach does not appear possible in lumbricid studies. The probability of sample contamination by secretions other than the one under investigation, also by coelomic fluid and nephridial excretory material, is high, paralleling the situation in mammalian gut where, even within a restricted area, different types of glands producing different kinds of glycoprotein contribute their secretions to the regional fluid (Gottschalk, 1963).

Even the discovery of the mechanism which causes release of the secretion from the lumbricid cell, so enabling selective stimulation, might not totally overcome this question of contamination.

Another major problem to be faced is that of making dynamic interpretations from the static evidence provided by the slide or micrograph. This is particularly acute when developmental sequences of gland cells are being established. At present it has only been possible to suggest that a particular image is probably the precursor or effete stage of a mature type. A more precise definition of the secretions than has been achieved in this study might then enable the use of autoradiographic techniques to determine with certainty such developmental sequences, but the choice of a sufficiently diagnostic label for a particular cell type might prove a problem.

Despite the limitations, the histochemical results have allowed certain speculations to be made about the three types of secretion in terms of their probable viscosity, and the surface film function of the metachromatic mucus is supported at the ultrastructural level. The orthochromatic mucus may well serve a generalised lubricative function and at the same time carry the ultrastructurally observed species recognition component. However, the product of the small granular, proteinaceous cells remains more obscure in functional terms.

The scope of the present study has not included embryological investigations which might well provide valuable information concerning the establishment of the secretory cells and the cuticle collagen layering. Nor has it included a systematic sampling of the species involved over a twelve month period to establish whether a seasonal variation occurs with respect to the secretory

cells as has been noted by Gondran (1954) for the epidermal mucous cells of Glossosiphonia complanata. The material for this present study was collected at varying times of the year, for the several purposes, and did not show differences sufficient to lead to the suggestion that a programmed survey would be fruitful.

The anticipation that a comparative study of lumbricid skin, over an ecological range of species, might reveal differences that could be related to the habitat has not been realised. The study has not revealed any features which self-evidently correlate with the known distribution of the species under review. Such adjustments as are necessary are presumably accomplished either by systems other than the epidermis or by biochemical means within the skin which have not been displayed by the techniques adopted or reflected in the microanatomy.

15 ENCHYTRAEID INTRODUCTION

The Enchytraeidae show a much greater ecological diversity than any other oligochaete family, having terrestrial, littoral and freshwater species. They also display, in certain species, a remarkable low temperature tolerance with Lumbricillus (Marionina) charae inhabiting the outflow stream of an arctic lake and Mesenchytraeus solifugus living on glaciers at zero or subzero temperatures. Stephenson (1930) suggested that the family had an arctic origin and certainly most temperate species breed during the coldest time of the year at these latitudes.

In temperate regions the number of terrestrial species is greater than that of the littoral species which, in turn, outnumber the freshwater species. If a freshwater origin of the Enchytraeidae is supposed, then the family displays considerable radiation. Supportive of this supposition is the genus Propappus which, on exclusively morphological grounds, is regarded as the most primitive within the family and this is a freshwater genus, though its forked chaetae have led Nielsen & Christensen (1959) to question its inclusion within the Enchytraeidae. The genus Lumbricillus, however, is also morphologically primitive (no gut appendages, chaetal shape) but has no temperate species occurring in unpolluted freshwater, being predominantly a littoral genus.

Christensen (1961) extended the study of enchytraeids by means of cyto-taxonomic work and confirmed the primitive status of Lumbricillus, along with Mesenchytraeus and Cernosvitoviella, by showing the chromosome number to be n = 16 or less in nearly all the species. Mesenchytraeus and Cernosvitoviella are also morpho-

logically primitive and are, like Lumbricillus, not predominantly freshwater genera. Cognettia, however, although morphologically simple and inhabiting limnic or very damp soil situations has a chromosome number of n = 54-160. Such results led Christensen (1961) to suppose that the enchytraeids might have originated in littoral situations, though he does not exclude the possibility of other permanently wet habitats.

Since no enchytraeid genus has species occupying terrestrial, littoral and freshwater habitats it was not possible to restrict the comparative study to selected species of a single genus and thereby avoid the complications of possible generic differences. Once committed to examining more than one genus, it was necessary not only to choose species with an 'ecological overlap' to provide the basis for comparisons but also to be aware of the relatively large size differences which occur within the family and take these into account in planning the survey.

It was decided to centre the study around the genus Lumbricillus for three reasons: for morphological and cytological reasons it is regarded as a primitive genus; it exhibits a considerable size range within its species; it has, although predominantly littoral and having no terrestrial species, one British species - L. rivalis - which occurs on sewage beds, in polluted streams and occasionally in freshwater outlets on the sea shore (many of the earlier records of L. lineatus from such situations are now thought to have been L. rivalis (Tynen, 1969a; 1972)). Therefore, observations could be made in the light of ecological and size variation within a single genus, ranging from the small littoral species georgiensis, mirabilis, vancouverensis, through the medium sized 'fresh' water rivalis to the very large, littoral reynoldsoni.

The absence of terrestrial members of Lumbricillus necessitated the study of other genera. Fridericia has species which occur very high up the shore (F. callosa) and ones which are truly terrestrial (F. bulbosa) and these small forms were chosen. A similarly sized species of Mesenchytraeus, taken from the same terrestrial habitat as F. bulbosa was also examined. In order to have a species of near comparable dimensions to L. reynoldsoni it was decided to include Enchytraeus albidus in the survey. Specimens of E. albidus were collected from its natural littoral habitat where it is widespread in driftline seaweed deposits, often occurring alongside L. reynoldsoni. Although E. albidus is known to be able to live in soil, and is frequently purchased from bait supplying agencies, there are no recent ecological reports of its natural occurrence in terrestrial situations, but abundant records from littoral sites.

Fairly detailed histochemical and ultrastructural studies were made on species of the genus Lumbricillus, particularly L. mirabilis, and the results of these form the greater proportion of this study. More limited studies were carried out on the selected species of Fridericia, Mesenchytraeus and Enchytraeus.

16 ENCHYTRAEID MATERIALS AND METHODS

i) Materials

Enchytraeids of the following species were studied:

Enchytraeus albidus Henle 1837

Fridericia bulbosa Rosa 1887

Fridericia callosa Eisen 1878

Lumbricillus georgiensis Tynen 1969

Lumbricillus mirabilis Tynen 1969

Lumbricillus reynoldsoni Backlund 1948

Lumbricillus rivalis Levinsen 1883

Lumbricillus vancouverensis Tynen 1969

Mesenchytraeus sp. (not mature enough for further identification)

E. albidus and L. reynoldsoni were collected from sites in Anglesey at the high-water mark; F. callosa, also from Anglesey, at sites above high-water mark; F. bulbosa and Mesenchytraeus sp. from coniferous litter at Keele University; L. georgiensis, L. mirabilis and L. vancouverensis at the high-water mark, in wrack beds, at Miracle Beach on the east coast of Vancouver Island, B.C., Canada (the type locality for these three species (Tynen, 1969b)); L. rivalis from sewage filter beds at Llangefni, Anglesey.

Within the genus Lumbricillus, mirabilis, reynoldsoni and rivalis were used for both histochemical and ultrastructural studies whereas georgiensis and vancouverensis were only examined at the electron microscope level. Specimens of the other enchytraeid species were processed both for light and electron microscopy.

ii) Methods

Because most of the chosen enchytraeid species reacted violently when placed in light microscope fixatives and were observed to react less drastically when placed in glutaraldehyde, a method of putting specimens of the smaller enchytraeid species into glutaraldehyde for a few seconds prior to the light microscope fixation was adopted. With specimens of L. mirabilis crystals of Xylocaine were floated on a dish containing the selected individuals in a minimal amount of 50% sea water, and the animals were fixed for light microscopy as soon as movement had ceased.

(a) Light microscopy

Although a more restricted histochemical programme was undertaken on the enchytraeid material, the methods employed were as for the lumbricid study (Section 2 iii a) with the exception of some specimens of L. mirabilis which were also fixed in Susa.

(b) Electron microscopy

L. mirabilis was fixed in 5% and 12% glutaraldehyde in three buffers - Millonig's, 0.1M phosphate at pH 6.3 and 0.1M phosphate at pH 7.1 - with 1% osmium tetroxide post-fixation in the appropriate buffer. L. georgiensis and vancouverensis were fixed in 5% glutaraldehyde in 0.1M phosphate at pH 6.3 and the British enchytraeids were fixed in 5% glutaraldehyde in 0.1M phosphate at pH 7.4, all with 1% osmium tetroxide post-fixation in the appropriate buffer.

Processing schedules for electron microscopy were the same as for the lumbricid material (Section 2 iii b) but for the fact that small post-clitellar pieces of whole worm were taken and not portions of the body wall.

17 ENCHYTRAEID HISTORICAL BACKGROUND (LIGHT & ELECTRON MICROSCOPY)

Little detailed information concerning the enchytraeid epidermis has appeared in the literature, consequently, the limited references to light and electron microscopical studies will be reviewed in this section and not separated as they were in the lumbricid part of this study (Sections 3, 9).

Light microscopy

The epidermal gland cells of many enchytraeids are prominent and are easily seen during microscopic examination of whole, living animals. This has resulted in their being detailed in recent species descriptions. In many of the smaller forms the arrangement of the cutaneous glands is in transverse rows (Nielsen & Christensen, 1959), and in certain species of Marionina the gland cells are reported as being highly coloured. In the larger species no such regular pattern exists (Backlund, 1948; Nielsen & Christensen, 1959).

In the original description of Lumbricillus reynoldsoni (Backlund, 1948) the mucous cells of narcotised, Bouin fixed, sectioned material were described as either being empty or containing granules which stained with the Aniline blue and Light green components of the Mallory and Masson techniques.

Electron microscopy

At the ultrastructural level, apart from the work of Hess & Menzel (1967) on the epicuticular particles of Enchytraeus fragmentosus and that of Djaczenko & Cimmino (1973) where the

cuticle and epicuticular projection dimensions of Enchytraeus albidus were given, there would appear to be little detailed information about the enchytraeid epidermis other than the work of Goodman & Parrish (1971) on the ice worm Mesenchytraeus solifugus.

They, acknowledging the regretable dearth of literature dealing with the comparative morphology of oligochaetes, studied the ultrastructure of the epidermis of M. solifugus "in an effort to assess whether the de facto specialisation of metabolic function in the ice worm is attributable to structural peculiarities resolvable under the electron microscope".

The cuticle was shown to be basically like that of Lumbricus, with unbanded fibres, microvilli and membrane bound bodies (= epicuticular projections). The basement membrane was composed of fibres which, although striated, were not entirely similar to those described for lumbricids in the literature and, furthermore, were shown to be oriented in crossed lamellae.

No secretory (mucous) cells were described in the epidermal epithelium which was characterised by the presence of a large number of pigment granules which presumably conferred the melanism diagnostic of this species. It was concluded that the pigmentation was an adaptation to intense light rather than low temperatures.

In the conclusion of this paper Goodman & Parrish (1971) referred to their separate survey of oligochaete cuticle and dermal epithelium. This has not been successfully traced in the literature. Their survey was on Aeolosoma sp., Chaetogaster sp., Enchytraeus albidus, Dero limosa and Branchiura sowerbyi and they list epidermal mitochondrial dimensions and show those of M. solifugus to be smaller. All had epithelial microvilli and epicuticular projections and the

three largest species (not named, but presumably B. sowerbyi, E. albidus and D. limosa) had cuticular fibres like M. solifugus, the next to smallest (? Chaetogaster sp.) revealed only wispy filaments whereas the smallest (? Aeolosoma sp.) had no detectable fibres. In addition, but this time without even a size guide to the species concerned, they stated "in three of the five species the epithelium consisted of a single layer of cells." The condition in the other two species was not mentioned.

18 ENCHYTRAEID HISTOLOGY

In broad terms the architecture of the epidermis of the species of Lumbricillus is like that of the Lumbricidae and consists of a cuticle covering an epithelium in which both secretory and non-secretory cells can be observed. However, points of difference occur. At the light microscope level only two types of secretory cell are recognised and when the histological appearance is linked to the histochemical findings these would seem to be very different from the lumbricid types. Also, the ratio of these two types varies with the species.

Prominent in the epithelium of L. mirabilis, slightly less so in L. rivalis and comparatively rare in L. reynoldsoni is a cell type the secretion of which is in the form of prominent granules, though some coalescence may be observed which results in small lengths of strand-like material. The secretory material (see Section 20) is strongly metachromatic and alcianophilic (Plate 43a, b) and therefore this cell type, although granular, is not homologous to the large granular cells of the lumbricids where the secretion was shown not to be metachromatic (Section 6).

The second type of secretory cell appeared virtually empty in material fixed for light microscopy, whereas 1 μm resin sections of the material processed for electron microscopy gave the impression of globular rather than granular contents. Again there was numerical variation with the species, this cell type occurring at a lower frequency than the metachromatic type (Plate 43a, b) in L. mirabilis, at the same or slightly higher frequency in L. rivalis and was the dominant secretory type in L. reynoldsoni. Also in

L. reynoldsoni the globular nature of the secretion was well preserved in material processed for electron microscopy and in the 1μm resin sections the central condensation of the material within each globule in the cell was prominent and strongly orthochromatic with TB. So it would seem that the histological appearance of these non-metachromatic cells is very different from the lumbricid situation.

The findings concerning L. reynoldsoni are not entirely consistent with those of Backlund (1948) in the original description of this species. Her light micrographs, of low magnification, showed an almost equal distribution of the empty and the granular types in the anteclitellar region.

No small granular type cells were seen at the light microscope level.

The other striking feature of the epidermis, especially in L. mirabilis and L. rivalis, is the regular and patterned arrangement of the secretory cells within the epidermis (Plate 43a, b). Superficial longitudinal sections show the gland cells to occur in rows around the animal, as stated in Section 17. When seen in truer L.S. than Plate 43a, b it can be detected that the epidermis is of uneven depth throughout any one segment, being almost twice as deep in the areas housing the mucous cells as in the regions where only supporting cells occur. High magnification showed that the circular muscle fibres do not form a continuous area within the segment as they do in lumbricids but are present as a series of small units situated directly under the supporting cells, being absent from the areas where the mucous cells occur. Such an arrangement is more effectively seen at the electron microscope level and will be more fully described there (Section 22). In

the larger L. reynoldsoni the secretory cells are not arranged in transverse rows, a feature noted in the original species description (Backlund, 1948).

No cells of the granular metachromatic type were observed in the epidermis of Fridericia, Mesenchytraeus or Enchytraeus. The sole secretory cell type appeared badly fixed in light microscope material, the contents not being well retained, and seemed equatable to the non-metachromatic mucus type of Lumbricillus.

19 ENCHYTRAEID CUTICLE HISTOCHEMISTRY

i) Results

The histochemical results for the cuticle of L. mirabilis are presented in Table XII, L. rivalis and L. reynoldsoni showing no differences. In general, the reactions are similar to those given in Table I for the lumbricid cuticle and the arguments for the conclusions that have been drawn will not be repeated (see Section 5).

The neutral mucopolysaccharide component is seen to respond to manipulation in exactly the same way as in the lumbricids and in the AB-PAS sequence the cuticle was PAS positive, indicating the predominance of periodate reactive material (Plate 43a). The generalised techniques for acid mucus gave results as for the lumbricid material. However, at the detailed level, four points of difference emerged.

There is stronger evidence of the acid mucosubstance component containing some sulphomucin than in lumbricids. The failure of the methylation-saponification sequence to fully restore TB metachromasia or alcianophilia was suggestive of the acid mucus not being wholly of the carboxylated type, and the AB at pH 1.0 technique gave a strong turquoise colouration indicative of the presence of sulphomucins. In the absence of manipulative techniques, not too much weight should be given to the Ald.F results (Plate 43b) as necessarily indicating sulphated mucosubstances. However, the deamination-AB sequence would seem to argue against the presence of sulphated groups as these are generally associated with amino groups in sulphomucins, and thus would be expected to be no longer available after deamination.

The Az. A results were unexpected. Whilst other tissues within the section responded, the technique failed, at any pH within the range 4.5 - 1.0, to give any positive staining at cuticular sites. Sulphated mucins retain staining properties well below pH 3.0, and most carboxymucins have their extinction point between pH 3.0 and 4.0. The question of masked azurophilia is a possibility. This is generally due to protein masking and although cuticular protein was not convincingly demonstrated other than by the WRF technique it remains as a possible explanation for these negative results.

The failure of hyaluronidase, even after 48h, to abolish TB metachromasia or alcianophilia points to the absence of a hyaluronic acid component susceptible to testicular hyaluronidase.

Whereas in the lumbricid cuticle study it was possible to detail differences between the bulk of the cuticle and the outer epicuticular region, it has not been possible to make such distinctions with the same degree of certainty in the lumbricillids. This might be due to the small size of the animals, especially L. mirabilis, and the thinness of the cuticle. Even in longitudinal sections cut at 3 μm the problem of the body curvature within the section thickness exists, and this might be responsible for the slightly more positive reaction observed at the free edge in some of the PAS techniques. Certainly, focussing alters the impression as to whether a definite epicuticular reaction is detectable or not.

The cuticle of the species of Lumbricillus studied histochemically is therefore such that it displays the presence of periodate reactive, diastase-resistant, vic glycol-containing neutral mucosubstance, and an acid mucus component which is not wholly of the carboxymucin type, some reactions pointing to the presence of a sulphomucin constituent.

The reactions of the other enchytraeid genera to the routine techniques of PAS, TB and AB were the same as those recorded for Lumbricillus.

ii) Discussion

Little can be profitably argued concerning the taxonomic or ecological significance of the presence of a not wholly carboxylated acid mucin component in the cuticle of the lumbricillid enchytraeids when compared with the lumbricid earthworms. But the absence of enzyme-susceptible hyaluronic acid again raises the question, discussed at length in the lumbricid section (Sections 5 and 7), of the significance of its presence as a viscosity factor. It was argued that its presence in the lumbricid cuticle might confer on the matrix such properties as would allow for efficient yet controlled flexibility within the cuticle. A lumbricillid such as L. mirabilis is extremely small and very flexible when compared with any lumbricid and its cuticle fibres are to be shown to be of much smaller cross-dimension (Section 23). It is conceivable that within such a fibre system there is less need for a viscous matrix, with the consequence that hyaluronic acid does not occur.

20 ENCHYTRAEID METACHROMATIC MUCOUS CELL HISTOCHEMISTRY

i) Results

This cell type was only observed within the genus Lumbricillus in the present enchytraeid survey (Plate 43a, b). The histochemical results of L. mirabilis are given in Table XIII and those of L. rivalis and L. reynoldsoni were similar, though some variation in the substrate digestion rate by the uronic enzymes was observed.

The results show the secretion to be of the carboxylated acid mucus type with identifiable uronic groups, and to contain a neutral mucosubstance moiety possessing demonstrable vic glycols. The protein moiety of the complex was not very convincingly exposed by the limited techniques employed, and the lipid technique gave negative results. The arguments for these histochemical conclusions are those detailed in the lumbricid Sections and will not be repeated.

The results of interest are those of the enzyme studies identifying uronic groups. Both testicular hyaluronidase and β-glucuronidase were employed, with differing effectiveness. Within the time limits of 2-6h given by Spicer, Horn & Leppi (1967) and Leppi & Stoward (1965) for hyaluronidase the enzyme was not effective in lessening or abolishing metachromasia or alcianophilia. Fullmer (1960) had, however, used an exposure time of 24-48h in his vertebrate mucosubstance studies. The present results show that prolonged exposure, although not abolishing the staining reaction used as a test, caused some breakdown of the secretion resulting in a weak staining reaction. β-glucuronidase was used

over the time scale suggested by Fullmer (1960) and showed partial digestion of the substrate after 24h and total negation of staining after 48h. The combined results of these two enzymes suggest strongly that the reactant group in the acid mucus component is a glucuronic one.

ii) Discussion

The enzyme results raise two points: the question of employing vertebrate enzymes to degrade invertebrate substrates of unknown biochemistry; the apparent lack of complete specificity of testicular hyaluronidase.

With the wide ranging functions ascribed to mucosubstances and their abundant distribution throughout the animal kingdom it is to be expected that chemical diversity might exist. Whereas it is easy to categorise mucosubstances as acid or neutral mucins, it is unlikely that more precise delineations would reveal exactly identical molecular complexes throughout the animal kingdom. Yet it is at this precise level that enzyme histochemistry operates. Negative results merely allow one to state that the substrate was not susceptible to a particular enzyme from a particular source but such results do not necessarily exclude the possibility of the existence of a fairly closely related substance, the molecular configuration of which is such that enzyme digestion, within the limits of exposure, is not successfully achieved.

Equally, positive results cannot be regarded as being unequivocal in the light of the known action of testicular hyaluronidase. This enzyme not only attacks hyaluronic acid but also chondroitin sulphates A and C (Stacey & Barker, 1962). Many of the claims for the presence of hyaluronic acid in invertebrates

have been based on unmanipulated metachromasia, alcianophilia or Hale's technique and the use of testicular hyaluronidase, and the substrate might equally have been a sulphated chondroitin. This would appear to be the case in the isopod work of Steeves (1965) where, in fact, pH extinction was also used and gave strong metachromasia at pH 2.8, well below the extinction point of hyaluronic acid and other carboxymucins.

In the present results there was no firm indication of sulphated mucus being present and this would appear to support the conclusion that the testicular hyaluronidase was affecting, though not totally degrading, uronic-containing material. Indirectly the absence of sulphated mucus strengthens the conclusion that a separate neutral mucus moiety exists, since chondroitin sulphates are also PAS positive and when present make the interpretation of any PAS positive results difficult.

The difference between the effectiveness of hyaluronidase and β-glucuronidase is difficult to explain, since the reactive group in hyaluronic acid is, in fact, the glucuronic acid unit of the repeated disaccharide sequence. Although our knowledge of the chemistry of hyaluronic acid stems from isolated vertebrate material, certain invertebrate sources such as Bombyx mori have been shown to resemble very closely authentic vertebrate hyaluronic acid in the saccharide component and to have ratios of glucuronic acid to saccharide approximating to those of vertebrate sources (Nishizawa, Yamaguchi, Handa, Maeda & Yamazaki, 1963).

Because testicular hyaluronidase has a wider substrate spectrum than the literature ascribes to β-glucuronidase, the molecular configuration of these two enzymes might well be sufficiently different as to cause the observed less successful

degradation of the uronic type carboxymucin in L. mirabilis by hyaluronidase.

Biochemical studies on the secretion of Lumbricillus would certainly provide more detailed chemical information about the metachromatic cell product but the practical difficulties raised in Section 14 concerning lumbricid secretion would operate here too, though the absence of dorsal pores (apart from the cephalic pore) in the genus Lumbricillus would eliminate one source of sample contamination.

The use of an invertebrate source hyaluronidase (not commercially available) might have produced less equivocal results. The presence of hyaluronidase has been demonstrated in many insect orders, nematodes and leeches where, in Hirudo medicinalis, its specificity appears to be greater than vertebrate source hyaluronidase (Hunt, 1970).

The neutral mucopolysaccharide results can be related to a distinct neutral mucosubstance within the complex, since uronic acid-containing acid mucopolysaccharides do not show PAS positivity. The techniques employed have not enabled molecular configurations within the complex to be established and so speculation regarding the relationships between the moieties cannot be usefully made.

Vertebrate hyaluronic acid is often in combination with protein, though the manner of the association is obscure. The histochemical identification of protein within a complex in which hyaluronic acid is identifiable does not necessarily indicate a close relationship within the complex. Conversely, the absence of protein cannot be assumed purely on grounds of failure of histochemical techniques to expose it, as the possibility exists that the linkages between the protein and acid mucopolysaccharide moieties

may be such as to exclude the dye-reactive groups from participation in the protein technique. Amino acids have been recorded in the hydrolysates of hyaluronic acid-containing secretions of invertebrates including polychaete worm tubes (Defretin, Biserte & Montreuil, 1949) but the degree of association with the acid mucopolysaccharide was not established. The techniques of Nishizawa, Yamaguchi et al. (1963) on Bombyx mori were such as to point to a strong association between the protein and hyaluronic acid moieties in this insect.

In the present study the protein results were feeble and the evidence for suggesting that the bonding between the protein and acid mucopolysaccharide may be such that dye reactive groups are blocked to such an extent that only weak reactions result is slender.

Arising from this study are several, perhaps interrelated, points which merit some consideration. These are: the possible function of a uronic acid type mucus; the absence of such a secretion in genera other than Lumbricillus; the metachromatic cell frequency differences in the species of this genus recorded earlier (Section 18); the differences in habitat of the selected lumbricillids; the size range of the chosen species.

The lubricative functions and viscosity properties of hyaluronic acid-containing complexes have been discussed earlier (Sections 5, 7) and it is difficult to reason, on these alone, the metachromatic cell distribution in terms of species ecology and size. Mucins are said to act as buffer defence mechanisms (Pease, 1966; Pinter, 1967), acid mucopolysaccharides are thought to act as selective ion barriers (Kantor & Schubert, 1957) and uronic acid-containing mucosubstances are stated as being

hydrophilic (Varute & Nalavade, 1970).

The spectrum of possibilities, in terms of the enchytraeids, would therefore appear to be dehydration prevention and, or, some involvement in the osmoregulatory mechanism. The former would seem to be unlikely in that the greatest anti-desiccation need must operate in the terrestrial forms and these have no metachromatic mucous cells. Equally, within the genus Lumbricillus the evidence is against such a function: mirabilis lives mainly on wrack beds and would receive some protection against water loss by virtue of this habitat; rivalis, on sewage filter beds, has no desiccation problems; reynoldsoni, however, lives in shingle above the weed line and might well face greater dehydration difficulties than the other species, yet has fewer metachromatic cells.

The possible osmoregulatory function of the metachromatic mucus is also difficult to argue, in detail, in the absence of full experimental data. L. mirabilis and reynoldsoni both occur in an environment in which they are exposed to wide extremes of salinities. Tynen (1969a) has shown that L. reynoldsoni has little or no means of controlling the passage of water in and out of the body and that its distribution near the top of the shore is partially, if not wholly, due to its preference for low salinities. Nothing is known about the water control or salinity preferences of L. mirabilis. It is not inconceivable that the hyaluronic type of secretion could play some role in the life of this enchytraeid where, because of its very small size, when compared with reynoldsoni, it is permanently exposing a relatively large surface area to an ever changing environment. But an argument based solely on size does not entirely hold in the light of the comparatively high frequency

of the metachromatic cell type in the medium sized rivalis which is exposed to a relatively constant environment of 'fresh' water on primary sewage filter beds. Nevertheless, it too must have osmoregulatory problems which a barrier film of hyaluronic type acid-containing mucus might help to alleviate. This argument, however, is not strengthened by the situation in E. albidus. This species is known to occur on sewage beds and also in shingle, alongside L. reynoldsoni, (which it approaches in size), yet has no secretory cells of the hyaluronic acid type.

Until more physiological data are available on water relations within these enchytraeids the present evidence can do little more than state the facts and draw attention to the factors, which may or may not be interrelated, which operate on these animals.

One further possibility concerning the mucous cell frequency in Lumbricillus, and an easier one to test, must be considered. The seasonal variation in the state of the secretory cells in the leech Glossosiphonia complanata recorded by Gondran (1954) has been noted in Section 14. The cells involved were of the metachromatic type and a build-up in numbers occurred prior to sexual reproduction.

The collection of enchytraeids for this survey was carried out during very limited periods of the year. L. mirabilis (and georgiensis and vancouverensis which, from electron microscope studies show the same frequency) was collected in October and December 1973, that is, during their reproductive season (Tynen, 1969b), though most of the specimens processed were not fully clitellate. L. reynoldsoni was collected during August 1974, as were L. rivalis and the other enchytraeids of this study. Several specimens of L. rivalis were clitellate at that time,

but no clitellum was observable in the specimens of reynoldsoni which, like rivalis, normally breeds in the colder half of the year. Little is known about the regression of the clitellum in enchytraeids and that of rivalis showed no signs of regression at the light microscope level. So it would appear that the species in which a high frequency of metachromatic cells has been recorded were collected either during the breeding season or at a time when at least some specimens were clitellate. The specimens of L. reynoldsoni examined by Backlund (1948) were clitellate and her light micrographs of an anteclitellar region showed almost equal frequency of the empty (non-metachromatic ?) and granular (metachromatic ?) forms. Without systematic sampling throughout the year further comment is not fruitful.

Irrespective of the cell frequency, only Lumbricillus of the four genera studied possesses metachromatic cells. The genus Lumbricillus is primarily littoral and is thought to have always been so (Christensen, 1961). That the occurrence of acid mucous cells is a generic character is a possibility, and one which is supported by their absence in E. albidus which shares the same ecological habitat as L. reynoldsoni and, on filter beds, L. rivalis. This weakens any argument raised to account for the acid cell type in ecological terms, though the evidence of the terrestrial species of Fridericia and Mesenchytraeus, where it is absent, would support it, and perhaps strengthens a generic one. Two further comments about E. albidus and F. callosa would seem appropriate, since both fall into ill-defined ecological categories. Although E. albidus is referred to as being terrestrial, it might be more accurate to say that white, terrestrial enchytraeids have been referred to as E. albidus, and there is little reliable documentation

of its occurrence at sites other than littoral ones or sewage filter beds. Any speculation involving a secondary migration to the littoral habitat from a terrestrial one, and so possibly accounting for the absence of metachromatic cells, is not borne out by its cuticle depth (Table XIV and Section 23) unless this had become decreased as a result of such a migration. The ecological classification is F. callosa is not straightforward. Tynen (1972) regards it as a littoral species, but it occurs well above stations at which L. reynoldsoni and E. albidus are found and is characterised by an extremely thick cuticle typical of the terrestrial species of this genus. Nielsen & Christensen (1961) do not regard it as littoral, finding it only in sandy meadows along the coast, and Ude (1901) recorded it at great distances inland.

Further discussion concerning the functional significance of the metachromatic cells within the genus Lumbricillus must await physiological data, but a more widely based survey of this genus, which contains sixteen good British species and four 'species dubiae' (Tynen, 1966) might prove profitable in establishing whether the possession of such a cell type is a generic character.

21 ENCHYTRAEID NON-METACHROMATIC MUCOUS CELL HISTOCHEMISTRY

i) Results

As stated in the histology section (18), this cell type nearly always appeared empty in light microscope fixed material. Occasionally the secretory product gave an extremely weak positive reaction with PAS and an equally weak HgBPB result. These two techniques, when applied to the 1 μm resin sections of the lumbricillids were again weak, as they generally are on resin sections even with prolonged timings, but in L. reynoldsoni, where the frequency of this cell type is high, were convincing enough, together with the TB orthochromasia of the central condensation within each globule, to enable identification of both a neutral mucopolysaccharide and a protein component. The results were such that further manipulations of these techniques were not undertaken. All tests for acid mucosubstances were negative.

ii) Discussion

The results would suggest that this cell type is involved in the production of a neutral glycoprotein, but were so feeble and erratic that further definition of the complex is not possible. The absence of further manipulations has led to the inability to state, with certainty, that this cell type is chemically identical throughout the species studied. Although the cells in resin sections of L. reynoldsoni showed strong orthochromasia sufficient to justify categorising them as orthochromatic cells, this was not consistently the case with the smaller lumbricillid species. For

this reason and the possibility that in the other genera the secretory cells grouped under this heading may not be of exactly the same type, it has been decided to refer to them as the non-metachromatic mucous cells, as this negative character is common to all of them and avoids the drawing of possibly unjustified parallels with the lumbricid situation.

22 ENCHYTRAEID EPIDERMAL ARCHITECTURE: ULTRASTRUCTURE

The enchytraeid epidermis differs from that of the lumbricid epidermis in five major aspects: the very unequal height of the cellular components in some species; the ratio of cuticle depth to supporting cell depth; the presence of a distinct intersegmental cell type in some species; the nature and arrangement of the layer subtending the epithelium; the frequency of the supporting cell mitochondria.

Whereas in the lumbricids the height of an epidermal cell depends on its position within the segment (Section 4), that of an enchytraeid epidermal cell, in a small species such as L. mirabilis, depends on the nature of the cell type and its position relative to the circular muscle fibres. Plate 44 shows the separate muscle units and the presence of the non-secretory, supporting cell type immediately external to them. In the areas between the circular muscle units are the secretory cells, the basal regions of which contact the basal lamina external to the longitudinal muscle fibres. Occasionally a secretory cell is found at the base of the 'slope' formed by the circular muscle fibre, in which case the cell is in contact with the basal lamina immediately above the circular muscle. Such arrangements result in the mucous cells occurring in rows as recorded in Section 18. Plate 45 is a low power electron micrograph of a superficial L.S. of L. mirabilis showing the lines of secretory cells and the intervening muscle fibres of the circular muscle system. A similar situation exists in L. rivalis (Plate 46), but in the larger L. reynoldsoni a much less exaggerated pattern exists. Here the depth of the epidermis is greater (see Table XIV), even at its narrowest points immediately above the circular muscle, and the gland cells

are not restricted to the regions between the muscle units and so have the irregular distribution noted earlier (Section 18) and by Backlund (1948). In the small species of the other genera the circular muscle is also split into units resulting in epidermal cells of varying heights, and again no secretory cell type is found immediately above the apex of a muscle unit. These observations suggest that a minimal epidermal depth is necessary for the housing of the gland cells, as it was in lumbricids (Section 4), and that in small or medium sized enchytraeids this depth obtains only between the muscle units, so resulting in regular rows of secretory cells.

Table XIV lists cuticular details of the enchytraeids under survey and two points emerge: the range of cuticle thickness, and the ratio between the cuticle and the supporting cell depth immediately external to the circular muscle. It is recognised that any variation in fixation contraction will affect these ratios and measurements when used in a comparative way.

In the littoral forms (excluding F. callosa) the cuticle depth is proportional to the size of the species, and purely on grounds of cuticle depth it would seem that the specimens of E. albidus examined were typically littoral. The terrestrial forms, Mesenchytraeus and Fridericia including F. callosa, are characterised by thick cuticles which can be regarded as ecological adaptations to the terrestrial environment. When compared with lumbricid species possessing similar cuticle depths (Table IX) the body size differences are very apparent. It is perhaps the small size, and its attendant surface problems, of the enchytraeid species that has necessitated a greater cuticle depth than is found in many lumbricids of much larger size.

The details of cuticle depth and the ratio of cuticle to epidermal supporting cell height at its narrowest point presented in

Table XIV show that the effect of small size operates much more on the height of the epidermis than on the thickness of the cuticle, the small species having a supporting cell depth of as little as 1 - 2 μm at its shallowest point and L. reynoldsoni of 8 - 9 μm.

Plate 47 is a superficial L.S. of L. mirabilis and shows the presence of a narrow, highly granular intersegmental cell type. This cell type is approximately 0.80 - 0.85 μm wide and the granules are, on average, 0.3 μm in diameter. Similar cells of like dimensions were observed in L. georgiensis and vancouverensis. Plate 48 shows this cell type in section in the larger L. rivalis where the cell width was 1.2 - 1.4 μm but the granule size was approximately the same as for the small littoral species. This cell type was not observed in the light microscope paraffin study and in the absence of histochemical information nothing can be said about the nature of the granules or their function.

Below the lumbricid epidermis is an amorphous basal lamina and below this the layer of banded collagen fibres which form the basement membrane of light microscope studies. The lamina and fibre layer form a single continuous sheet following the body contour and is penetrated by the blood capillaries and nerve fibrils which enter the epidermis. The material subtending the enchytraeid epidermis differs from this in its size, ultrastructural nature and topology.

It is approximately 150 nm deep and is amorphous, there being no collagen fibre components present in any of the enchytraeids studied, and appears equatable to the basal lamina proper of lumbricids. Because of the comparative thinness of the circular muscle layer in enchytraeids and its prominent fragmentation into discrete units within each segment, the basal lamina follows a sinuous course outlining the muscle arrangement (Plate 44). A basal lamina of similar

size and fine structure exists between the circular and longitudinal muscle coats and at points where the circular muscle is absent it is this basal lamina (external to the longitudinal muscle) that subtends the epidermis (Plate 44).

The occurrence of two laminae was recorded by Goodman & Parrish (1971) for Mesenchytraeus solifugus, but in the ice worm the upper lamina (termed basement membrane by these authors) was fibrous and consisted of 10-18 cross-lamellae each several fibres deep, the individual fibres of each lamella being banded at a periodicity of 70Å. From their micrographs this layer appears slightly thicker than the cuticle and is calculated to be just over 1 μm, the cuticle being approximately 0.9 μm. The second lamina, between the muscle coats, was much thinner (no pictures or dimensions given) and contained 2-4 lamellae. The species under review in this present study did not reveal such size disparity between the layers or such ultrastructural detail, all of them showing two equally deep amorphous layers. Goodman & Parrish (1971), having only lumbricid literature with which to make comparisons, did not associate this arrangement in M. solifugus with the habitat and life pattern of this enchytraeid.

Very prominent in the supporting cells of L. georgiensis, mirabilis, rivalis and vancouverensis are mitochondria (Plates 48, 55a). These organelles feature less obviously in L. reynoldsoni and E. albidus, and in the terrestrial forms they occur at a lower frequency and one which approximates to the condition in the supporting cells of lumbricids.

It is immediately admitted that the above statements are subjective and are made without any statistical backing, morphometric techniques not having been applied to the micrographs and the tissue blocks not having been sampled with this in mind.

Measurements of the mitochondria show them to be of a width (250 - 300 nm) well within the range given by Goodman & Parrish (1971) for a selection of oligochaetes, but during the examination of the material in the electron microscope and from a study of micrographs an impression was gained that they were of greater length than those observed in the lumbricid epidermis. It is acknowledged that this might be due to the enchytraeid cells being of smaller size and therefore there being a greater chance of the section plane passing along that of a mitochondrion. Plate 49 shows one such mitochondrion in L. mirabilis measuring at least 3.5 µm in length, and it is conceivable that the portions of mitochondrion above it may belong to the same organelle, the intermediary portions not being in the plane of the section. Such a length greatly exceeds the upper range dimension of 1.1 µm given by Goodman & Parrish (1971) for one in the tubificid Branchiura sowerbyi.

The invertebrate literature reveals little concerning the numbers of mitochondria associated with particular tissue types, or required by a cell of a certain size for adequate functioning. In vertebrate studies Berger (1973) has produced data to suggest that the numbers of mitochondria of the two morphologically different types in rat liver cells vary so as to maintain a relatively constant mitochondrial volume/cytoplasm relationship within the cell gradient of the tissue. Whilst recognising that the supporting cells of L. georgiensis, mirabilis and vancouverensis are of small size it cannot be argued, in the light of the lower frequency in the terrestrial species with cells of comparable size and an equal frequency in L. rivalis where the cells are much larger, that small cells per se have or require a higher energy output and therefore possess more mitochondria. No obvious mitochondrial/cytoplasm relationship appears to exist in these enchytraeids.

Nevertheless, the reported distribution, although not substantiated statistically, leads to speculation concerning a possible active transport function of the epidermis in the small littoral forms and the freshwater form studied. It is thought probable that an active salt uptake through the skin occurs in Lumbricus terrestris when in fresh water (Dales, 1967) and this might well be the case in these enchytraeids. However, it is precisely these forms that possess a type of mucus, rare in L. reynoldsoni and absent from the terrestrial species and E. albidus, which might be involved in osmoregulation (Section 20). In the absence of facts other than the present histochemical and ultrastructural ones it is not thought profitable, at this stage, to develop hypotheses involving a possible barrier and pump mechanism in those enchytraeids with a hyaluronic acid-containing mucus and numerous supporting cell mitochondria.

The integrity of the epidermis is maintained, as in the lumbricids, by several ultrastructural features which, by virtue of the small size of the enchytraeids, are more easily observed in their entirety in this microdrile family.

The intimate attachment of the epidermis to the cuticle and to the basal lamina will be discussed in Section 23. Lateral integrity is achieved by means of junctional complexes and the intercellular bridges (septate desmosomes) are prominent apically (Plate 64). Below this region extensive interdigitation characterises the mucous cell-supporting cell junction (Plate 59). This is clearly seen in the cross section through this sub-apical region in Plate 50.

23 ENCHYTRAEID CUTICLE ULTRASTRUCTURE

i) Observations

At the ultrastructural level the cuticle of all the enchytraeid species examined has an organisation similar to that of the lumbricid earthworms (see Section 10) (Plate 51a).

The collagen fibres are unbanded and are arranged in an orthogonal cross-lamella pattern. Whereas the number of fibre layers was porportional to the size of the worm in lumbricids, Table XIV shows that this is not the case in the enchytraeids, L. reynoldsoni, for example, having fewer than the much smaller L. mirabilis. The overall depth of the cuticle has already been discussed in Section 22 and a comparison between L. mirabilis and F. callosa shows that the thicker cuticle of the latter has, in fact, fewer fibre layers than the considerably thinner cuticle of L. mirabilis, but the fibre thickness measurements (Table XIV) show that the fibre size is four times greater in F. callosa than in the lumbricillid. Even when the cuticle depth is similar, differences occur. L. georgiensis and vancouverensis have fewer fibre layers, each of greater size, than mirabilis, although all three have approximately the same body size and occur in the same ecological situation.

The microfibrillar matrix of the enchytraeids can, as with that of the lumbricids, be divided into the four zones described in Section 10, with the exception of the truly terrestrial F. bulbosa and Mesenchytraeus sp. In these two species the outer electron dense zone - the surface zone - is not apparent in most of the tissue blocks sampled, nor is the pale zone which subtends it in other enchytraeids and lumbricids. In place of these two zones is a vaguely triple

layered area with the middle portion slightly less electron dense than the other two (Plates 51b, 77b). The measurements of the surface and pale zones for the other enchytraeids are given in Table XIV.

Microvillar extensions from the epithelial surface occur as in the lumbricids, each arises from an ovoid base which possesses a pair of shoulders (Plate 52a, b). From each shoulder an arc of microfibrils arises and terminates among the fibrils of the basal zone of the cuticle (Plates 52a, 53a). The fine structural detail of the shoulder is as that described for the lumbricids (Section 10) and the prominent tonofilaments extend into the epidermal cells. Whereas it was presumed that these might extend the full depth of the epidermal cell and terminate in the hemidesmosomes at the base of the cell in the lumbricids, it was not convincingly demonstrated that they did so, because of the depth of the cell and the very slight chance that the section plane would lie exactly parallel to the course of a single tonofilament bundle. In the enchytraeids the epidermal cells are much shallower and Plate 53a shows a bundle of tonofilaments running from a shoulder to the cell base. The ascending microvilli of the enchytraeids also contain prominent tonofilaments (Plate 53b) 10 nm in diameter, and the microvilli decrease in diameter as they approach the free edge of the cuticle as in lumbricids (Section 10).

In contrast to these striking similarities at the fine structural level, it must be recorded that the microvilli appear to be less regularly arranged than in the lumbricids, many more 'gaps' existing in the pattern and, on occasions, more than one microvillus occurring within a fibre-space (Plate 53b). Where a regular array is present, the average distance between the microvilli of a row, in L. mirabilis,

is 170-190 nm and the distance between the rows 80-90 nm.

Epicuticular projections are a constant feature of the enchytraeid cuticle and Table XV lists their dimensions. In general they are slightly narrower and longer than those of the lumbricids and have a slight waist at one third of the distance from the top (Plate 54b). The definite substructure of two parallel electron dense discs recorded in the lumbricids (Section 10) has not been observed in the enchytraeids and the dense lining and pale core arrangement was only clearly observed in the terrestrial forms and F. callosa (Plate 54a).

The origin of the epicuticular projections from the exposed microvillar tips is as described for the lumbricids and is shown, together with a branching microvillus, in Plate 54b,c.

Trapped between the epicuticular projections, and extending a variable distance beyond them, are the fine strands of material forming the equivalent of the lumbricid supracuticular mucoid coat.

The cuticle is interrupted by the secretory cell pores, the lining of which is formed of material similar to the pale zone of the cuticle matrix. A circlet of mucous pore microvilli exists around the metachromatic pores in the species of Lumbricillus (Plate 62), as in lumbricids, but the mucous pore particles observed in the latter have not been detected in any of the enchytraeids.

Prominent in the cuticles of the species of Lumbricillus and in E. albidus are membraned structures 180-200 nm in diameter as shown in Plates 48, 55a, 73a. These lie between the collagen fibres (Plate 55b) and take their orientation. Even at high magnification (Plate 56a, b) no organised substructure can be ascertained, other than a less electron dense core. Plate 56a, b show such structures very close to the epidermal cells and suggest that they might arise from them. It has not been possible to follow one such structure for its entire length, but Plate 57a shows that this might be

considerable. Their distribution is not regular, some portions of some sections not containing any. This was not obviously relatable to any other somatic feature. The membraned structures were not observed in the cuticles of Fridericia or Mesenchytraeus.

ii) Discussion

The basic organisation of the cuticle is similar throughout the Enchytraeidae under survey and is seen to be like that of the lumbricids (Section 10) and the published reports of other oligochaete species (Hess & Menzel, 1967; Krall, 1968; Potswald, 1971; Goodman & Parrish, 1971; Djaczenko & Cimmino, 1973). It also resembles that of many polychaetes (Storch & Welsch, 1970; Michel, 1972) and at least one leech (Damas, 1969). A matrix housing the cross-lamella pattern of unbanded collagen fibres and ascending microvilli giving rise to epicuticular projections may therefore be considered as annelid features.

The greater thickness of the cuticle in the genus Fridericia is achieved by increased fibre thickness rather than by a greater number of fibre layers, and such an arrangement is almost certainly responsible for their 'stiff' movement when compared with the littoral lumbricillids of similar size. Enlarged fibre thickness cannot be automatically associated with the terrestrial habit since Mesenchytraeus sp. has a moderately thick cuticle composed of a large number of small diameter fibres of approximately the same size as those described in the ice worm M. solifugus (Goodman & Parrish, 1971).

The present results for E. albidus differ from the data published by Djaczenko & Cimmino (1973), which will be converted to nm units here for ease of comparison. They reported a mean fibre diameter of 400 nm in E. albidus, though the 6.25 and 18.5 nm periodicity

was only observed in fibres 80-100 nm thick. No fibres approaching 400 nm were seen in the cuticle of the specimens of E. albidus used for this present study. The largest, mid-cuticular fibres were about 200 nm in diameter. Two possible explanations for this apparent discrepancy can be put forward. The basis of the work of Djaczenko & Cimmino (1973) was their special fixation technique and this might conceivably account for these differences. However, arguing against this explanation is the fact that their lumbricid fibre measurements are of the same order as those of this present study where conventional fixation techniques were used (see Section 10) and the linear measurements of the epicuticular projections of E. albidus are the same as those given here (Table XV). It would be surprising if their fixation affected only the fibres of E. albidus. The other possibility is that their material of E. albidus was from a terrestrial source. They did not give location details, and because they did not state the overall cuticle depth nor show micrographs of the tissue, further comment cannot be made other than to point out thatTynen (personal communication) suspects that terrestrial forms named E. albidus might well not be the same species as the littoral forms of that name.

Since no functional explanation has been put forward for the dense surface zone of the matrix when it has been reported or depicted in micrographs by other workers (Goodman & Parrish (1971) on M. solifugus; Djaczenko & Cimmino (1973) on Tubifex tubifex) or when described in Section 10 on lumbricids, it is not possible to advance any ideas concerning the departure from this widely occurring detail in Mesenchytraeus sp. and F. bulbosa. Again, it may be the consequence of small size combined with a terrestrial existence, and would appear not to be a taxonomic feature since a very definite surface zone is reported for M. solifugus by Goodman & Parrish (1971) and one exists in F. callosa. However, the size and ecology interpre-

tation is difficult to sustain in the face of the details given for F. callosa, which is a species of small size, has a cuticle of 'terrestrial' thickness, and lives sufficiently high up the shore as to be much more a terrestrial species than a littoral one.

The ascending microvilli are similar to those of the lumbricids but for the less regular arrangement. This might be used to argue against their role in determining the cuticle fibre orientation as presented in Section 10. Although the distances between microvilli within a row are smaller than in lumbricids, the distances between the rows are within the lumbricid range, and this arrangement is again such that 540 nm long tropocollagen molecules would preferentially take up the orthogonal orientation. The occurrence of two or more microvilli very close together would not interfere with the pattern, but the large number of 'gaps', if present at the developmental stage, might be expected to have done so. However, the orthogonal pattern of fibres is regular, from which it might be suspected that either no 'gaps' were present during the embryological stages and that microvilli have been subsequently lost, or that the 'gaps' are randomly enough arranged as not to affect the general pattern of fibre orientation. The study provided no evidence of withdrawal or degeneration of microvilli as suggested by Potswald (1971) in Aeolosoma.

The epicuticular projection dimensions, whilst thinner and longer than those recorded for lumbricids, are similar in range to those given for other enchytraeids (converted to nm): E. fragmentosus 50 x 200 (Hess & Menzel, 1967); M. solifugus 50 x 180 (Goodman & Parrish, 1971); E. albidus - length 210 (Djaczenko & Cimmino, 1973); and for other microdriles: Dero 45 x 140 (Krall, 1968); Aeolosoma 30 x 100 (Potswald, 1971); Tubifex - length 160 (Djaczenko & Cimmino, 1973); and from observations, outside this present study, on the naids Pristina idrensis, Stylaria lacustris, Dero obtusa and the tubificid T. tubifex.

No complex attachments as described by Hess & Menzel (1967) for the epicuticular particles of E. fragmentosus (written Enchytraesus by these authors) consisting of four basal extensions which branched to give a fenestrated membrane (= surface zone) were seen in any of the enchytraeids under survey. Nor were the fine mucoid filaments seen to be arranged in six groups at four levels around the projections, as detailed by Hess & Menzel for E. fragmentosus (1967).

In all cases, including the lumbricids (Section 10), the projections are narrower than long (1: 2.5-3 in lumbricids; 1: 3-4 in enchytraeids) and this appears, from published micrographs and drawings, to hold for polychaetes and leeches too. Although no tested function has been ascribed to these projections in annelids it is tempting to consider the possibilities in terms of the very uniform shape which the literature and this study have revealed. A large number of small oblong pegs would provide a good anchorage for a permanently present supracuticular mucoid coat.

However, the apparent universality of the oblong shape is not maintained by observations, outside the range of this present report, on Stylodrilus heringianus belonging to the microdrile family Lumbriculidae which, together with the Haplotaxidae, is considered to be primitive within the Oligochaeta (Stephenson, 1930; Brinkhurst & Jamieson, 1971). Plate 57b shows the epicuticular projections of S. heringianus which are 170-180 nm wide and 220-230 long. They arise from the ascending microvilli, which are of the same order of size as in other microdriles, but become very wide and establish the complex substructure before finally being pinched-off from the microvillus. As yet, no explanation can be advanced for the possession of almost cuboidal epicuticular projections by this silt

dwelling, freshwater microdrile, but the complexity of its epidermal cells when compared with tubificids and naids make it a promising candidate for further study.

The membraned structures lying in the cuticle of all the species of Lumbricillus studied in this survey and in E. albidus present problems of interpretation. They are 180-200 nm in diameter and of indeterminate length and reveal no substructure to indicate their nature, origin or function. Despite the large numbers, or portions, observed, the condition in Plate 56b showing what might be their epidermal origin was rare. In size they closely approximate to that of the cilia of the epidermal sense organs and one possible explanation might be that they are degenerate cilia, remnants either of an early developmental stage, or of a ciliated ancestry.

Their occurrence within the genus Lumbricillus, which is recorded as being primitive within the family, might appear to support the latter speculation, but their presence in E. albidus, a genus which on morphological and cyto-taxonomic grounds is not regarded as primitive, fails to do so, and their absence in Mesenchytraeus sp. (and they were not recorded by Goodman & Parrish (1971) for M. solifugus), another primitive genus, does likewise. The literature does not reveal records of similar structures in other oligochaetes and it is therefore difficult to sustain such a phylogenetic argument for members of a family which has never been regarded as near the ancestral oligochaete stock (Stephenson, 1930; Christensen, 1961; Clark, 1969; Brinkhurst & Jamieson, 1971), unless these structures have acquired some secondary role within the cuticle which has allowed them to be selectively retained within these enchytraeids, and lost in all other forms.

That they are degenerate remnants of an early developmental stage is also difficult to argue fully, in the absence of fine

structural information regarding such stages in enchytraeids. It is not easy to envisage at what stage in the ontogeny such structures would have a role to play in the life of the animal. For them to have been functional epidermal cilia, later to be trapped by the cuticle and then degenerate, would necessitate their active life to be at a time prior to full cuticle formation. Presumably the cuticle is fully developed, as in the lumbricids, at hatching, and so it becomes necessary to argue for the possession of cilia during the early cocoon stages in the aquatic (littoral and freshwater) forms where they have been observed. At present, there is nothing known to support this. Also, if the degenerate cilia interpretation is to be sustained it must account for the retention, in disorganised form, of the distal part of the organelle and the complete disappearance of the basal portions from the epidermal cells. One would imagine that any degeneration and resorption process would involve both the proximal and distal portions of the cilia.

Some explanation of the erratic distribution of the structures within the cuticle is also demanded. Enchytraeids emerge from the cocoon with considerably fewer segments than are present in the mature individuals, newly hatched forms having twelve or fewer segments. There is an increase in segment number until maturity is reached (Nielsen & Christensen, 1959) though no detail appears in the literature as to how this is achieved. If embryological cilia had been trapped during cuticle development and post-hatching growth and segment formation was shown to occur along the whole length of the worm, then such cilia would have an irregular and unpredictable distribution in the mature worm, as was the case. On this basis, more sampling of tissue might reveal a more generalised occurrence of these structures in enchytraeids.

Until more information is available concerning the distribution of these structures within the family, and developmental studies have been undertaken, they must remain as recorded features of unknown function and origin.

24 ENCHYTRAEID METACHROMATIC MUCOUS CELL ULTRASTRUCTURE

i) Observations

These cells, occurring only in the genus Lumbricillus and showing a frequency variation described earlier (Sections 18 and 20), extend the full depth of the epidermis and lie in the areas between the circular muscle units in the smaller species (Plate 44). When distended with secretion the cells are ovoid in shape, the nucleus and secretory apparatus being located basally and the apical pore surrounded by pore microvilli (Plate 58).

The secretion is in the form of spherical or oval globules, each membrane-bound (Plate 59), but a distinct goblet cavity is not present and remnants of cytoplasm occur between the globules.

Secretory globule ultrastructure

The globules, in the five species of Lumbricillus, are characterised by a distinctive patterning consisting of strands of electron dense material, 40 - 50 nm wide, which form, in many section planes, a parallel arrangement giving rise to a fingerprint-like configuration (Plate 58). In other planes, a more honeycomb-like pattern is revealed (Plate 60). The matrix material between the strands is finely fibrous and of medium electron density and is similarly arranged in parallel areas, 50 - 60 nm wide, in the fingerprint formations. There is evidence of condensation of this matrix material at the globule edges resulting in an array of finely radiating fibrils (Plate 59).

The secretory process

The granular endoplasmic reticulum and polarised Golgi systems are located basally and appear typical of secretory cells (Plate 61a).

No electron dense areas are observable within the cisternae of the endoplasmic reticulum. The first manifestation of the patterning occurs towards the mature face of the Golgi apparatus, the mature face vesicles coalescing to form the large globules described above.

Action of pronase

Pronase treatment of ultrasections of L. mirabilis did not obliterate the patterning of the globules, though some 'smudging' was apparent and the electron dense strands were less well defined than in control material (Plate 61b).

Occlusor mechanism

Apically the metachromatic cells have a ring of 13 - 15 pore microvilli (Plate 62), these being supplied with tonofilaments.

In L. mirabilis, georgiensis and vancouverensis a distinct occlusor mechanism is present in the form of sphincter-like tonofilaments. These are arranged in two groups. The inner sphincter ring lies within the slightly thickened cytoplasmic lining of the apical region of the mucous cell, the top of the sphincter being 92 - 95 nm below the cell-cuticle interface and the sphincter being 90 - 100 nm deep. The ring consists of approximately 50 distinct filaments, each of which is 5 nm in diameter, a size which would classify them as microfilaments rather than tonofilaments (Helper & Palevitz, 1974). The outer sphincter ring lies within the adjacent supporting cells, two or three of which abutt on to each metachromatic mucous cell. The top of this ring is located 170 - 180 nm below the cell-cuticle interface and the filaments are again 5 nm in diameter. Plate 63 is a median longitudinal section through the pore region and both sphincters are seen in cross section. Plate 64 is a higher magnification of the sphincter region. Because the two

rings are not positioned at the same level, surface sections across this area have failed to show both sphincters in their entirety in the same section. Plate 65 is a very slightly oblique transverse cut through the occlusor region and shows the inner sphincter entire and the section plane is sufficiently angled as to reveal traces of the outer sphincter at one side. Plate 66 is a much more oblique cut, showing portions of both sphincters. It also reveals that the filaments of the inner ring are in contact with those that run vertically in the mucous pore microvilli. The outer sphincter is not continuous through the intercellular junctions of the supporting cells, but there is evidence that the filaments of this outer ring are in continuity with the vertically oriented tonofilaments of these supporting cells.

In L. rivalis only the inner ring, within the metachromatic mucous cell, is present and Plate 67 is an off-centre section through this region. The position, number and size of the filaments is the same as for this sphincter in L. mirabilis. The region of the surrounding supporting cells where the outer ring of L. mirabilis is positioned is devoid of tonofilaments. In L. reynoldsoni (Plate 59) there is no trace of sphincter tonofilaments in either of the two positions described for L. mirabilis.

Mucous cell control

No nerve terminals were observed abutting on to the metachromatic mucous cells, though the epidermis is rich in nerve fibrils, many of which lie close to the base of the secretory cells. Plate 68 is a deep surface slice of L. rivalis, taken at a level where the circular muscle units are in the section, and the intimate positioning of the epidermal nerve fibrils and secretory cells is clearly seen. At higher magnification (Plate 69), neurosecretory granules and

vesicles are apparent, but the latter are concentrated away from the mucous cell and towards the muscle.

ii) Discussion

The patterning of the metachromatic globules in the species of Lumbricillus revealed no detectable species variation as did that of the orthochromatic globules of lumbricids (Section 11). However, the ultrastructural details of the polarised secretory apparatus are similar in that the point of visualisation of the pattern is, as in the lumbricid orthochromatic mucous cells, at the mature face of the Golgi. It would therefore appear that only the final elaboration of the complex is susceptible to fixation in such a way as to produce the observed patterning. The failure of pronase to obliterate this patterning is consistent with the histochemical findings (Section 20) where the protein tests were weak.

The fingerprint-like patterning is very different from the ultrastructural representation of the metachromatic mucous cells in lumbricids (Section 12), the histochemistry of which has been shown to be different (Section 7) from that of these enchytraeids.

Fingerprint acid mucous cells have been described ultrastructurally by Kryvi (1972b) in Sabella, but the micrographs and text details show these epidermal cells to be different from those of Lumbricillus, the globules having a much finer and less pronounced pattern of dense components and a closer packing of the strands. His histochemical study did not define the nature of the acid mucus sufficiently for further comparisons to be made.

The coelenterate literature (Chaet, 1965; Philpott, Chaet & Burnett, 1966; Davis, 1973), however, contains histochemical and ultrastructural accounts of glandulomuscular cells in the basal

disk of Hydra which parallel the findings in the lumbricillid cells under discussion in three important ways.

Histochemically the secretion in Hydra was shown to be PAS positive, AB positive, metachromatic and sensitive, to some degree, to testicular hyaluronidase (Philpott, Chaet & Burnett, 1966; Davis, 1973) and the authors claim that this hyaluronic acid-containing mucus provides the adhesive agent which attaches the animal to the substrate. Thus, in histochemical terms, this secretion would appear to be similar to that of the metachromatic mucous cells of Lumbricillus.

At the ultrastructural level this similarity is maintained, the micrographs of Chaet (1965), Philpott, Chaet & Burnett (1966) and those of the Type III droplets in H. oligactis and H. pirardi (Davis, 1973) showing spherical or oval globules displaying alternating dense and less dense strands of material forming a fingerprint-like pattern.

Finally, Davis (1973) records, together with details of the longitudinally oriented myonemes present in the gladulomuscular cells of the basal disk, myoneme filaments oriented radially with respect to the gland pores and suggests that their contraction and relaxation may regulate the opening and closing of the pores. Although filaments of three diameters were described, the details of those associated with the pores are neither given nor illustrated.

The histochemical and ultrastructural similarities between the basal disk cell type of Hydra and the metachromatic mucous cell type of Lumbricillus are striking. They suggest that the chemical nature of the two secretions is sufficiently alike as to reveal, on fixation, similar ultrastructural representations, but it must be suspected that other differences, possibly physical ones, exist since the secretion in Hydra functions to form an adhering surface and this is clearly not the case in the enchytraeids.

The occlusor mechanism described in L. mirabilis, georgiensis, vancouverensis and L. rivalis is of interest firstly because no such mechanism was observed in any of the secretory cell types in lumbricids, nor has been recorded in secretory cells of other annelids, and secondly because of its less elaborate arrangement in L. rivalis and absence in L. reynoldsoni.

The arrangement of the tonofilaments is such that contraction would bring about a closure of the pore in a typical sphincter or purse-string manner, in the same way as the "contractile ring" microfilaments operate in dividing cells of echinoderms (Schroeder, 1969) and mammals (Schroeder, 1970). Presumably, however, in lumbricillids the filaments are also capable of opening the pore by their relaxation, a function not required of the microfilaments in dividing cells. Whereas it might be argued that the initiation of microfilament contraction in dividing embryo cells could arise from a pressure threshold being reached when the cell attained a certain size, such an explanation cannot be advanced for the lumbricillid situation. Although the cells involved in the sphincter apparatus are fully differentiated and have presumably reached their mature size, that is not to say that their shape remains constant. During locomotion epidermal adjustments have to be made and consequently variations in pressure at particular points within any one cell occur. To explain the functioning of the sphincters purely in these terms would be to link their controlling influence directly and solely to the locomotory activities of the animal. The present observations have not provided any direct ultrastructural evidence to support an idea of independent control of the sphincter filaments.

Millott (1948) demonstrated intracellular fibrillae at the light microscope level in the cells surrounding the intestinal gland

cells of Lumbricus terrestris and figured certain of these in continuity with a ring of like fibrillae at the cell apex which formed an occlusor ring around, but not within, the gland cell. His anatomical studies failed to reveal nerve endings within the surrounding cells but physiological studies led him to believe that gland secretion was under nervous control.

The order of size of the sphincter rings in lumbricillids is such that they were not observed at the light microscope level, but the ultrastructural information shows that a basic arrangement as described by Millott (1948) exists concerning the outer ring in L. mirabilis. The epidermal nerve net is well developed in lumbricillids and it is not inconceivable that impulses, on reaching the supporting cells, could operate on the supporting cell tonofilaments and so affect the outer, supporting cell sphincter in L. mirabilis. Since no structural system of connecting filaments exists between the outer and inner sphincter, and it is presumed that the two sphincters work synergistically, the state of the outer ring could be relayed to the inner ring by means of pressure changes.

Such a hypothesis might explain the control and co-ordination of the two sphincters in L. mirabilis, but the absence of an outer ring in L. rivalis necessitates an interpretation which must rely on more general pressure changes.

The part played by the mucous pore microvilli might well be supplementary to the functioning of the sphincters, in that when the mucous cell pore is open they serve as a physical barrier to excessive loss of mucus and may also act as a mucus 'spreading factor' as suggested by Kryvi (1972b) for the pore microvilli of Sabella. The apparent association of the mucous pore microvilli filaments and those of the inner sphincter could result in the microvilli altering

position depending on the pore aperture and thereby being more effective as a sieving apparatus when the pore is open.

The presence or not of sphincters and the extent to which they are developed in the metachromatic mucous cell type is shown to vary within the genus Lumbricillus, as does the frequency of this cell type. In Section 20 it was argued, on the basis of the nature of the secretion, that perhaps both the size of the species and the demands of the environment were interrelated factors. The same arguments could be advanced with respect to the degree of pore control evidenced by this ultrastructural study. The small L. mirabilis, georgiensis and vancouverensis, being exposed to widely fluctuating environmental conditions, perhaps require a more effective control mechanism than the larger L. rivalis which lives in surroundings that are more constant, and the even larger L. reynoldsoni where the cell type frequency is low. A suggestion linking mucus flow to environmental salinity is upheld by the facts recorded for certain migratory fish. A more complete understanding of the situation in the lumbricillids must await more broadly based ultrastructural studies and physiological data concerning the water relations of these forms.

The nerve fibrils, with their neurosecretory granules and vesicles, though in close topographical relationship to both the metachromatic and non-metachromatic cells (Plate 68), would appear not to be directly involved in the processes of mucus production and extrusion, since the discharged vesicles are concentrated away from the secretory cells and towards the basal lamina, beyond which lie the circular muscles. Such an orientation would seem to imply a sub-epidermally situated target, controlled from the epidermis.

Similar nerve fibrils with granules and vesicles were observed in lumbricid tissue, but never within the epidermis; they were

situated in the sub-epidermal connective tissue strands, often immediately below the basal lamina and collagen fibre layer. They, too, showed a polarised substructure, with the vesicles concentrated nearest the muscle tissue.

It would appear, therefore, that it is only in their positional siting that they differ in the two oligochaete families. This could well be a consequence of body size, which has already been seen to reflect itself in the circular muscle arrangement and the lack of a fibrous sheath subtending the basal lamina in enchytraeids. Within the economy so demanded by small size, it might be that any hormonal control of the circular body musculature is directed from the epidermal nerve plexus in enchytraeids, rather than from a separate sub-epidermal one as in the lumbricids.

25 ENCHYTRAEID NON-METACHROMATIC MUCOUS CELL ULTRASTRUCTURE

i) Observations

The frequency variation of the non-metachromatic cell type within the genus Lumbricillus has already been documented (Section 18) and Plates 45 and 46 show the distribution of this type in L. mirabilis and L. rivalis respectively. The secretory product is markedly different from that of the metachromatic cells, many of the globules appearing almost empty. The cells extend the full depth of the epidermis, have a basal nucleus and secretory apparatus, and occur in the areas between the circular muscle units in all but L. reynoldsoni. Because the non-metachromatic cells failed to show a consistent ultrastructural form in all the enchytraeid species under survey, the observations will be presented for each species.

In L. mirabilis the secretory globules, measuring 1.0 - 1.2 μm, have intact membranes and lie amongst cytoplasmic remnants within the secretory cell. The globule material is dispersed and has a flocculated appearance (Plate 70a). Nearer the basally located secretory apparatus the globules are smaller (0.6 - 0.7 μm) and the secretion is more evenly distributed within the globules (Plate 70b). In some cells the globules showed greater electron density and uniformity in these formative areas.

L. georgiensis and vancouverensis were similar to L. mirabilis and also showed the occurrence of small, denser globules (0.7 μm) near the base of the cell, together with larger globules in which the material was more dispersed.

In L. rivalis (Plates 46, 68, 71) the globules (1.2 - 1.4 μm) contain very little electron dense material, appearing either

uniformly electron pale or very finely flocculated. Occasionally sections contained portions of cells housing slightly smaller globules (1.0 - 1.2 μm) where the secretory material was a little more electron dense and uniformly distributed. However, cells containing both types of globules were not observed.

In L. reynoldsoni the non-metachromatic cell is the dominant secretory cell type in the epidermis (Plate 72a) and presented a consistent and characteristic form. The globules (1.3 - 1.6 μm) have intact membranes and the secretory material condensed into a single, electron dense sphere (Plate 72b), such spheres staining orthochromatically with TB in the 0.5 and 1.0 μm resin sections.

The main secretory cell in E. albidus has globules (1.0 μm) with intact membranes and finely dispersed contents (Plate 73a), even at the level of the mature Golgi face (Plate 73b) though the Golgi lamellae show a greater electron density than the vesicles of the mature face. Also present, and shown in Plate 73a, are cells with smaller globules (0.7 μm) of uniform and medium electron density. No intermediates between these two types were observed.

Secretory cells were not abundant in Mesenchytraeus sp. but both cells with flocculated globule contents (globules 0.9 μm) and ones with denser condensations within the globule limit (0.65 μm) were observed. No intermediates were seen.

Material of F. bulbosa showed good fixation of the cuticle and muscle but the epidermal cells appeared very distorted. However, secretory cells with homogeneous globules of medium electron density and measuring 0.5 μm were observed.

In F. callosa two types of secretory cell occur and Plate 74 shows both the type with flocculated globules averaging 0.7 μm and the type with more uniform contents housed in globules of about 1.0 μm. No cell containing both types of globule was observed.

ii) Discussion

The ultrastructural study of the non-metachromatic secretory cell type has shown variations within and between species, and in the absence of detailed histochemical findings it must remain a possibility that the cell types described are not, of necessity, of exactly the same chemical type.

The flocculated appearance of the globule material in many of the species might suggest that the secretion, in life, is highly hydrated and that the fixation and processing is such as to remove much of the secretion leaving the dispersed fragments seen in this study. Light microscope processing caused a similar loss of product (Section 18) and rendered this cell type unsuitable for detailed histochemical investigation (Section 21).

The ultrastructural appearance of the non-metachromatic cell type in L. reynoldsoni was unlike that of any of the other species of that genus, the material condensing into a single sphere and no flocculation being observed. The globule size was larger than in any other species, but the uniformity of the condensation, reminiscent of the homogeneous orthochromatic globules of some lumbricids (Section 11), suggests that this secretory product has at least different physical properties from those of the other lumbricillids and might well be of a different chemical nature, as yet undetected. Because it is the dominant secretory type within the epidermis of L. reynoldsoni, there being few metachromatic cells, its secretion forms the bulk of the slime produced by this enchytraeid. This is in contrast to the other lumbricillids where the non-metachromatic type accounts for 50% or less of the secretory cells. It would therefore not be surprising to find, in further studies, that the secretions of the non-metachromatic cell type within the lumbricillids were not homologous.

There is evidence that failure to maintain a uniform distribution of material within the globule limit might be associated with larger globule size. This was shown in several species, but the occurrence of overlap and the presence of intermediate forms was rare. The small, more uniformly dense globules near the Golgi of L. mirabilis and the size difference linked to the ultrastructural appearance in L. georgiensis could be used to support such an argument. The generalisation holds for E. albidus when whole cells are considered, but does not appear to do so at the level of the mature Golgi face in the dispersed cell type, the vesicles containing material similar to that of the larger, more mature globules. It does not hold for F. callosa. Here the secretory material is more dispersed in cells with smaller globules and is uniformly distributed in those with larger ones.

When compared with the results of the enchytraeid metachromatic cells and the three types of lumbricid secretory cell, the observations on the enchytraeid non-metachromatic cells are less than satisfactory. They have failed to show conclusively that all the representations described relate to the same cell type. The common denominators would appear to be lack of metachromasia in paraffin and resin sections, and lack of electron density of the globules other than that produced by severe condensation, neither of which alone, or in combination, justify any categorical statement.

Further studies directed towards better fixation of this cell type for both light and electron microscope studies might prove profitable, though the present results are of some value. A fixation procedure which allows comparisons and contrasts of other epidermal features to be made and, at the same time, reveals the recorded differences in the non-metachromatic cells must be reflecting real

differences in the secretory product. The future problem is to detect whether these are physical or chemical or both.

Also, further sampling of the tissue might reveal intermediates between the forms described and so relate the types and allow for discussion concerning the ageing of the secretory cells, as it did for one of the lumbricid cell types.

26 ENCHYTRAEID SMALL GRANULAR CELL ULTRASTRUCTURE

i) Observations

Within the epidermis of species of Lumbricillus there occurs, at a very low frequency, a secretory cell type characterised by small, electron dense granules which range in size, when in good section and with clearly defined membranes, from 0.5 - 0.6 μm in diameter. Plate 75a shows, in L. mirabilis, one such cell extending the full depth of the epidermis and resting on the basal lamina external to the longitudinal musculature. The cell is without a distinct secretory cavity. Apparent at that magnification is the nucleus and the electron density variability of the granules, some of which are compressed. This is more clearly seen in Plate 75b which is a higher magnification of the basal third of this cell. At even greater magnification (Plate 76a) the granule material in the less dense granules has the appearance of consisting of fine, randomly arranged tubules, but none of these is in good enough cross section for accurate measurements to be made of their diameter. In L. reynoldsoni the shape of the granules is less well preserved (Plate 76b), as was the case in L. rivalis.

Plate 77a shows the apex of a small granular cell in L. mirabilis and part of the ring of pore microvilli can be seen.

The cells are rich in granular endoplasmic reticulum cisternae, the contents of which are amorphous and of pale to medium electron density. Polarised Golgi stacks occur but are not prominent.

In the species other than those belonging to the genus Lumbricillus no cell type showing all these features was observed, but fewer blocks of tissue were sampled and, as stated above, the

small granular cell type occurs very infrequently in the lumbricillids where considerably more tissue was examined. However, in Mesenchytraeus sp. cells with dense granules approximately 0.4 μm in diameter were seen (Plate 77b).

Action of pronase

Ultrasections exposed to pronase digestion show this cell type, in L. mirabilis, to be unaffected by the enzyme treatment (Plate 77c).

ii) Discussion

The small granular cell type in the genus Lumbricillus is shown to be ultrastructurally unlike the metachromatic and the non-metachromatic mucus types (Sections 24, 25). It is also shown to be, in general terms, very like that of the lumbricid small granular, proteinaceous cell described in Section 13. The lumbricillid granules are of the same order of size and show the same range of electron density as that noted in the lumbricids. At the detailed ultrastructural level the 16 - 18 nm wide tubules recorded in the Lumbricidae were not observed though the material had a fine tubular appearance.

These similarities, together with the presence of pore microvilli, lead to the cautious suggestion that this cell type may be equatable to the small granular, proteinaceous type of the lumbricids. This suggestion might have been strengthened if this cell type had been observed at the light microscope level and histochemical techniques applied, but this was not the case, and the proteinaceous nature of the secretion in lumbricillids has not been established. Furthermore, the failure of pronase to affect the ultrastructural appearance of the granules might indicate that they were not proteinaceous, or, at least, that the fixation has rendered the complex not susceptible to the enzyme administered in a concentration and for a duration

known to digest the protein moiety in lumbricid orthochromatic cells (Section 11). It must be admitted that true parallels on this point cannot be made, since the large number of lumbricid ultrasections subjected to pronase digestion unfortunately did not contain any small granular type cells, the distribution of which is infrequent and unpredictable.

If it were to be presumed that the small granular cells of the genus Lumbricillus were equivalent to those of the lumbricid epidermis, and the ultrastructural evidence is strong, little can, however, be stated about the functional significance of this, no firm function having been advanced for this cell type in the lumbricidae (Sections 8 and 13). Nevertheless, one point of interest arises, namely that this cell type was only observed in the lumbricillid enchytraeids and these species are alone, among those under survey, in possessing metachromatic mucous cells, as do the lumbricids. The earlier speculation (Section 8) that the small granular secretion might affect the viscosity of the other epidermal secretion could also be made with respect to the small granular cells within the genus Lumbricillus. With the absence of the metachromatic and the small granular type in other enchytraeids, it could possibly be that a functional interrelationship exists between these two types.

The cell type shown in Plate 77b in the epidermis of Mesenchytraeus sp. is not sufficiently similar to those described in Lumbricillus to warrant regarding the two types as assuredly the same. The granule size (0.4 μm) more nearly approaches that of the dense granules of the intersegmental cells of lumbricillids (0.3 μm, Section 22), but the cell width of 3.3 μm greatly exceeds that of the lumbricillid intersegmental cell and the mesenchytraeid cell is seen not to be in an intersegmental position. The present evidence

seems weighted in favour of not regarding the cell type as homologous to the small granular form described for the genus Lumbricillus.

The use of a wider range of light microscope fixatives in future studies on the enchytraeids might produce material on which histochemical tests could be performed and so provide additional information about the small granular cell type in these microdriles.

27 ENCHYTRAEID DISCUSSION

The present study, whilst encompassing several genera within the family, also embraced forms of varying size and ecological habitat. While the diverse results, both histochemical and ultrastructural, would appear to suggest that generic, size and distribution factors operate in combination on the epidermis of these forms, it has rarely been possible to relate any set of observations to one of the above factors in isolation.

The occurrence of metachromatic mucous cells and small granular secretory cells in the genus Lumbricillus could be a generic character, but could equally reflect the fact that this genus is not found in terrestrial situations and is faced with osmoregulatory problems which the secretions of these cell types might help to alleviate. The numbers of supporting cell mitochondria and the mucous pore occlusor mechanism might be interpreted as supporting the idea that the epidermis is involved in regulatory processes in the species where these features occur.

Future structural studies could be directed towards a fuller survey of this genus to establish the distribution of the metachromatic cell type, and physiological information concerning the water relations of these forms could provide a basis for discussion about the function of this cell type.

A full account of the non-metachromatic secretory elements within the enchytraeid epidermis must await further work aimed at achieving a more satisfactory fixation, at light and electron microscope levels, of these cells. The present work has been unsatisfactory and has failed to show that the cells placed in this category are unequivocally homologous.

28 FINAL DISCUSSION

The present work has revealed that the basic organisation of the epidermis, consisting of an epithelium containing secretory cells overlain by a cuticle, is similar in the two families studied. Furthermore, the details of the cuticle show it to be structurally the same in these megadriles and microdriles and the literature shows its pattern of organisation to be similar throughout the annelids, whether they be terrestrial, freshwater or marine. The basic features of the cuticle would therefore appear to be related to the annelid level of complexity and mode of life. The cross-lamella pattern of the cuticle fibres might well reflect the locomotory patterns that are so closely linked with their metameric organisation.

Such universality does not hold for the nature of the secretory elements and this was partly to be expected. The secretion forms the interface between the worm and the environment, and since this is different for aquatic and terrestrial annelids in terms of locomotory friction, osmoregulatory and desiccation pressures, it is not surprising that the nature of the secretion also differs.

Within the Lumbricidae studied a uniformity of secretory types was recorded, all the genera showing the same histochemical and basic ultrastructural forms. The lumbricids are terrestrial, and the nature of the epidermis of the secondarily semi-aquatic form Eiseniella tetraedra has not been investigated in this survey. With this exception, the British lumbricids, as a group, are subjected to the same basic environmental pressures, ecological preferences for different soil types having been shown not to be reflected in the structural or histochemical nature of the epidermis and its secretory cells.

The literature indicates that at the histochemical level parallels do not exist between the mucous cells of the megascolecids and those of the lumbricids, though both families contain large species and are predominantly terrestrial. It would therefore seem that the nature of the epithelium in lumbricids cannot be solely related either to their size or ecology, and the reported differences might be the result of the early divergence of the megadrilid stock into the two major groupings, be they the Series of Clark (1969) or the Superfamilies of Brinkhurst & Jamieson (1971). Both these recent assessments of oligochaete phylogeny stress the separate origins of the Megascolecidae and the Lumbricidae. At the same time, however, they both underline the close affinity of the Lumbricidae and the Glossoscolecidae, a megadrile family with both terrestrial and freshwater species. A literature search has not revealed any sufficiently detailed accounts of the glossoscolecid epidermis to allow serious comparisons to be made. Brinkhurst & Jamieson (1971) report only one type of gland cell, the nature of which is unspecified, in the epithelium of Biwardrilus (Criodrilus) bathybates, but state that the epidermis of Callidrilus ugandaensis closely resembles that of Lumbricus in possessing "large-grained" gland cells (presumably equivalent to the large granular cells of lumbricids) and vacuolated cells "identical with the albuminous gland-cells of Lumbricus" (equivalent, as argued in Section 8 iv, to the lumbricid metachromatic type). Without substantial histochemical and ultrastructural information this parallel cannot be taken further.

The enchytraeid epithelium proved less uniform than that of the lumbricids, the genus Lumbricillus alone showing three secretory cell types which could, broadly, be equated to the types described for the earthworms. But at the detailed level, both histochemical

and ultrastructural, the comparisons did not hold for the mucous cells, particularly the metachromatic ones. This cell type was absent from the other genera studied and, as indicated in earlier discussion, no firm explanation can be advanced to account for this.

As a family, the enchytraeids show the greatest ecological diversity within the Oligochaeta, having freshwater, littoral and terrestrial species. Although the present work has recorded differences within the epidermis of the enchytraeids studied, it has not been possible to relate these directly to a single factor, be it size, ecology or relationships within the family.

This lack of epidermal uniformity is not completely surprising when other somatic organs within the Enchytraeidae are examined. The chaetae show variation with respect to their number per bundle, their shape, the presence or absence of a nodulus, and, in certain species, the occurrence of coelomic chaetae. Nephridia may have associated interstitial tissue or this may be lacking, blood pigmentation varies within the family, the gut may or may not have peptonephridia, and the coelomocytes show a range of forms. Certain of these characters link up vaguely with the cyto-taxonomic studies of Christensen (1961) but, by and large, little correlation can be established between these variables and the taxonomy, and none with the ecology.

This somatic versatility appears to extend to the secretory elements of the epidermis and contrasts strongly with the relative conservatism shown by the Lumbricidae in their somatic organisation.

A study of oligochaete phylogeny casts little light on the origins of the Enchytraeidae or their relationships with other oligochaetes. Clark (1969), following Michaelsen (1930) clearly

distinguishes them from the other oligochaetes by placing them, alone, in a separate order. Christensen (1961) relates them to the tubificids, naids and phreodrilids, and Brinkhurst & Jamieson (1971) place them, together with the tubificids and naids, in the suborder Tubificina (order: Haplotaxida). Such authoritative differences underline the obscurity of the enchytraeids.

One of the original aims of this work (Section 1) was to discover if a relationship existed between characteristics of the epidermis of the selected oligochaetes and their surroundings. Within the narrow range of environmental variation facing the chosen lumbricids, no relationship was observed. The enchytraeids studied were from widely disparate habitats and their epidermes showed considerable variation, but the present study failed to display a firm correlation between the epidermal characteristics and the environment. However, sufficient information has accured to prompt, and to form a basis for, more extended studies on this most neglected of oligochaete families.