THE STRUCTURE AND FUNCTION OF THE SUBVENTRAL GLAND IN *OESOPHAGOSTOMUM VENULOSUM* (NEMATODA: STRONGYLIDAE)

by

ANNE THERESA JONES
B.Sc.Hons. (A.N.U.)

DEPARTMENT OF ZOOLOGY
THE UNIVERSITY OF ADELAIDE

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Master of Science. May 1980.

Awarded November 1980

SUMMARY

This study examined the structure and function of the subventral gland, associated with the so-called excretory system of nematodes. *Oesophagostomum venulosum*, a strongyloid parasite of sheep, was chosen as the experimental animal.

The work commenced with a light microscope study of the excretory system of *O. venulosum* and an electron microscope investigation of the ultrastructure and intracellular compartmentation of the subventral gland in this nematode. The results suggested that at least some of the secretory products of the subventral gland might pass across the membrane of the gland into the pseudocoelomic fluid for direct use in this matrix or be transported by it to other parts of the worm. This hypothesis was tested experimentally using a number of techniques.

In seeking a productive experimental approach the work became, to some degree, an exploration of methodology - encountering difficulties similar to those met by other workers in trying to devise sound physiological experiments for namatodes. Eventually two major experimental approaches were used: (1) radiotracer techniques, (2) polyacrylamide gel electrophoresis. Some of the results obtained supported the hypothesis that the subventral gland may export secretory products into the pseudocoelomic cavity as well as releasing secretions to the exterior through the excretory pore as previously supposed.

DECLARATION

This thesis contains no material previously submitted by me for a degree in any university.

To the best of my knowledge it contains no material written or published by another person except where due acknowledgement is given.

ACKNOWLEDGEMENTS

I wish to acknowledge the help given by many people during the course of this project. Dr. R.S. Sommerville, Department of Zoology, University of Adelaide, originally suggested and supervised the project. Supervision was completed by Professor W.D. Williams, Department of Zoology, University of Adelaide. Mr. R. Norton and Mr. J. McGarey, of the Waite Agricultural Research Institute, Adelaide, provided and maintained the sheep used during the project. Dr. W. Southcott and Mr. T. Lyle, C.S.I.R.O. Pastoral Research Station, Armidale, N.S.W., assisted in establishing the *O. venulosum* culture and provided infective larvae whenever they were needed. Dr. A.F. Bird, C.S.I.RO. Division of Horticulture, Adelaide, advised me on the fixation of nematode tissue. Dr. J. Wells, Department of Biochemistry, University of Adelaide, advised me on cell fractionation and differential centrifugation. Mr. M. Schwinghammer, Department of Biochemistry, University of Adelaide, taught me how to prepare "Laemli" gels. Professor W.P. Rogers, W.A.R.I., lent me the electrophoretic apparatus which I used. Mr. K. Crocker, Electron Microscope Unit, University of Adealide, assisted me with photography. Dr. A.F. Bird, Dr. W.L. Nicholas, Department of Zoology, A.N.U., and Professor W.P. Rogers all helped me in developing ideas on the function of the nematode subventral gland.

Finally I must thank my parents, Max, Kate, Wendy and Rosie for providing practical assistance, encouragement and shoulders to weep on during this long ordeal.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 NEMATODES AND THEIR EXCRETORY SYSTEMS

1.2 THE SCOPE OF THE PRESENT INVESTIGATION

1.1 NEMATODES AND THEIR EXCRETORY SYSTEMS

The Nematoda is the largest pseudocoelomate phylum and, after the Insecta, the most numerous, ubiquitous and diverse Metazoan group. It is, nevertheless, surprisingly uniform in superficial anatomy, an indication of the evolutionary success of the nematode form. Adequate accounts of the basic structure of nematodes are given, among others, by Bird (1971) and Nicholas (1975).

The so-called excretory system occurs with great diversity, and undergoes radical differentiation during development in some species. It has been described for many species, and several good reviews on this subject have been published (Chitwood and Chitwood, 1950; Weinstein, 1960; Lee, 1965; Bird, 1971). Although the structures which have been designated excretory certainly seem, on morphological grounds, to constitute a functional unit, an excretory function has not been clearly established for them.

Nematode excretory systems are usually divided into groups, and the principal division of the Nematoda into two classes - the Secernentia and the Adenophorea - is based, in part, on the type of excretory structures present (Chitwood and Chitwood, 1950). In the Secernentea, the excretory system is usually described as glandular and consists of a single large cell lying in the pseudocoelomic cavity. This cell is lined with cuticle only at the end which typically swells into a small vesicle or ampulla just before opening to the exterior through a pore (Bird, 1971). The more elaborate "tubular" excretory systems of the Adenophorea consist of lateral chords which may be linked by a transverse canal, in turn connected to the median excretory pore by a cuticularised duct. Large cells, single or paired, may be associated with these tubular excretory systems. Many variations of the two main types of excretory system, particularly of the tubular system, have been observed. Chitwood and Chitwood have classified the excretory systems and their nomenclature is shown in Figure 1. Some nematodes

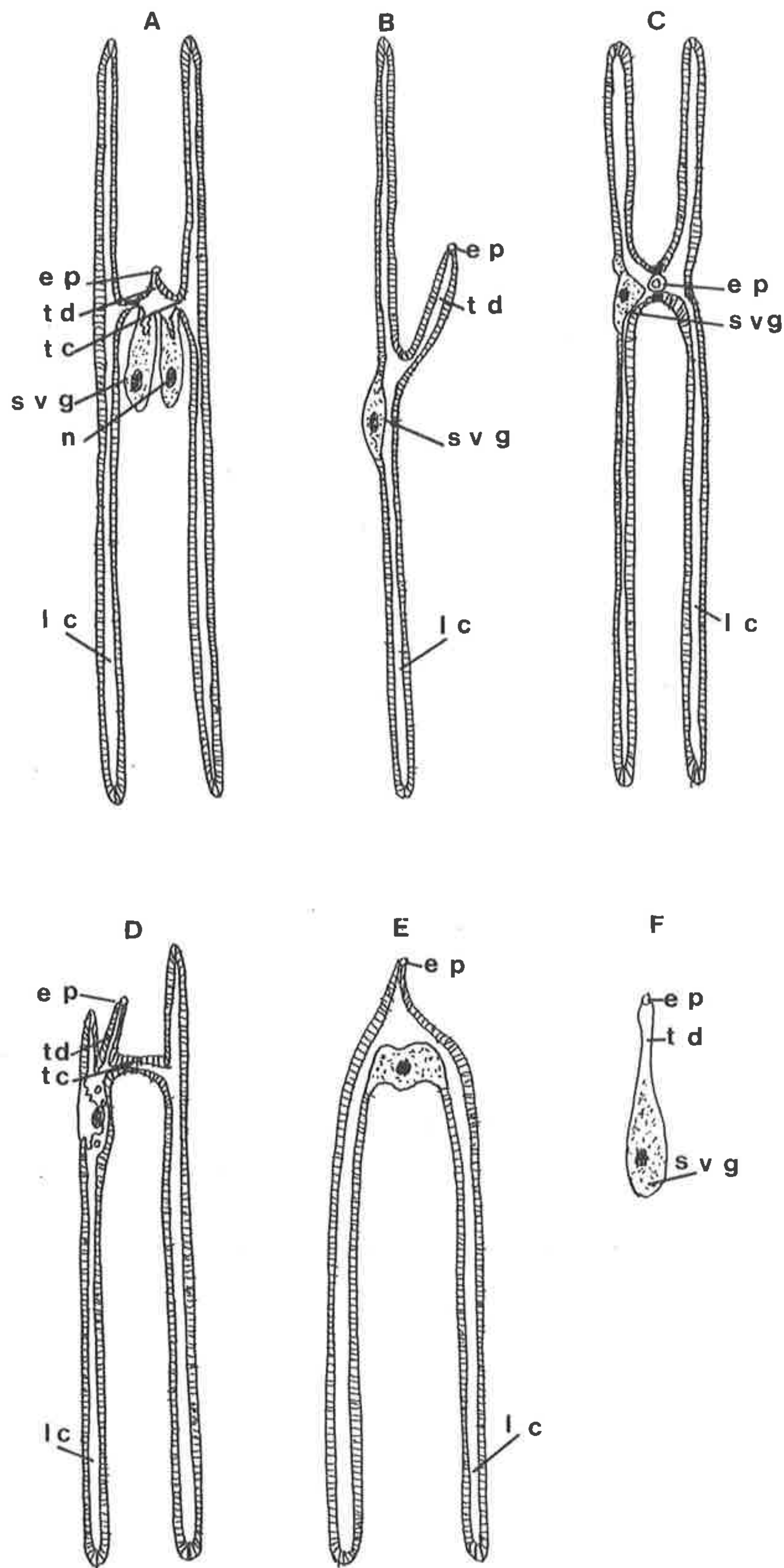


Fig. 1. Nematode Excretory Systems. Representative types, after Chitwood and Chitwood (1950).
Tubular Excretory Systems. (a) Rhabditoid type, H-shaped with a lateral canal in each lateral chord and two subventral glands. (b) Tylenchoid type, asymmetric with only one lateral canal and one subventral gland. (c) Oxyuroid type, H-shaped without subventral glands. (d) Ascaroid type. (e) Cephalaboid type.
Glandular Excretory Systems (f) Single subventral gland.

ep, excretory pore; es, excretory sinus; lc, lateral canal; n, nucleus; svg, subventral gland; td, terminal duct; tc, transverse canal.

apparently lack any type of excretory structure and others have only extremely reduced systems (Crofton, 1966; Bird, 1971). The excretory pore appears to be the most ubiquitous element of the nematode excretory system. Table 1 lists the types of excretory structures observed in the various nematode orders.

Much work has been done on osmoregulation and excretion in nematodes (see reviews by Weinstein, 1960, and Lee, 1965), but osmoregulatory and excretory mechanisms are only imperfectly understood for the group. The tubular components of the so-called excretory system have been implicated in either osmoregulation or excretion in a few species of nematode. For example, Weinstein (1952) showed that in the larvae of *Nippostronglus muris* and *Ancylostoma caninum* the ampulla of the excretory system contracted at a rate proportional to solute concentration in the medium. The evidence for an excretory role for the excretory system includes the observed formation of precipitates at the excretory pore of *Nippostrongylus* placed in immune serum and the collection of dyes in the lateral canals and their expulsion through the excretory pore (Lee, 1965). Overall there is little direct experimental evidence to support an excretory function for the excretory system, and indeed, there is much evidence to suggest that other organs in the nematode also play such a role, for example the gut and cuticle (Weinstein, 1960; Lee, 1965).

There is no evidence to suggest that the glands associated with either the glandular or tubular excretory systems have any osmoregulatory or excretory function. These glands have been designated "excretory" merely because they are morphologically juxtaposed to the tubular parts of the excretory system and apparently have access to the exterior through the excretory pore. Waddell (1968) has shown that the lateral canal, transverse canal and excretory sinus are developmentally and structurally distinct from the subventral glands in the tubular system of *Stephanurus dentatus*. His conclusions concerning ultrastructural differences between the glands and the "tubular" parts of the system have been confirmed by other workers (e.g. Lee, 1970; McLaren, 1974). Narang's (1970)

TABLE 1. Nematode "Excretory" Structures According to Order and Habitat. (Chitwood and Chitwood, 1950; Hyman, 1951; Croll and Matthews, 1977).

SECERNENTEAN ORDERS	HABITATS	"EXCRETORY" STRUCTURES PRESENT
Rhabditida	terrestrial; sheathed juvenile may be transported in an invertebrate	symmetrical, tubular, "H-type"; lateral and transverse canals; with or without 2 glands
Strongylida	free living larvae; adults parasites of vertebrate gut	"H-type"; tubular system; with 2 glands
Ascarida	parasites of vertebrate intestine	"H-type"; lateral canals reduced anteriorly; 2 glands
Spirurida	parasites of many vertebrate tissues	"U-type"; tubular system; glands present only in some juveniles (microfilaria)
Tylenchida	phytoparasites	assymetrical, tubular; lateral canal in 1 chord; 1 gland
ADENOPHOREAN ORDERS		
Trichinellida	mammalian parasites	reduced, absent or unknown
Dioctophymatida	vertebrate parasites	reduced, absent or unknown
Enoplida	marine	glandular
Dorylaimida	freshwater, soil	reduced, pore seen in some species
Chromadorida	marine	glandular or unknown
Monohysterida	freshwater, soil, marine	glandular or reduced

work on the ultrastructure of the excretory system in the Adenophorean nematode, *Enoplus brevis*, suggests that this glandular excretory system is also comprised of ultra-structurally distinct glandular and tubular components, analogous to those seen in the tubular excretory systems of the Secernentean nematodes. Thus, although the tubular components of nematode excretory systems may have an osmoreg-ulatory or excretory function, it is possible that associated glands have a quite different function.

Until recently, the role of the glands has been ignored. However, a number of workers has examined them during the last ten years and clearly established their secretory, "glandular" nature. This work will be reviewed below; here it suffices to note merely that their function is unknown.

The glands associated with the tubular and glandular excretory systems have been referred to by several names - renette, excretory cell, excretory sinus, exodigestive gland, subventral gland, etc. Since the glands from both types of system are ultrastructurally similar (Narang, 1970), and may therefore be functionally alike, I shall refer to all such glands as "subventral glands" (Waddell, 1968). This name is convenient and implies only what is definitely known about the glands, and no more.

1.2 THE SCOPE OF THE PRESENT INVESTIGATION

The work reported here consisted of a study of the structure and function of the subventral gland in *Oesophagos-tomum venulosum*, a stronglyloid parasite of sheep and goats. The aim was two-fold. Initially it was to describe the general morphology of the excretory system and ultrastructure of the subventral gland (it had not previously been described for this species). This morphological study was then to form the basis of the other part of the project, an investigation of the function of the subventral gland.

O.venulosum was chosen because: (1) its host is easily maintained and is economically important, and (2) because the worm and its subventral gland are large and would, it was thought, permit the use of various experimental techniques which involved dissection of nematodes and manipulation of isolated glands.

Section 11 of this thesis describes the morphological studies made on the subventral gland of *O. venulosum*. In Section 111, the experimental work which tested various hypotheses suggested by the morphological work is described. Section IV is a general discussion of the results obtained during the present study and their significance to the question of the function of the subventral gland.

CHAPTER 2: THE EXPERIMENTAL ANIMAL, *OESOPHAGOSTOMUM VENULOSUM*, AND ITS MAINTENANCE IN SHEEP

2.1 DESCRIPTION OF *O. VENULOSUM* AND ITS LIFE CYCLE

2.11 Description of Adult Worm

2.12 Life Cycle

2.2 THE MAINTENANCE OF *O. VENULOSUM*

2.21 Maintenance of Host

2.22 Infection of Sheep

2.23 Egg Counts

2.24 Collection of Worms

2.25 Preparation of Larval Cultures

2.26 Identification of Larvae

2.1 DESCRIPTION OF *O. VENULOSUM* AND ITS LIFE CYCLE

2.11 Description of Adult Worm

Oesophagostomum venulosum belongs to the Strongylidae. The oesophagostomes or nodular worms are common strongyloid parasites of the intestines of pigs, ruminants and primates (Hyman, 1951). Characteristically, they damage the intestinal wall by causing the formation of swellings or nodules where juvenile stages encyst in the gut mucosa. *O. venulosum* is less pathogenic than *O. columbianum*. However, it is thought that moderate infestations of *O. venulosum* may be responsible for stunted development and malnutrition - especially on poor pastures - and that the adult stage may produce a toxaemia similar to that caused by adult *O. columbianum* (Seddon and Albiston, 1967). *Oesophagostomum* and related genera are distinguished by the presence of a pronounced groove on the ventral surface of the cervical region (Hyman, 1951). The cuticle just anterior to this "cervical groove" is inflated to form what is known as a "cervical vesicle".

Adults are rather large nematodes and have been described by Goodey (1924) and Gevaur (1951). The male is 12-13 mm long by 0.45-0.46 mm wide. The female is 15-17 mm by 0.5-0.6mm (Goodey, 1924). The worms display typical strongyloid features: cuticularised buccal capsule, upstanding mouth collar and external leaf crown in both sexes, and, in the male, characteristic strongyloid bursa (Hyman, 1951).

2.12 Life Cycle

The life cycle has been described by Goldberg (1951) and is outlined in Fig. 2. Briefly, the eggs shed by adult worms are passed in the host's faeces. Under laboratory conditions, the eggs hatch 24 hours after passing out of the host. There are two free-living juvenile stages. The third juvenile stage is infective and it retains the second-stage cuticle as a protective sheath. The third-stage larvae appear 3-5 days after hatching. Third-stage larvae may be kept under refrigeration for 2-6 months in faecal cultures or water. In the field,

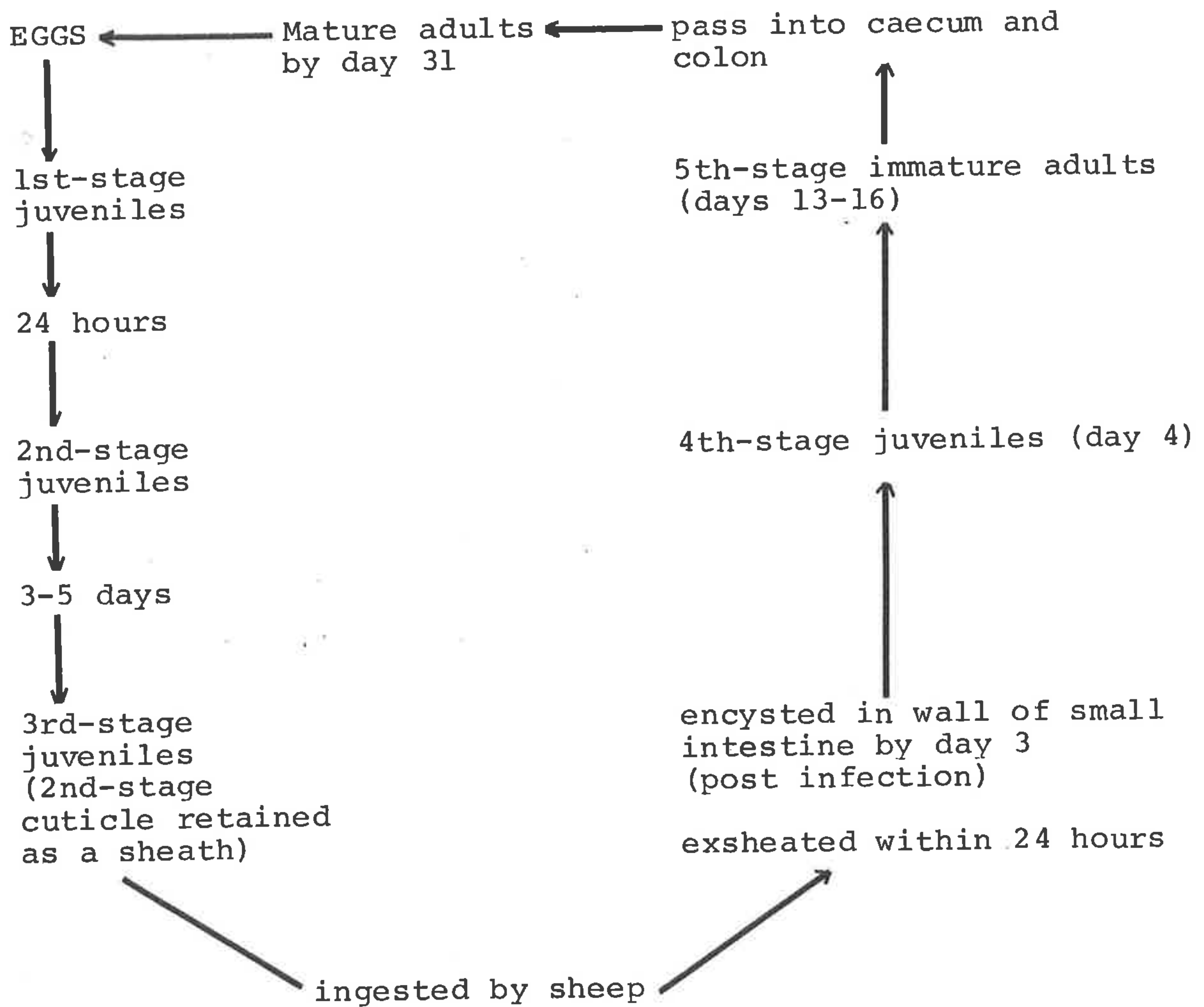


Fig. 2 Life Cycle of *Oesophagostomum venulosum* (after Goldberg, 1951).

third-stage larvae are ingested by sheep and exsheath in the intestinal lumen within 24 hours. The worms undergo two moults during their migration through the alimentary tract of the host. Mature adult worms - fifth-stage - are found in the caecum and the first three feet of the colon (99% were found in the caecum and colon 31 days after infection in Goldberg's studies). The presence of fertile females on day 31 suggested that most worms were mature by this time. The duration of different phases of the life cycle probably varies with environmental conditions. For example, Goldberg reported that the prepatent period was 28 days and that maximum egg production occurred about day 60-65. Dash (1973), however, found that the prepatent period lasted about 35 days, and that the patent period lasted about 90 days.

2.2 THE MAINTENANCE OF *O. VENULOSUM*

2.21 Maintenance of Host

Adult worms were raised in Merino-crossbred lambs. The sheep were housed in individual, indoor, elevated pens with slatted floors. They were fed 800 grams of lucerne chaff daily and unlimited water. Sheep were kept at the Waite Institute for Agricultural Research, Glen Osmond, South Australia.

During this study, it became evident that the optimum age for host sheep at the time of infection was 4-6 months (i.e. newly-weaned lambs). Initially 15 month old lambs were used as hosts and only about 38% of infections were successful (Goldberg used 2-11 month old lambs, apparently with success).

A comprehensive investigation into the optimum conditions for infection of sheep with *O. venulosum* would not have been appropriate during the present study. However, trial and error indicated that infections were more successful with younger lambs, When newly-weaned four month old lambs were infected with approximately 500 larvae, 98% of infections were successful. Under these conditions an average of 100 worms (range 30-200) were recovered. The necessity for keeping larval doses small

has been stressed by several workers (Goldberg, 1951; Dash, 1973). This need was demonstrated in the present study when attempts to infect young lambs with large doses of larvae (1000-1500) failed. In these cases only very few adult worms, 1-10, were recovered from the infected sheep. Attempts at reinfection were invariably unsuccessful.

The difficulties encountered in attempts to establish infections of *O. venulosum* in older lambs, or to reinfect, suggest the presence of some kind of age or immune protective mechanism. There is little information available on immunity to oesophagostomes. However, there has been a report of the existence of cross immunity between *Chabertia ovina* and *Oesophagostomum venulosum* (Hörchner, 1968). *C. ovina* is a common parasite of sheep in South Australia, whereas *O. venulosum* is not (Seddon and Albiston, 1967; unpublished observations). It is possible that the older sheep's experience with *C. ovina* enabled them to build up immunity to *O. venulosum*. Or perhaps, the total environment within and outside the sheep in S.A. is unsuitable for *O. venulosum* in some way.

2.22 Infection of Sheep

Lambs were usually drenched with 10ml of Thiabendazole before infection but when newly-weaned (4 month old lambs) were used, they were not drenched. Each lamb was infected once with 500 larvae of *O. venulosum* suspended in 10ml of water. It was administered orally by inserting a 10cm length of polythene tubing (10 mm diameter) down the sheep's throat and then pouring the dose down through a funnel. Fifty ml of water was used to flush the larval dose into the lamb's rumen.

2.23 Egg Counts

The development of the worms within the host was followed by regular examination of the sheep's faeces for the presence of the parasites eggs. Small quantities of faeces were removed from the rectum and egg counts were made according to the McMaster technique for enumerating eggs. The particular method used was originally devised for cattle faeces (Roberts and

Sullivan, 1950). It was selected for this study because it is particularly useful for counting small numbers of eggs. For each lamb, a 3gm sample of faeces was soaked in 20ml of tap water overnight in an airtight container at $4^{o}C$. The next day, after thorough stirring, 10ml of each faeces/water sample were mixed with 10ml of saturated NaCl solution. The chambers of a McMaster slide (McMaster slide for cattle faeces, H.V. Whitlock and Co., Sydney) were then filled with faeces/salt solution. Any eggs present in the suspension rise up through the salt solution and lie just below the cover of the chamber where they can be easily counted under a binocular microscope. The mean egg count from counts of 3 chambers was used to estimate the number of eggs per gram of faeces. *O. venulosum* eggs could be easily recognised. The only other eggs which were consistently present were those of *Ostertagia circumcincta* identified from Kates and Shorb (1943). Although the mean number of eggs per gram for a group of lambs gives an indication of the progress of the infection, a single count on an individual lamb does not indicate whether the infection is light or heavy (See Fig. 3). The great variation in counts obtained on each day from 12 lambs, (Fig. 3) probably reflects non-random distribution of eggs in space (in the faeces; Roberts and Sullivan, 1950) or in time. Since, in a study like the present one, it is not practicable to overcome the problem of egg count variation by performing more counts, there must always be some uncertainty as to the success of an individual infection. In the present study under ideal conditions the prepatent period appeared to be 35-40 days and infections lasted up to 120 days. The number of adult worms recovered from infected sheep appeared to diminish in older infections. Adult worms were invariably found at the blind end of the long caecum, hardly ever in the colon. The only other helminths ever seen in the caecum were "whipworms", *Trichuris ovis*. The length of the prepatent period and the site at which adult worms were found, in the present study, differed somewhat from the published descriptions (see Sections 2.12 and 2.21). Such small variations may be manifestations of the

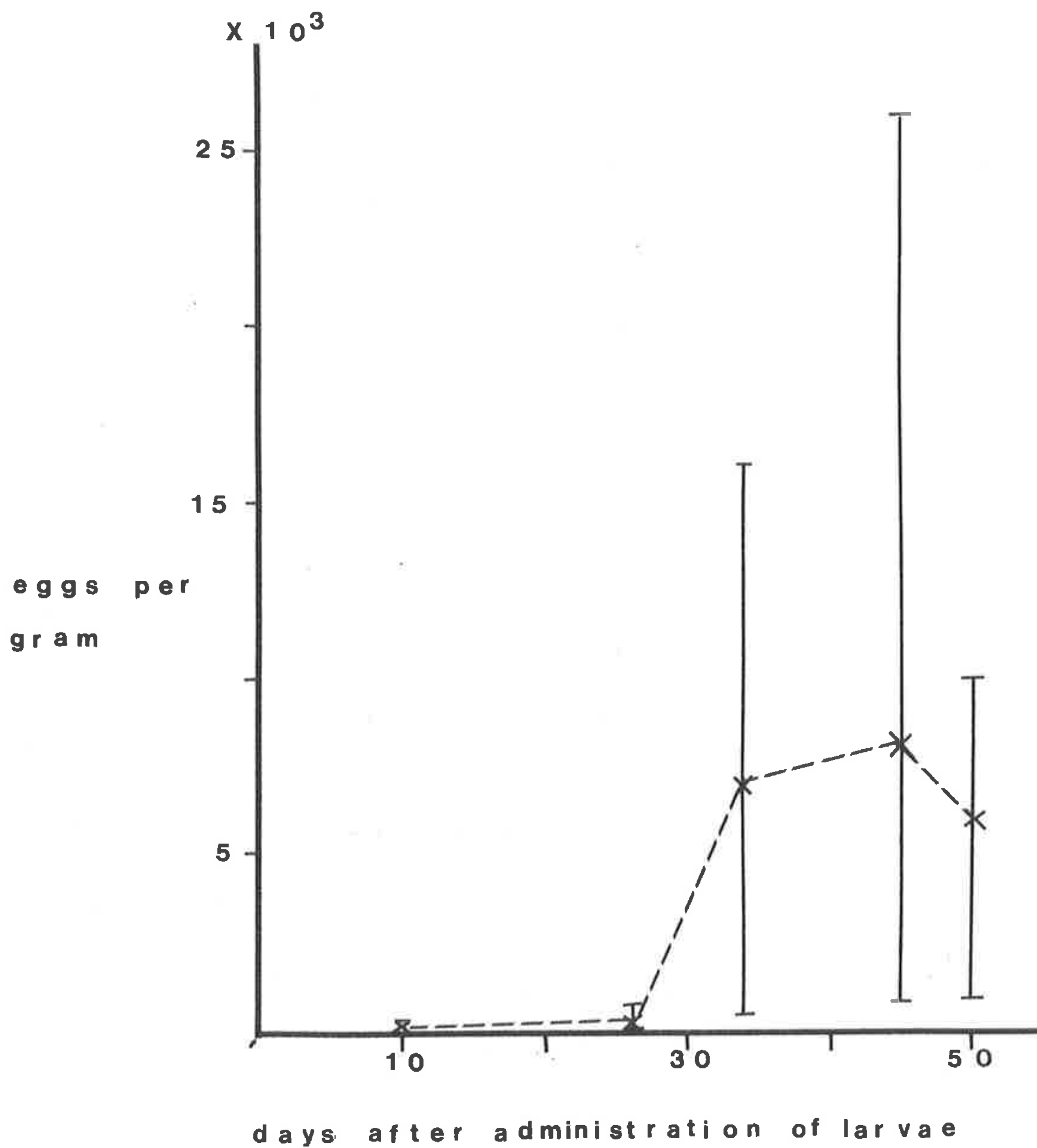


Fig. 3. Egg Counts.
Range and mean number of eggs per gram of faeces from 12 lambs infected at 4 months with 500 larvae of *O. venulosum* each. Counts were made 10, 26, 34, 45 and 50 days after infection. The wide variation in number of eggs per gram of faeces from different lambs is obvious.

X, mean; I, range.

effects of different geographic and host environments on the life cycle of *O. venulosum*.

2.24 Collection of Worms

Sheep were slaughtered at the Waite Institute, the abdomen immediately opened and the caecum removed. Worms were either removed from the caecum immediately or the caecum was ligatured and transported back to the laboratory in a vacuum flask before removal of the worms. In either case, worms were removed from the caecum with forceps, washed in Krebs-Ringer Phosphate (KRP), and kept in KRP at either 4°C or 37°C until needed.

2.25 Preparation of Larval Cultures

O. venulosum is not common in S.A. and could not be found here. In order to establish a culture of larvae, I visited the CSIRO Pastoral Research Laboratory, Armidale, N.S.W., an area where the species is endemic (Seddon and Albiston, 1967). There adult worms were obtained from sheep and identified as *O. venulosum* by comparison with Goldberg's description. The gravid females were squashed to release fertile eggs which were cultured on helminthologically sterile sheeps' faeces to produce infective larvae. These were used to infect sheep and so a culture was established.

Infective larvae were brought back to Adelaide and a stock established in sheep. Shortage of space made it difficult to maintain the culture in Adelaide so the Armidale culture was usually used as a source of infective larvae for experimental sheep. But when larvae were cultured in Adelaide, wethers infected with adult *O. venulosum* were "bagged" overnight to obtain faeces containing *O. venulosum* eggs. These faeces were crumbled and 2 litre preserving jars were filled with crumbled faeces to a height of 4cm. The cultures were moistened with tap water, covered and left for 7 days. After this period, aggregations of larvae could be seen ascending

the moist sides of the glass jars. The jars were inverted and the larvae carefully washed out with fine streams of tapwater from a pasteur pipette. Larvae were usually stored in screw top bottles at 4°C and used over a 2 month period.

2.26 Identification of Larvae

Larvae were killed and stained for counting with a drop of 0.1% iodine in 0.1% potassium iodide and examined under a binocular microscope.

The cultures obtained from Armidale were almost pure *O. venulosum*. The third-stage larvae of *O. venulosum* were easily identified and distinguished from those of *Trichostrongylus colubriformis; Ostèrtagia circumcincta* and *Haemonchus contortus* which were occasionally present (Dikmans and Andrews, 1933). They have approximately 32 light yellow-green intestinal cells and a rounded tail. The sheath forms a distinctive long pointed tail. The average length of the whole ensheathed larva is 0.89mm and its width is 0.030 mm at the base of the oesophagus (Goldberg, 1951). Infective larvae of *O. venulosum* are virtually indistinguishable from those of *Chabertia ovina* (Dikmans and Andrews, 1933; Wetzel and Marholott, 1955). However, *C. ovina* was only present in the Armidale cultures on one occasion.

CHAPTER 3: THE MORPHOLOGY OF THE EXCRETORY SYSTEM

3.1 INTRODUCTION

3.2 METHODS

3.21 Collection of Worms

3.22 Fixation and Embedding

3.221 Schedule for fixing and embedding *O. verrulosum* tissue

3.222 Modification of schedule for light microscopy

3.3 RESULTS

3.4 DISCUSSION

3.1 INTRODUCTION

All Strongyloid nematodes examined possessed an excretory system of the "Rhabditoid" or "H-type" (Chapter 1). This type is characterised by the presence of "... a terminal cuticular duct, a sinus connected with a pair of lateral canals, and a pair of subventral glands" (Waddell, 1968). There have been major studies of three Strongyloid excretory systems in recent years - *Stephanurus dentatus* (Waddell, 1968), *Nippostrongylus brasiliensis* (Lee, 1970) and *Necator americanus* (McLaren, 1974). All three display the typical rhabditoid features and are similar ultrastructurally. However, they differ in details such as the degree of cuticularisation of the terminal duct, the number of nuclei in the tubular parts of the system, and the presence or absence of ducts in the subventral glands.

In general, strongyloid excretory systems appear to be composed of two components which are distinct morphologically, ultrastructurally and developmentally: (1) the paired subventral glands, and (2) the tubular network which comprises the excretory pore, excretory canal, sinus, paired lateral canals and transverse canals. The lateral canals are embedded in the lateral hypodermal chords, terminating blindly. They are linked by transverse canals extending from the sinus which opens ventrally into the excretory canal and so into the ventral excretory pore. The connection between the subventral glands and the tubular system is made by the transverse canals or the sinus, which pass over or through the subventral glands and are intimately associated with them at the point of contact. In *Stephanurus dentatus*, Waddell found that the two morphologically distinct components of the excretory system developed separately until the fourth juvenile stage when the subventral glands became associated with the sinus.

The ultrastructure of the excretory system was similar in each of the three species examined. The tubular components contain few organelles other than a small number of mitochondria, a few strands of aggranular endoplasmic reticulum and some small golgi complexes. In the subventral glands,

however, the presence of abundant secretion granules, mitochondria, golgi complexes and granular endoplasmic reticulum implies a high degree of metabolic activity. The lateral canals are elongated cellular structures, each with a convoluted intracellular lumen along its length. The central lumen is lined by plasma membrane. Numerous membrane-bound vesicles and canals are clustered around the lumen, apparently opening into it. The subventral glands lie free in the pseudocoelomic cavity except for a loose attachment to the gut by one or two pseudocoelomocytes (Waddell, 1968).

It was thought that an understanding of the structure of the subventral gland and its relationships with other compartments within *O. venulosum* would suggest hypotheses which could be tested experimentally. Since the secretory nature of the gland is well known (Chapter 5) the morphological studies concentrated on examining routes by which secretory products might leave the gland, and by which their precursors might enter.

3.2 METHODS

The same fixation and embedding methods were used for producing thick sections for light microscopy and ultra-thin sections for electron microscopy (Chapter 4). Fixation in formaldehyde and osmium tetroxide and embedding in epoxy resin are described below.

3.21 Collection of worms

Sheep were slaughtered early in the morning. The caecum was quickly removed and opened to reveal the adult worms which were picked out gently with forceps and dropped into warm, 37°C, balanced salt solution (BSS) in McCartney bottles. The BSS used here and throughout the entire study was Krebs-Ringer phosphate pH 7.3. The McCartney bottles were transported back to the laboratory in a vacuum flask. Here the worms were washed in warm BSS and kept in fresh BSS in a covered petri dish at 37°C until used. For all microscope work the worms were fixed as soon as possible. The time that elapsed between slaughter of sheep and fixation of the worms varied between 1 and 2 hours.

3.22 Fixation and Embedding

Nematode tissues, whether whole worms or excised sections of them, were fixed in buffered formaldehye, post-fixed in buffered osmium tetroxide and embedded in Spurr low-viscosity embedding medium (Polysciences, Inc.). The schedule was developed with advice from Dr. A.F. Bird (see also Bird, 1971).

3.221 Schedule for fixing and embedding *O. venulosum* tissue

1) Whole worms or dissected pieces were prepared for fixation in a drop of warm BSS on a microscope slide and then drawn into an adjacent drop of hot, 42°C, buffered formaldehyde; prepared by dissolving 4% paraformaldehyde in Millonig's phosphate buffer at pH 7.3 and 80°C.

2) After 10 minutes, the tissue was transferred to cold, 4°C, buffered formaldehyde and left in it for approximately 170 minutes and then given three 10 minute washes in cold Millonig's phosphate buffer.

3) The tissue was post-fixed in buffered osmium tetroxide at 4°C, prepared by dissolving 0.1 gm of osmium tetroxide in 10ml of Millonig's phosphate buffer plus 1ml H_2O and 0.05 gms of glucose. After 45 minutes the tissue was washed in cold Millonig's buffer for 5 minutes.

4) The tissue was dehydrated in ethanol - 2 x 15 minutes successively in 30%, 50%, 70%, 80% and 90% and 4 x 15 minutes in 100%.

5) Tissue was infiltrated with Spurr embedding medium - 50% ETOH: 50% Spurr for 24 hours under vacuum at room temperature, followed by fresh epon for 24 hours under vacuum at room temperature.

6) Moulds were prepared by prepolymerising a thin layer of resin on the base of each mould for 2-3 hours. The specimens were carefully aligned on these bases, covered with fresh epon and left under vacuum for 12 hours at room temperature. The blocks were polymerised in an oven at 60°C for 48-96 hours.

3.222 Modification of schedule for light microscopy

The schedule was applied to light microscopy in the following way. Whole worms were placed in drops of warm BSS on a microscope slide. Each worm was drawn into an adjacent drop of hot formaldehyde. After the worm had begun to stiffen it was cut through behind the subventral glands. This method allowed fixative to penetrate the specimen well and there was little distortion of the nematode's internal organs because the pseudocoelomic fluid was partially fixed before cutting and so did not gush out of the punctured worm. The anterior sections of the worms were subsequently fixed and embedded as previously described.

After polymerisation the specimen blocks were trimmed and sections, approximately 1mμ thick, were cut with a Reichert Om U3 ultramicrotome.

The sections were mounted in drops of water on glass slides lightly smeared with Haupt's adhesive (Steedman, 1960). The slides were dried on a hot plate, 70-80°C, for about ten minutes and then stained with 1% toluidine blue in 1% borax (Pease, 1964). The staining method used was a modification of the Azure 11: Methylene blue method described by Bird (1971). Drops of periodic acid were left over the dried slides for 5 minutes at room temperature, and then washed off with distilled water. After drying, slides were covered with toluidine blue solutions on the hot plate, 70-80°C for about ten minutes. The slides were then washed in distilled water, dried, cleared in xylene for one minute, and mounted in a netural mounting medium (DePeX). Prepared slides were examined under an Olympus VCY175 microscope and photographed with an Olympus PM6 camera and Ilford 125 ASA film.

3.3 RESULTS

Figures 4 and 5 are diagrammatic representations of the dorsal and lateral aspects of the anterior ends of the excretory system in *O. venulosum*. Plates 1 to 6 are photomicrographs illustrating details of the excretory system. Figure 6 is a representation of the entire excretory system of *O. venulosum*.

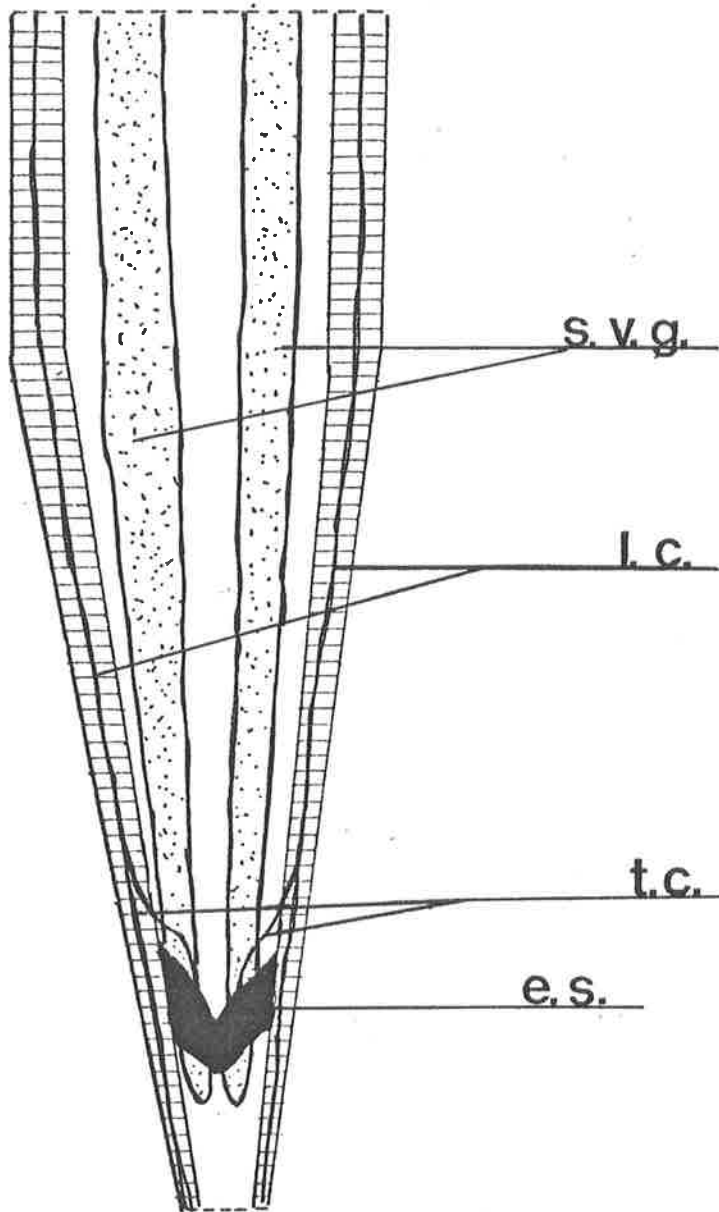


Fig. 4. Dorsal aspect of the anterior end of the excretory system of *O. venulosum*.
es, excretory sinus; lc, lateral canal; svg, subventral glands; tc, transverse canal.

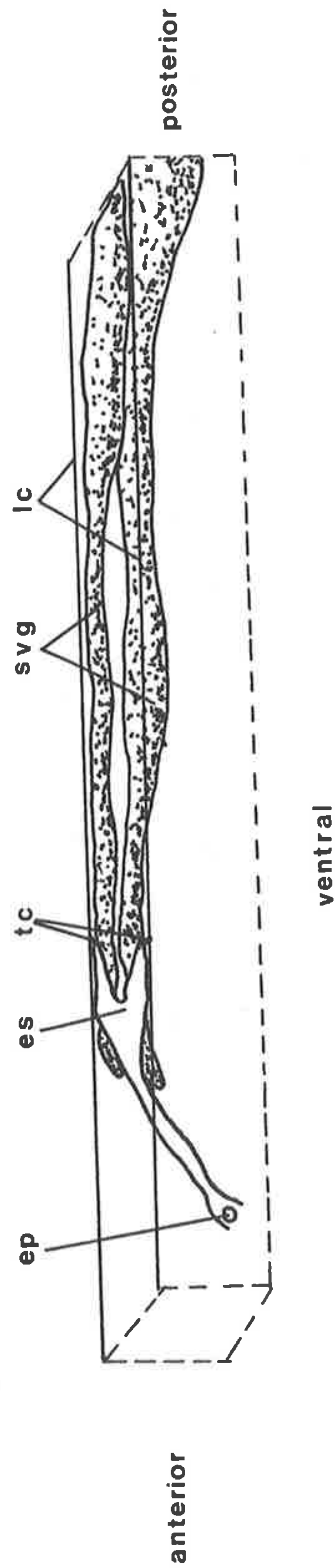


Fig. 5. Lateral aspect of the anterior end of the excretory system of *O. venulosum*.

es, excretory sinus; ep, excretory pore; lc, lateral canal; svg, subventral gland; tc, transverse canal.

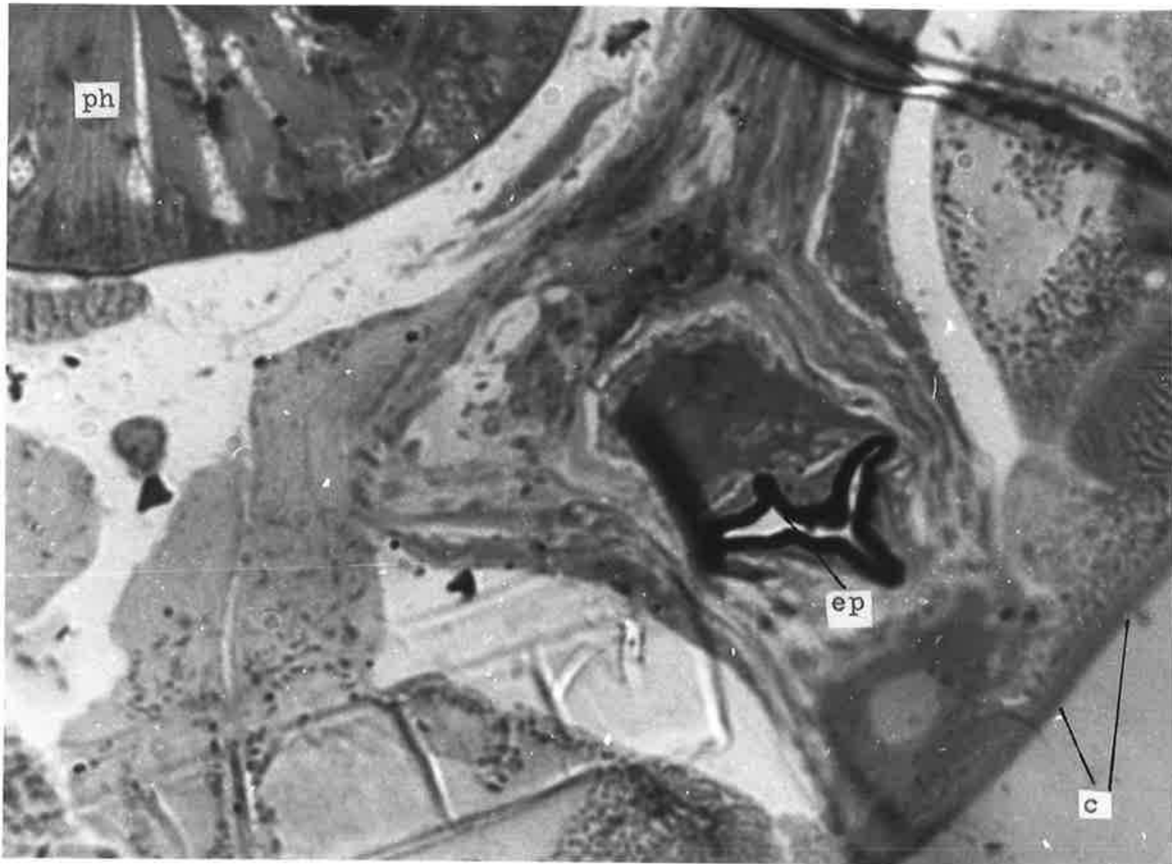


Plate 1. The heavily cuticularised excretory pore of *O. venulosum*.

c, cuticle; ep, excretory pore; ph, pharynx. X 1,500

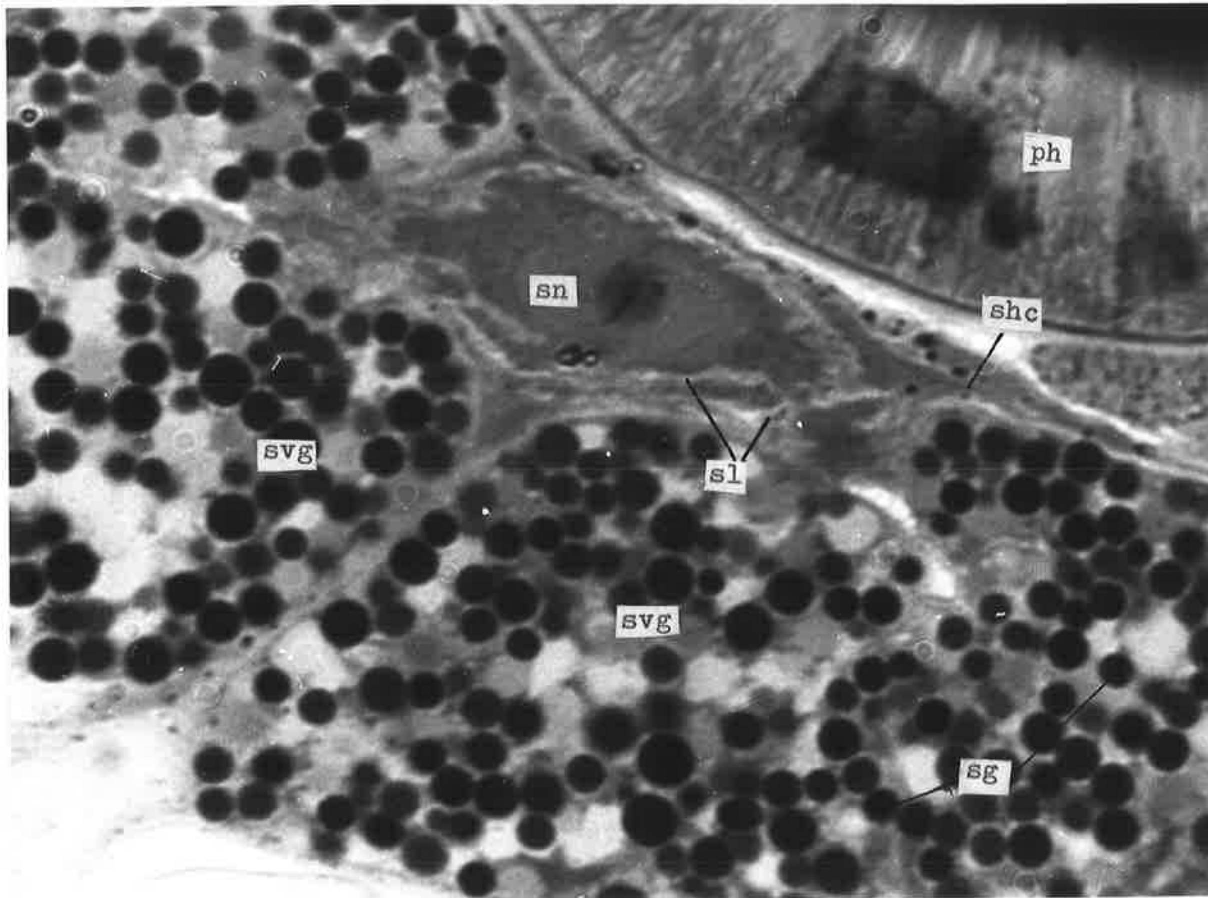


Plate 2. The large median nucleus of the sinus seen in the excretory sinus, lying over the conjunct anterior ends of the subventral glands. The attachments which link the sinus and the hypodermal chords are visible. Note that the subventral glands are packed with secretion granules and contain no ducts.

ph, pharynx; sg, secretory granule; shc, sinus/hypodermis connection; sl, sinus lumen; sn, sinus nucleus; svg, subventral gland.

X 1,500

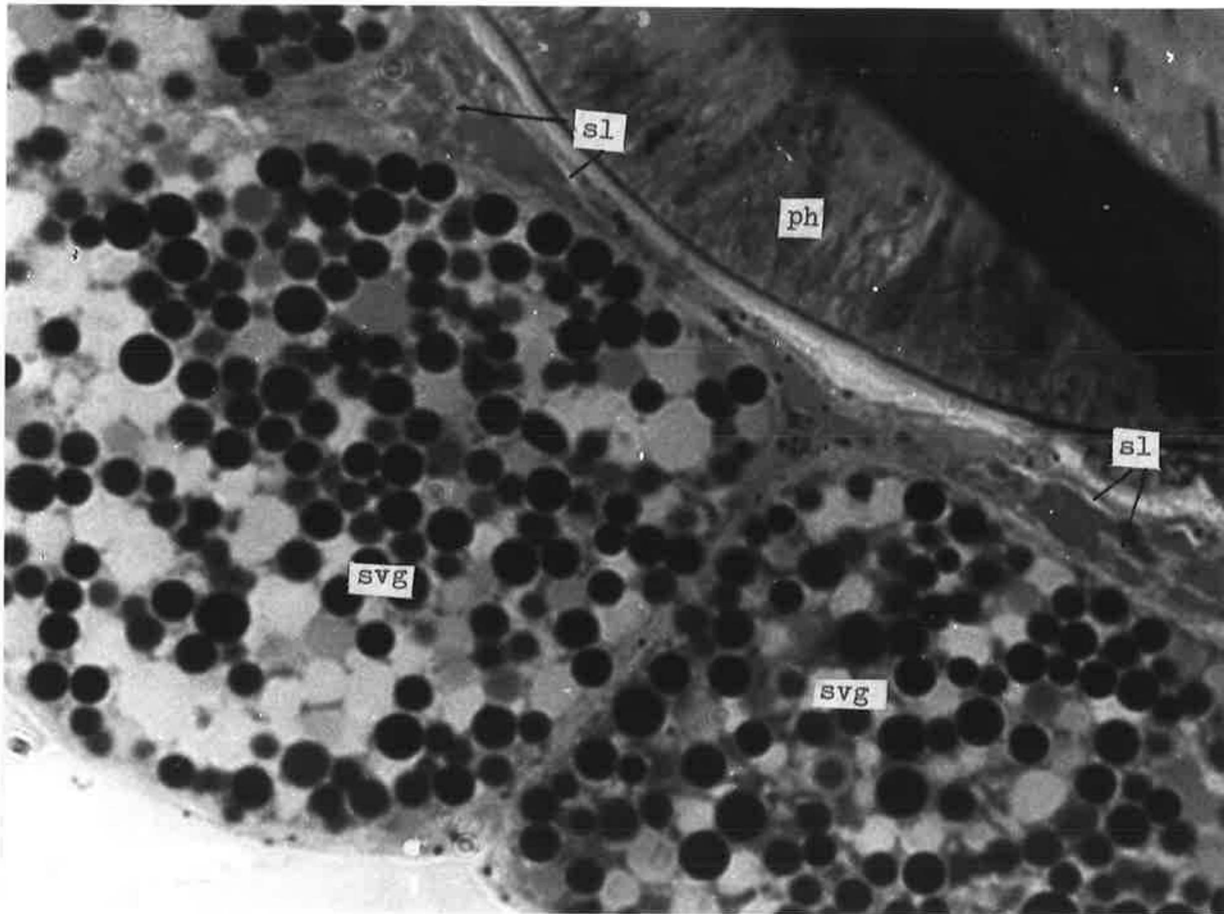


Plate 3. Division of the excretory sinus into two posterior branches, each associated with one subventral gland. Convoluted sinus lumen can be seen.

ph, pharynx; sl, sinus lumen; svg, subventral gland.

X 1,500

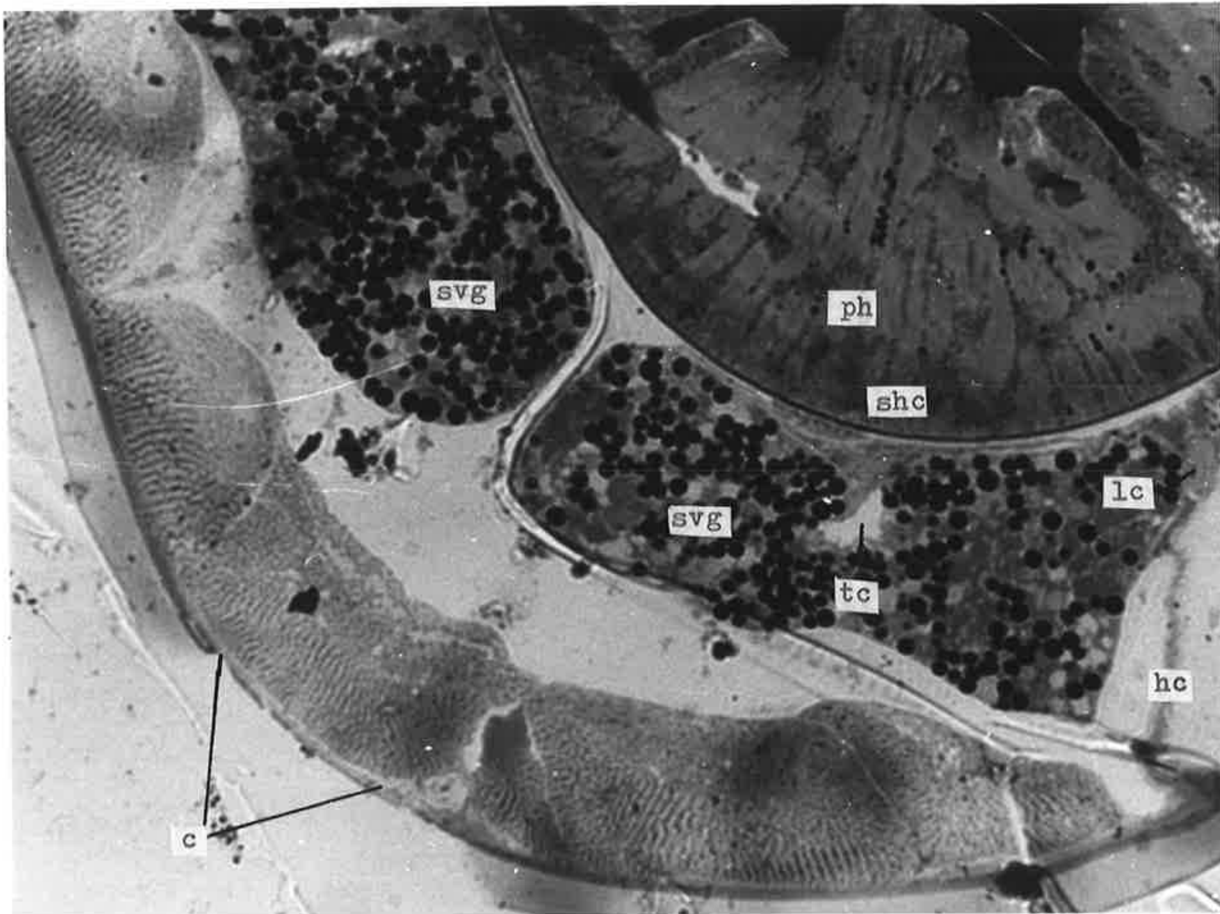


Plate 4. The transverse canal embedded in one of the subventral glands can be seen lying beneath a posterior branch of the sinus. The attachments between the sinus and the hypodermal chord, and one lateral canal are visible.

c, cuticle; hc, hypodermal chord; lc, lateral canal; ph, pharynx; shc, sinus/hypodermis connection; svg, subventral gland; tc, transverse canal.

X 600

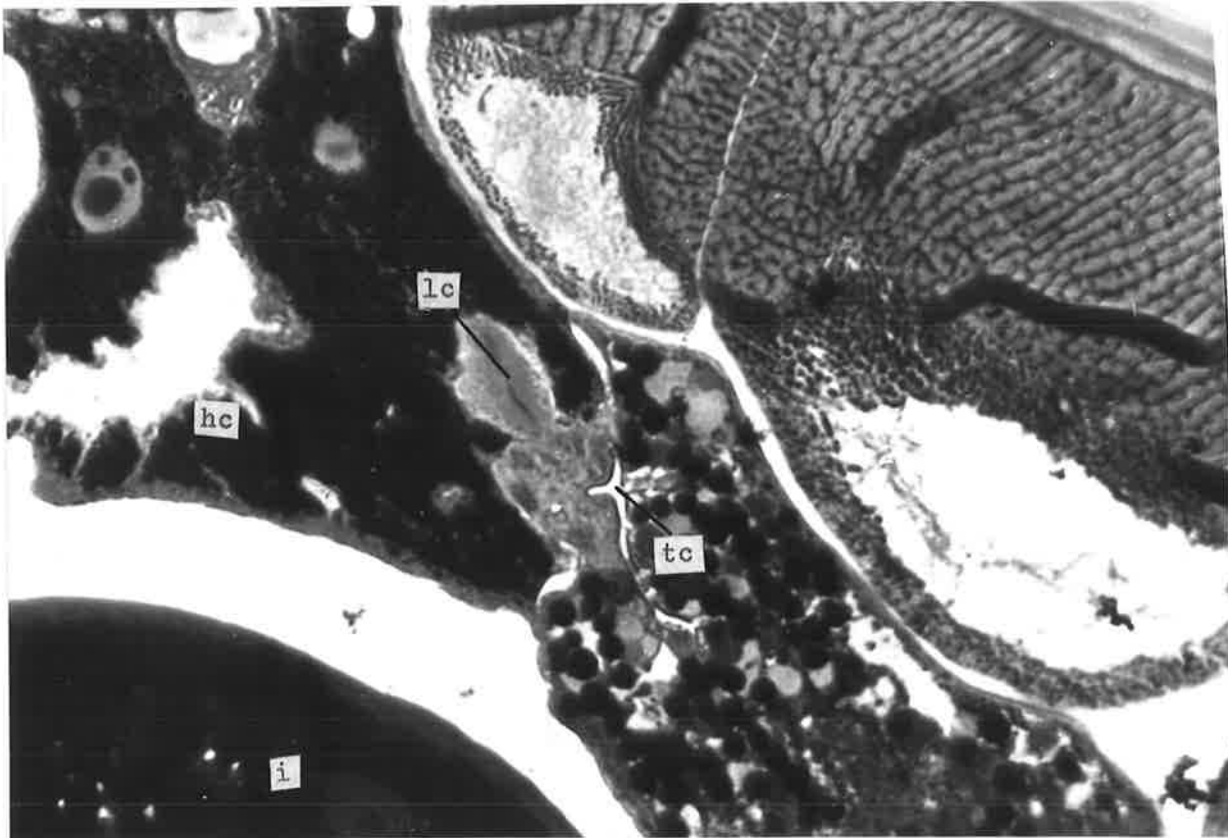


Plate 5. One subventral gland aligned against the adjacent hypodermal chord. The transverse canal is now associated with the lateral canal.

hc, hypodermal chord; i, intestine; lc, lateral canal; svg, subventral gland; tc, transverse canal.

X 1,500

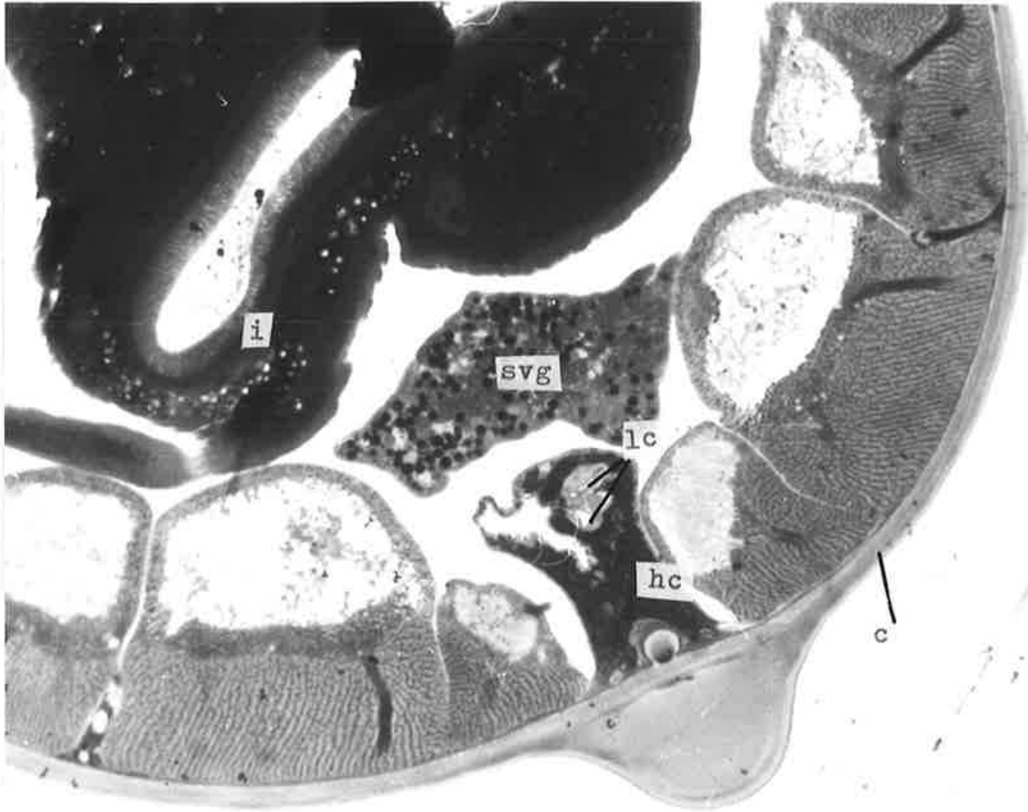


Plate 6. A subventral gland lying free in the pseudocoelomic cavity. Two branches of the lateral canal can be seen in the hypodermal chord.

c, cuticle; hc, hypodermal chord; i, intestine; lc, lateral canal; svg, subventral gland.

X 400

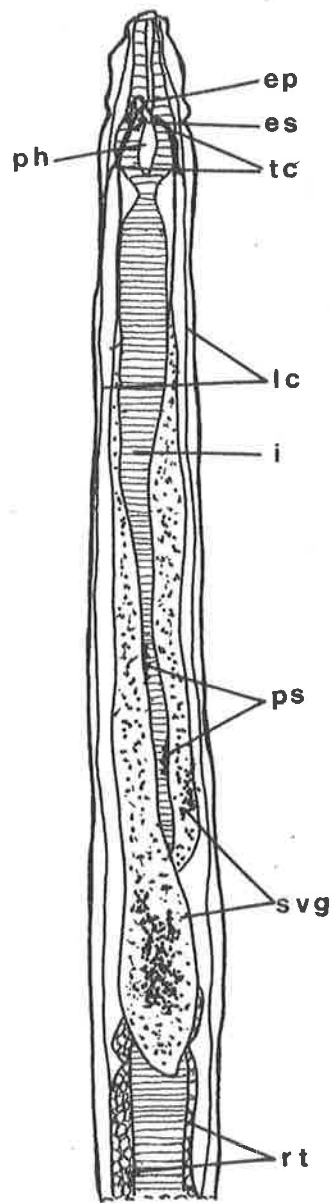


Fig. 6. The whole excretory system of *O. venulosum*.

ep, excretory pore; es, excretory sinus; i, intestine; lc, lateral canal; ph, pharynx; ps, pseudocoelomocytes; rt, reproductive tract; svg, subventral gland; tc, transverse canal.

The excretory system of *O. venulosum* (Fig. 6) consists of a cuticularised excretory pore (Plate 1) connected to an excretory sinus; there are two lateral canals lodged in the hypodermal chords and an asymmetrical pair of subventral glands. Transverse canals embedded in each subventral gland connect the lateral canals with the excretory sinus. All of these structures are intimately associated at the anterior end of the worm (Figs. 4 and 5).

Examination of thick sections did not lead to a complete understanding of the excretory system of *O. venulosum*. In particular, the connection between the excretory pore and the sinus, certain features of the relationship between the sinus and the hypodermis, and the structure of the posterior ends of the lateral canals are not known. Nevertheless, this work did produce an overall picture of the excretory system.

The subventral glands of *O. venulosum* are two large, ductless, sack-like cells which taper anteriorly (Plate 2). The larger member of each pair is approximately 0.5 mm long in the female and slightly smaller in the male. The anterior ends of the subventral glands are attached to the "tubular" components of the excretory system. The foremost tips of the two glands are contiguous and aligned in the midline of the worm beneath the pharynx. Here they are closely associated with the excretory sinus (Figs. 4 and 5 and Plate 2).

The excretory sinus is a large, flat, branching cell with a large median nucleus (Plate 2) and an extremely convoluted internal lumen (Plate 3). The sinus extends dorsally from the ventral excretory pore and comes to lie between the pharynx and the subventral glands, closely adhering to the dorsal surface of the glands (Plates 2, 3 and 4). The large nucleus of the sinus almost fills the anterior portion of the sinus, lying above the conjunct anterior ends of the two subventral glands. Posterior to this, the glands separate and the sinus bifurcates, one posterior branch of the sinus being associated with each subventral gland and the transverse canal embedded in it (Plates 3 and 4). There is also a fine attachment extending

from each side of the sinus to the corresponding hypodermal chord. With light microscope magnification it is not possible to ascertain whether this attachment is supportive, allows transfer of material or has some other function. As the connection between the sinus and the subventral glands disappears posteriorly, each gland moves closer to the adjacent hypodermal chord and the transverse canal embedded in each gland becomes associated with the lateral canal in the hypodermal chord (Plates 5 and 6). The precise nature of the connection could not be seen.

The lateral canals lying in the hypodermal chords are thick-walled tubes which end blindly, anteriorly. The transverse canals, embedded in the subventral glands, connect the lateral canals to the excretory sinus. Plate 4 shows a cross-section through the anterior end of one lateral canal. Plate 5 shows a lateral canal and its associated transverse canal both embedded in the hypodermis. The transverse canal, having branched off the lateral canal, runs parallel to it for a short distance before emerging from the hypodermis to connect with the subventral gland. Each transverse canal is embedded in a deep groove or invagination in the dorsal surface of the subventral gland. Anteriorly, this groove is overlaid by and intimately associated with the excretory sinus (Plate 4); posteriorly, it is closely attached to the hypodermal chord (Plate 5). The nature of the connections formed between the lateral canal and the excretory sinus and hypodermis could not be determined at the magnifications used. The tissues of the sinus and hypodermis overlie and perhaps line the transverse canal. It was impossible to discern whether there was a demarcation between the sinusoidal and hypodermal tissues.

Posterior to the junction between the subventral glands and the tubular excretory system, the lateral canals continue in the hypodermal chords for an unknown distance. The glands are wrapped loosely about the intestine and are usually attached lightly to it by one or two pseudocoelomocytes. The entire excretory system of *O. venulosum* is shown in Figure 6.

3.4 DISCUSSION

O. venulosum has a typical rhabditoid excretory system, very like that described by Waddell (1968), Lee (1970) and McLaren (1974). The present study has shown that a number of structural relationships are formed between the various organs comprising the excretory system. These are summarised schematically in Figure 7. From a light microscope study it is impossible to decide finally whether these relationships are merely structural or whether they are also functional. A considerable amount of ultrastructural and experimental work would be required to characterise precisely each one of the anatomical links indicated by an arrow in Figure 7. However, some speculative conclusions may be advanced.

The accepted interpretation of the structure of the tubular excretory system is that material is collected into the lateral canals, transferred through the transverse canals to the excretory sinus, and thence expelled to the exterior through the excretory pore. There is some evidence to support this view. However, the reason for the movement of material is unknown (Chitwood and Chitwood, 1950; Waddell, 1968; Bird, 1971).

The connections formed between the subventral glands and the tubular system are even more difficult to understand because less is known about them. The association of the subventral glands with the excretory sinus, lateral canals and transverse canals certainly provides the gland with routes by which (1) secretory products of the glands may be passed to the exterior through the excretory pore or into the hypodermis; (2) material collected in the lateral canals or sinus may be taken up by the subventral glands. The association may also have some supportive function.

The relationship of the glands and tubular system to other compartments in the nematode is also important. For the purpose of the present study, the association of the subventral glands with the pseudocoelomic fluid and pseudocoelomocytes is particularly significant. The link between the glands and the pseudocoelomic fluid will be discussed

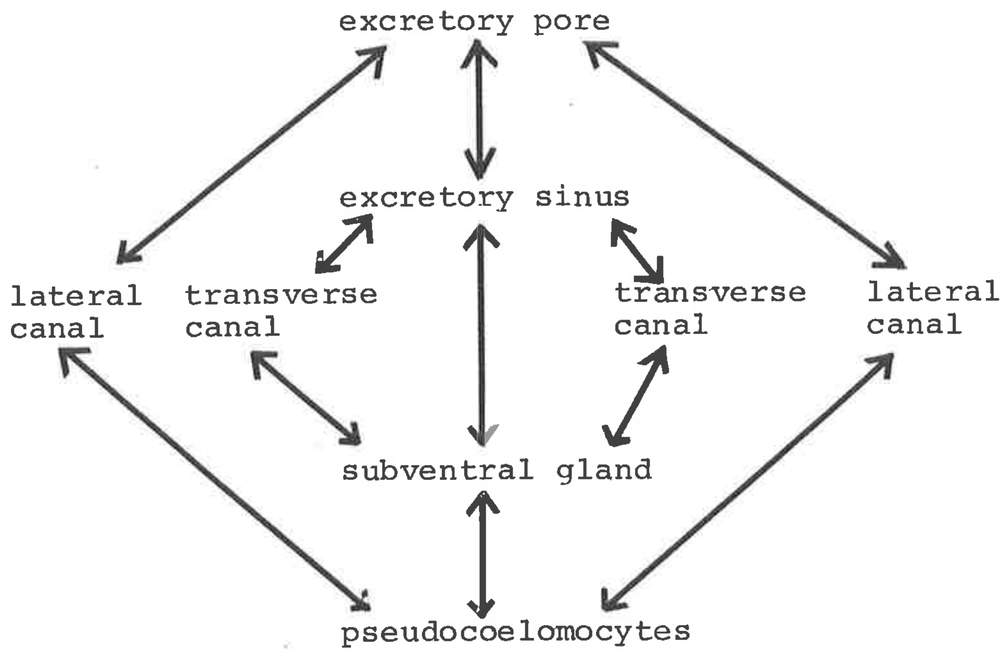


Fig. 7. Structural relationships between organs in the excretory system of *O. venulosum*.

in detail later. The pseudocoelomocytes may be supporting the large subventral glands structurally or they may be involved in the glands' functions. The few facts that are known about the various types of nematode pseudocoelomocytes support both hypotheses (Grassĕ, 1965; Peregrine, 1972; Boghen and Davey, 1975).

Although the anatomy of nematode excretory systems raises many interesting questions, they will not be further discussed here. This study of the excretory system of *O. venulosum* is intended, primarily, to provide an introduction to the structure of the rhabditoid excretory system and a framework within which the ultrastructural studies discussed in Chapter 4 and the experimental work described in Section 111 of this thesis can be understood.

CHAPTER 4: ULTRASTRUCTURE OF THE SUBVENTRAL GLAND

4.1 INTRODUCTION

4.2 METHODS

4.21 Preparation of Subventral Glands for Electron Microscopy

4.3 RESULTS

4.31 Ultrastructure of the Subventral Gland

4.32 Compartmentation in the Subventral Gland

4.4 DISCUSSION

4.1 INTRODUCTION

During the past decade, several studies on the ultrastructure of nematode subventral glands have been published (Table 2). Most have investigated the glands associated with the tubular excretory systems of Secernentean nematodes, and members of almost all Secernentean orders have now been examined. Although the glands in adult nematodes have been most thoroughly studied, various juvenile stages have also been investigated. The only Adenophorean gland examined so far is that of the adult *Enoplus brevis* (Narang, 1970). All studies have indicated that the subventral gland is usually an extremely active secretory cell, containing numerous secretion granules, golgi complexes, mitochondria and large amounts of endoplasmic reticulum. These morphological studies and experimental work associated with them have produced several hypotheses on the function of the subventral gland (see Chapter 5). However, no hypothesis has yet been validated, mainly because of the difficulty of devising effective physiological experiments (see Chapters 5 and 8).

The electron microscope work described in this chapter was intended to produce a description of the ultrastructure and intracellular compartmentation of the previously undescribed subventral gland of *O. venulosum*. It was proposed on the basis of this work, along with the morphological work described in Chapter 3 and a consideration of previous studies on the gland, to design new experimental approaches suitable for examining the function of the gland in *O. venulosum*.

4.2 METHODS

4.21 Preparation of Subventral Glands for Electron Microscopy

Subventral glands were fixed in isolation to ensure rapid penetration of the fixative. Even so, the glands were difficult to fix well, possibly on account of their thick cell coat (see 4.3). The fixation method used was developed by trial and error with advice from Dr. A.F. Bird (C.S.I.R.O. Division of Horticulture, Glen Osmond, S.A.).

Table 2. Ultrastructural Studies on Nematode Subventral Glands

SECERNENTEAN ORDERS	SPECIES	STAGE	REFERENCE
STRONGYLIDA	*Stephanurus dentatus*	adults	Waddell (1968)
	Nippostrongylus brasiliensis	adults	Lee (1970)
	Necator americanus	adults	McLaren (1974)
SPIRURIDA	*Breinlia sergenti*	micro-filaria	Kanasuntheram, Singh, Ho and Chan (1974)
RHABDITIDA	*Panagrellus redivivus*	adults	Narang (1972)
TYLENCHIDA	*Ditylenchus myceliophagus*	adults	Narang (1972)
	Ditylenchus dipsaci	4th-stage juveniles	Narang (1972)
	Heterodera rostochiensis	2nd-stage juveniles	Narang (1972)
ASCARIDA	*Anisakis sp.*	juveniles	Lee, Chen and Lin (1973)
	Phocanema decipiens	4th-stage juveniles	Davey and Sommerville (1974)

ADENOPHOREAN ORDERS	SPECIES	STAGE	REFERENCE
ENOPLIDA	*Enoplus brevis*	adults	Narang (1970)

Adult worms were obtained from infections which had been maintained for 35-90 days. Records were kept of the age and sex of each worm used.

Whole worms were placed in drops of warm BSS on microscope slides as previously described. Each worm was cut through just posterior to the subventral glands and the anterior section of the worm was immediately drawn into the adjacent drop of formaldehyde. The preparations which consisted of anterior sections of worms with the subventral glands extruding from them were processed as previously described. The specimens were trimmed in the last change of 100% ethanol and a single isolated subventral gland was aligned in each block of epon. Ultra-thin ("silver") sections were cut with a Reichert Om U3 ultramicrotome and mounted on copper grids coated with carbon and formvar. Grids were double-strained in warm, 60°C, 1% uranyl acetate and then in lead citrate. They were examined and photographed in a Seimens Elmiskop I electron microscope.

4.3 RESULTS

To obtain an accurate picture of the ultrastructure of this very large cell, many sections were cut and photographed. There were no discernible differences between glands taken from worms of different ages and sexes. The members of each asymmetric pair of glands appeared to be identical. As mentioned in Section 4.2, difficulties were encountered with producing good fixation of the gland. The micrographs were less clear than desired but nevertheless do give the necessary information for constructing an ultrastructural description of the gland.

The ultrastructure of the gland will be described in two sections. In the first, the general features of the gland's fine structure will be outlined and illustrated in a series of electron micrographs. In the second, special features of the compartmentation of the gland will be discussed and illustrated. Figure 8 is a drawing of a sagitally sectioned subventral gland. The regions indicated will be referred to when the compartmentation of the gland is described.

4.31 Ultrastructure of the Subventral Gland

In general appearance the gland has the typical ultrastructure of an active secretory cell (see Plates 8 and 9). It is packed with secretion granules, golgi complexes, mitochondria, granular endoplasmic reticulum and other organelles associated with secretory activity.

The nucleus of the gland is extremely large, 0.05 mm long and irregularly shaped. It has an "active" appearance, being composed mainly of lightly staining euchromatin dotted with masses of denser heterochromatin. The double membrane and nuclear pores can be seen (Plate 9). The irregular shape of the nucleus allows large tracts of cytoplasm to be closely associated with the nuclear contents. Finger-like protrusions from the nucleus can be seen ramifying the surrounding cytoplasm.

The most obvious and distinctive organelles seen in the gland at both light and electron microscope level are the great numbers of secretion granules. Secretion granules are defined here as membrane-bound structures, usually spherical in shape, which contain granular material presumed to be secretory product of the subventral gland (see Plate 8). They include (1) conventional densely-stained spherical secretion granules, 3-5 µm in diameter in profile; (2) irregularly-shaped and less densely stained secretion granules of similar size; (3) large pale pools of granular material, 10-50 µm in diameter in profile and irregular in outline. The granular contents of the pools may be distributed evenly in them, condensed into small aggregations or accumulated into one large mass or inclusio (Plate 10). The large inclusions often seen in the pools resembl the irregularly-shaped secretion granules in size, shape and density of staining (Plate 9). In some parts of the cell, the pools unite to form a continuous reticular array (Plate 20). The membranes surrounding all types of secretion granules are often indistinct (Plates 10 and 11) but can be seen clearly in some sections (Plate 12).

The subventral gland contains numerous mitochondria which, from their profiles in the micrographs (Plates 10 and 11), seem

to be dumbell-shaped, 5-8 μm long and to contain a few longitudinally aligned clavate cristae. Many golgi complexes can be seen in the gland and they vary in size and electron density. Their profiles are mostly small, 3-5 μm. The cisternae contain granular material and small vesicles can be seen budding off the complexes (Plate 10). No aggranular endoplasmic reticulum was seen in the subventral gland and granular endoplasmic reticulum appeared to fill every space in the cell not occupied by other organelles (Plates 7, 8 and 11). Its distribution like that of the other organelles varies from region to region within the gland (see 4.32).

Numerous large, longitudinally aligned arrays of microfilaments occur in the cytoplasm (Plate 13). Under high power (Plate 14) these arrays were seen to include individual microfilaments and striated bundles of filaments, similar to collagen in appearance. The arrays are located throughout the gland but are particularly common near the plasma membrane and nucleus.

The gland is enclosed by a cell membrane, elaborated to various degrees in different regions. The elaborations consist of microvilli and other protuberances (Plates 15 and 17). Many "canals" extend from the elaborate membrane into the cytoplasm for a short distance (Plate 16), surrounded by vesicles and canals. A smooth external coat or glycocalyx apparently encapsulates the entire gland, adhering closely to the tips of the microvilli (Plates 15, 16 and 17). This glycocalyx has a filamentous appearance (Plate 17) and fine convoluted strands of granular material seem to adhere to it at points along the cell surface (Plate 15).

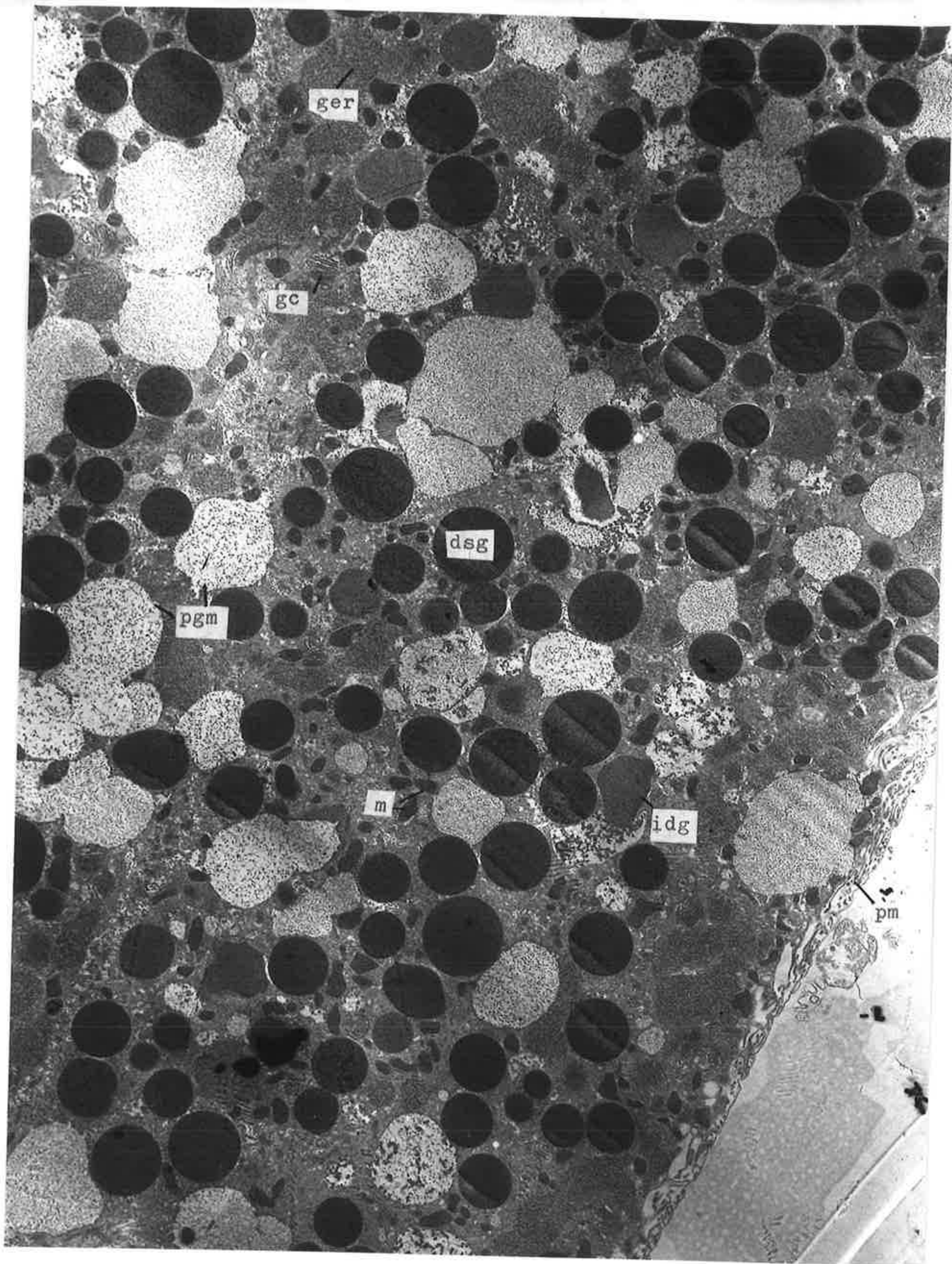


Plate 7. Typical organelles of the subventral gland: secretion granules, mitochondria, golgi complexes, granular endoplasmic reticulum.

dsg, dense secretion granule; gc, golgi complex; ger, granular endoplasmic reticulum; idg, irregularly-shaped dense granule; m, mitochondrion; pgm, pool of granular material; pm, plasma membrane.

X 3,000

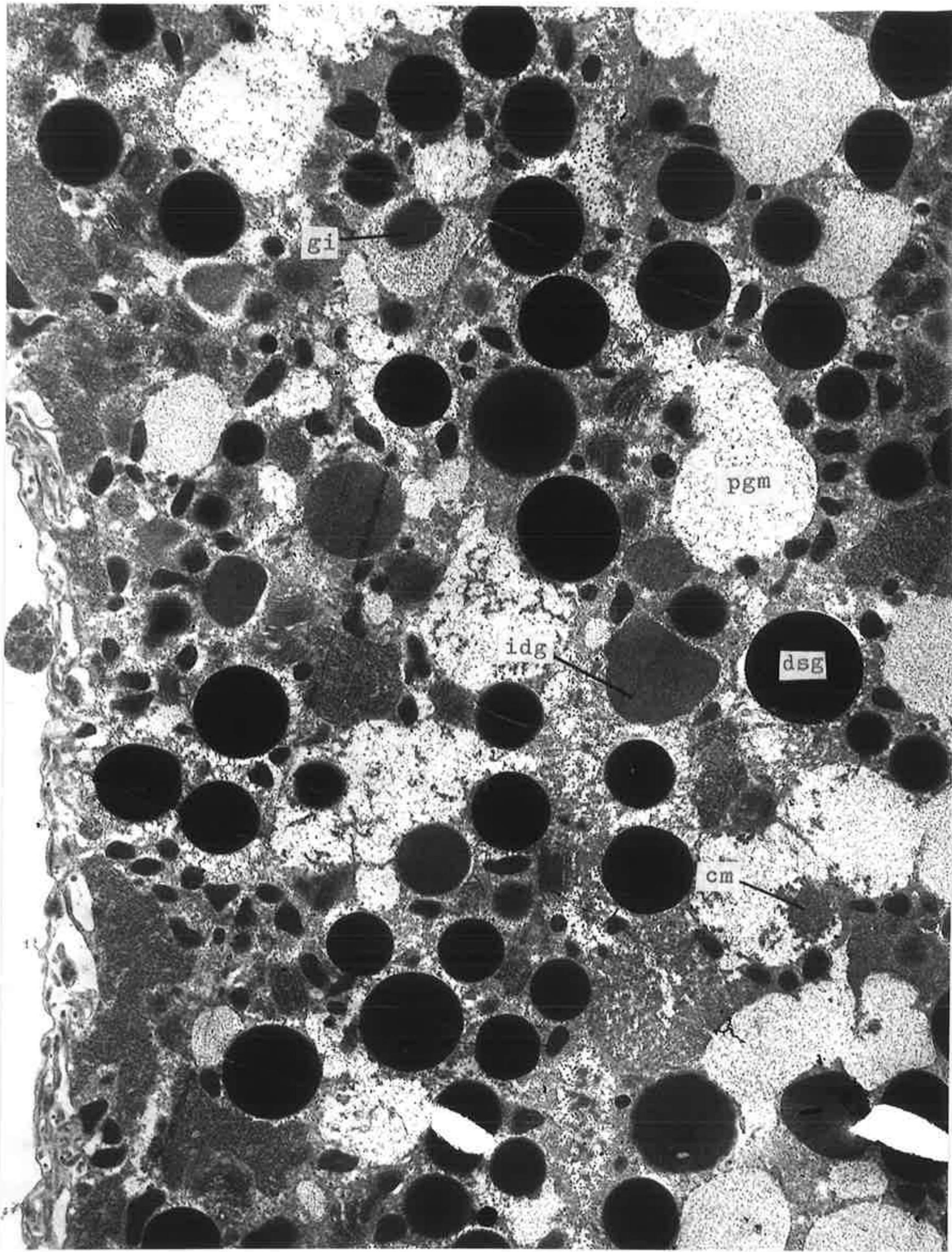


Plate 8. Membrane-bound secretion granules of the sub-ventral gland; pools of granular material; pools containing condensing material and large granular inclusions; irregularly-shaped dense granules; dense, spherical secretion granules.

cm, condensed material; dsg, dense secretion granule; gi, granular inclusion; idg, irregularly-shaped dense granule; pgm, pool of granular material.

X 3,000

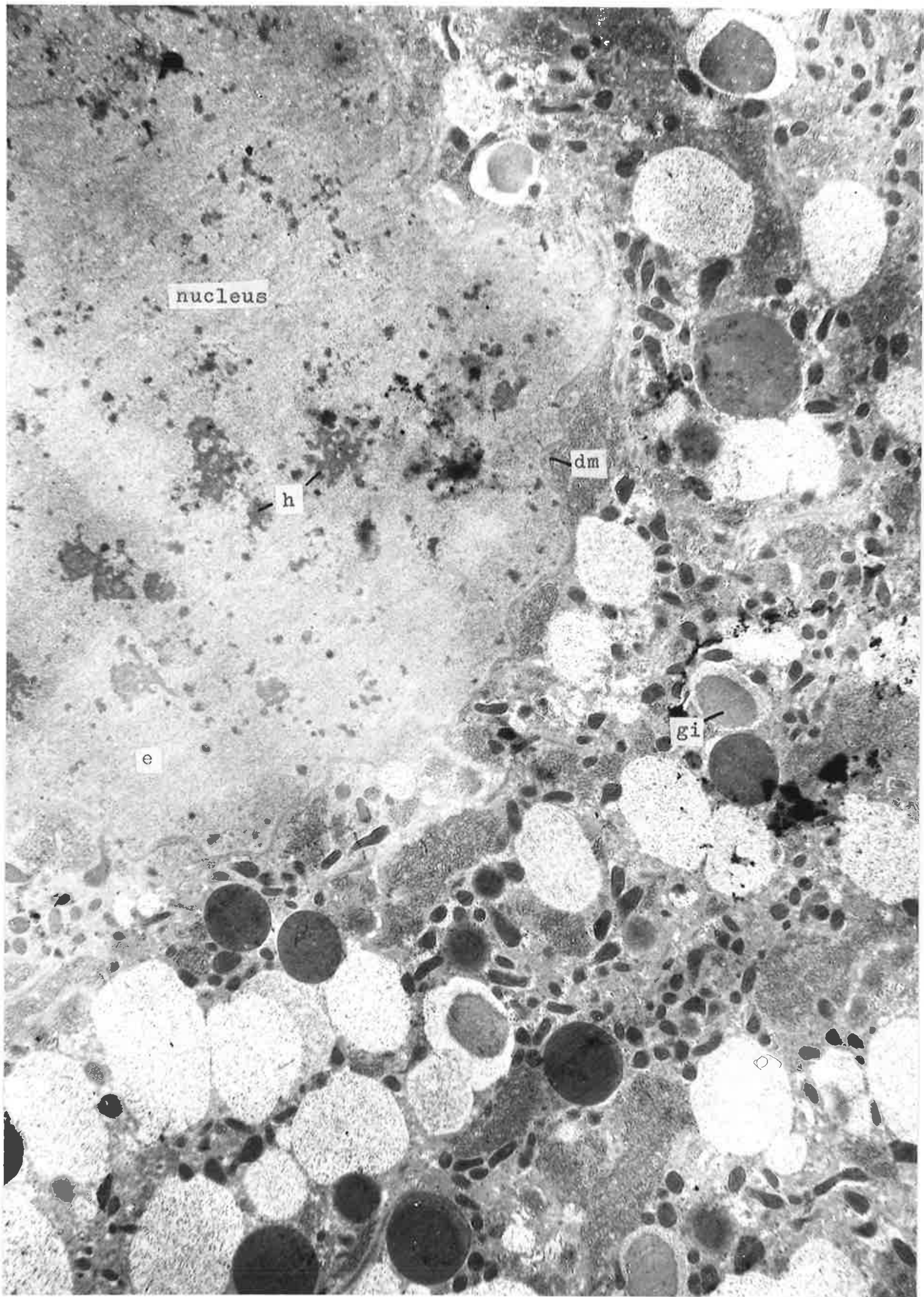


Plate 9. A section of the large irregularly-shaped nucleus of the subventral gland. Note the lightly stained euchromatin and the scattered aggregations of darker heterochromatin. Note that inclusions in pools resemble irregularly-shaped secretion granules.

dm, double membrane; e, euchromatin; gi, granular inclusion; h, heterochromatin; n, nucleus.

X 3,000

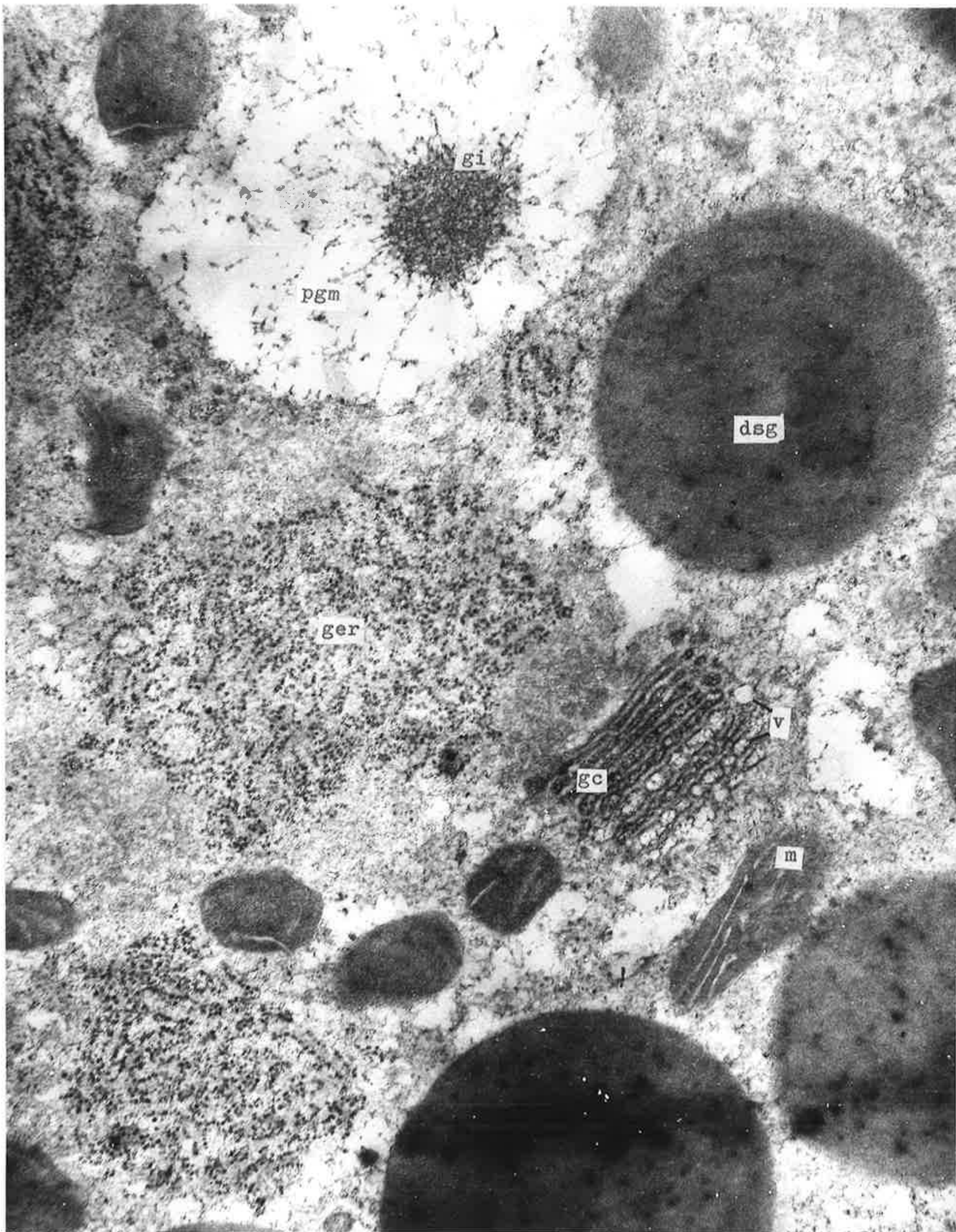


Plate 10. High power electron micrograph showing subventral gland organelles: granular endoplasmic reticulum, golgi complex budding off vesicles, dense secretion granules, pool with large granular inclusion.

dsg, dense secretion granule; gc, golgi complex; ger, granular endoplasmic reticulum; gi, granular inclusion; m, mitochondrion; pgm, pool of granular material; v, vesicle.

X 30,000

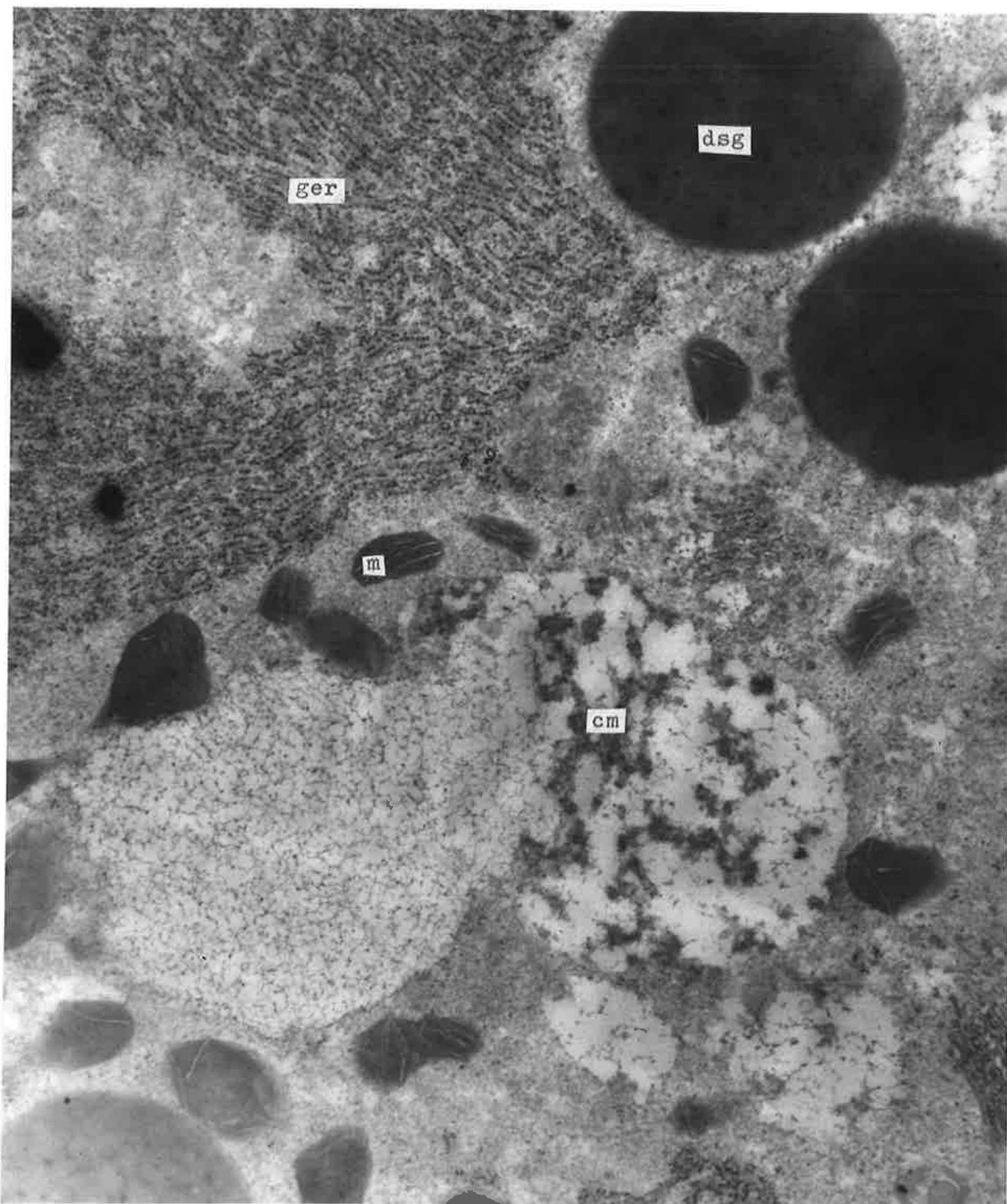


Plate 11. High power picture of subventral gland organelles. Note pool with homogeneous material at one end and condensed masses at the other.

cm, condensed material; dsg, dense secretion granule; ger, granular endoplasmic reticulum; m, mitochondrion.

X 24,000

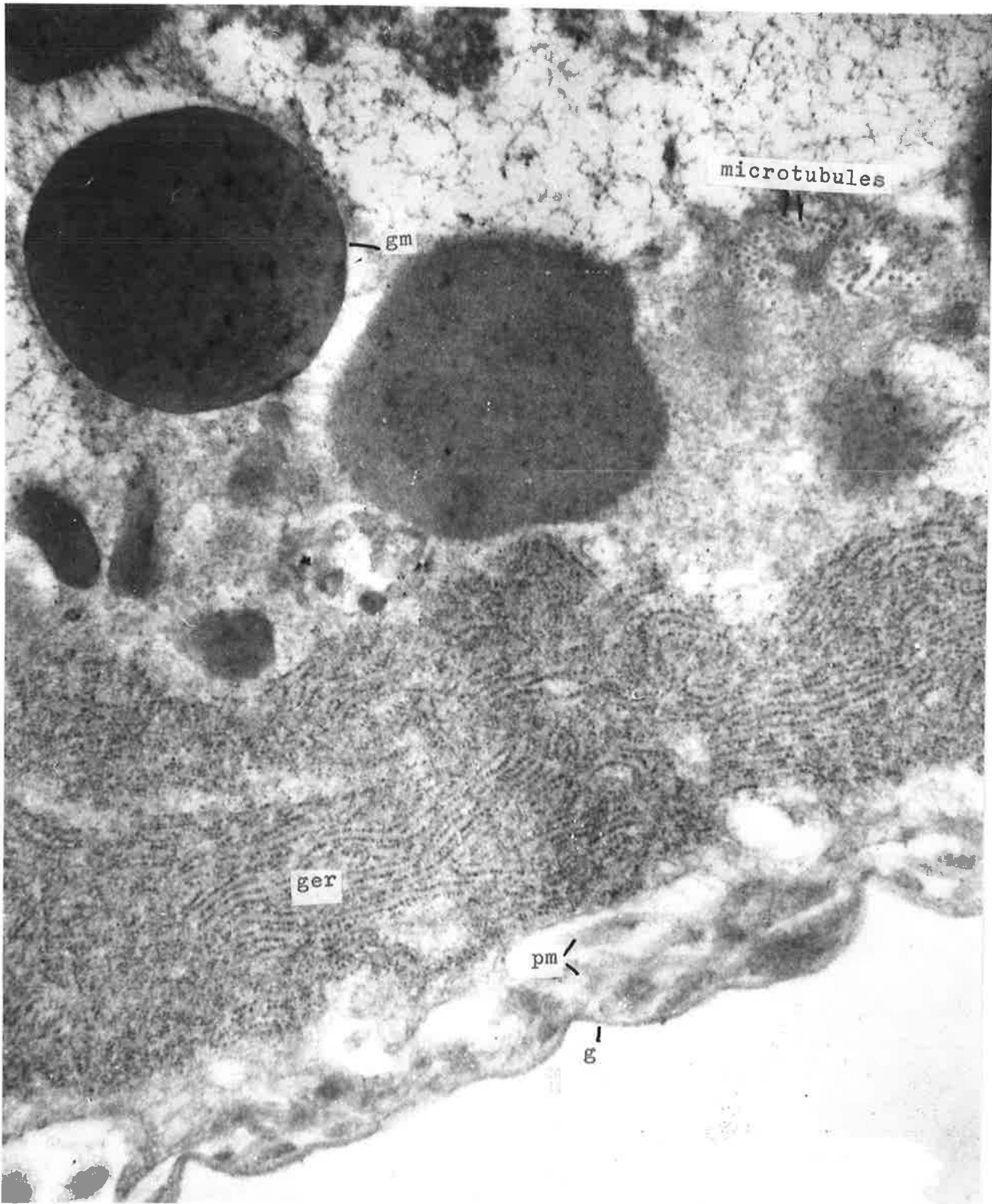


Plate 12. High power picture of subventral gland showing convoluted plasma membrane, glycocalyx, microfilaments and a sheet of granular endoplasmic reticulum such as is often seen just inside the cell membrane. The limiting membrane can be seen on one of the dense secretion granules.

g, glycocalyx; ger, granular endoplasmic reticulum; gm, granule membrane; m, mitochondrion; pm, plasma membrane.

X 30,000

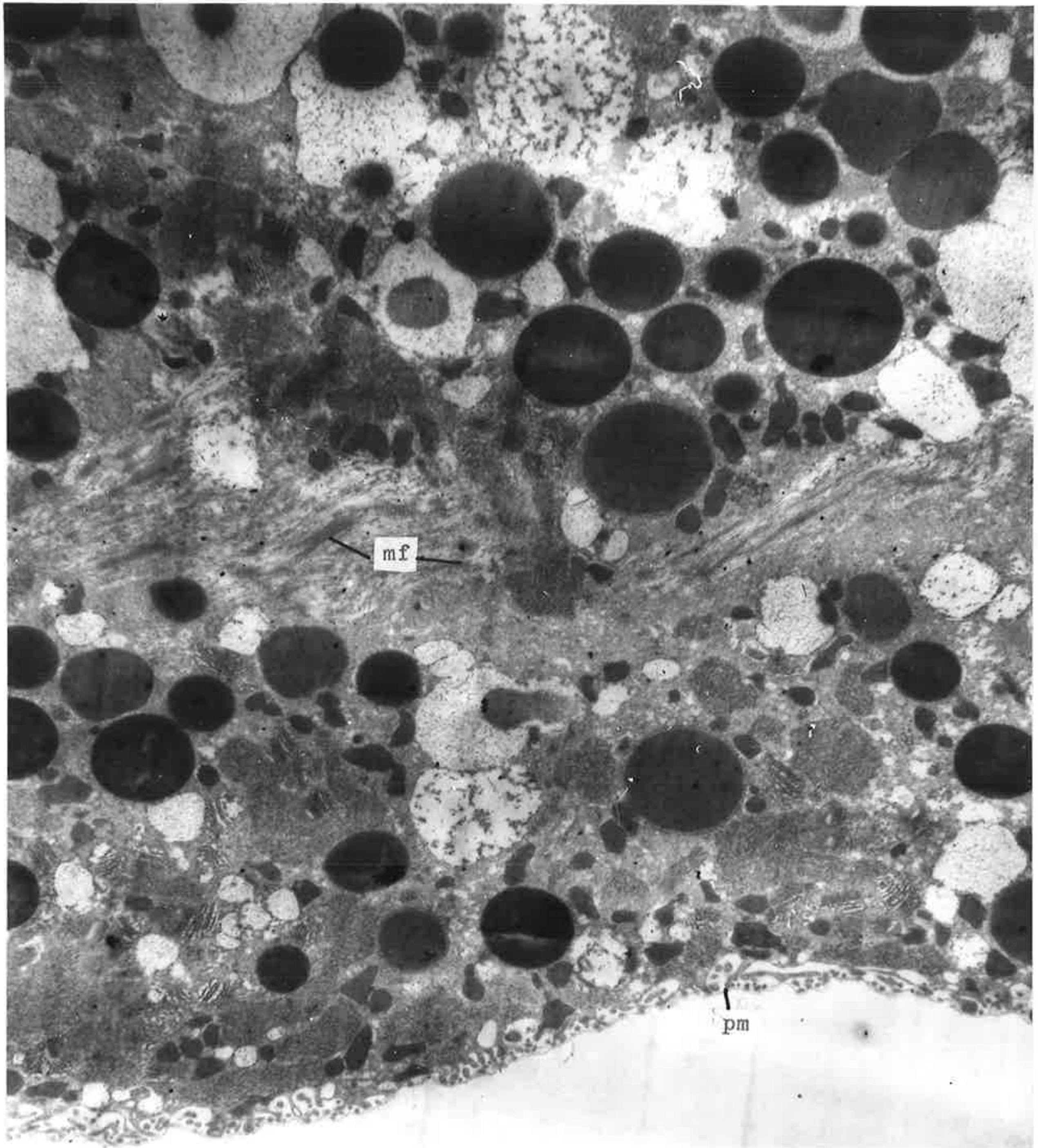


Plate 13. Arrays of microfilaments as they are often seen quite close to the membrane of the gland.

mf, microfilament; pm, plasma membrane.

X 3,000

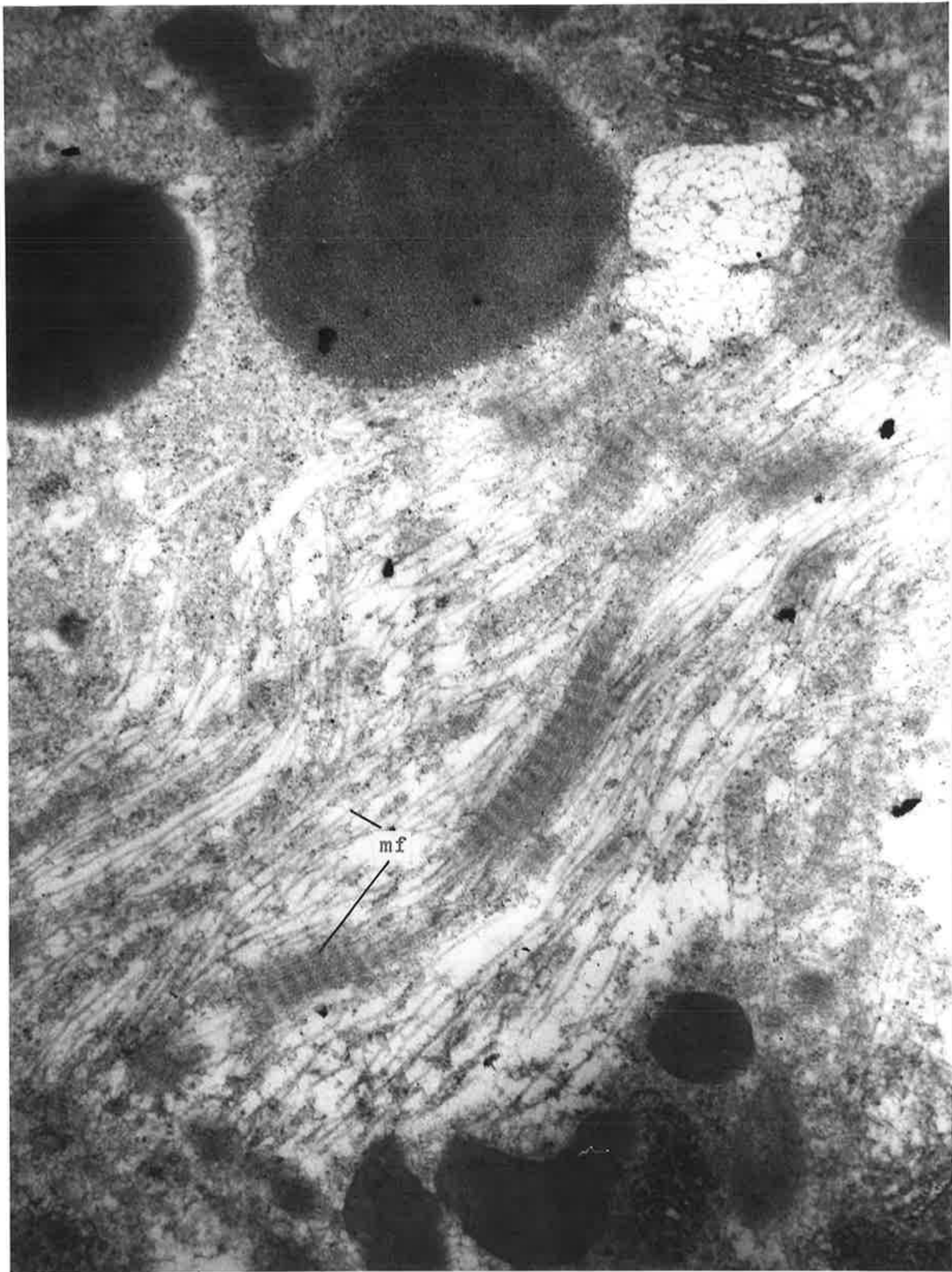


Plate 14. High power picture of an array of microfilaments showing the characteristic banding pattern of the bundles. Each band is about 0.1 μm wide.

mf, microfilaments.

X 30,000

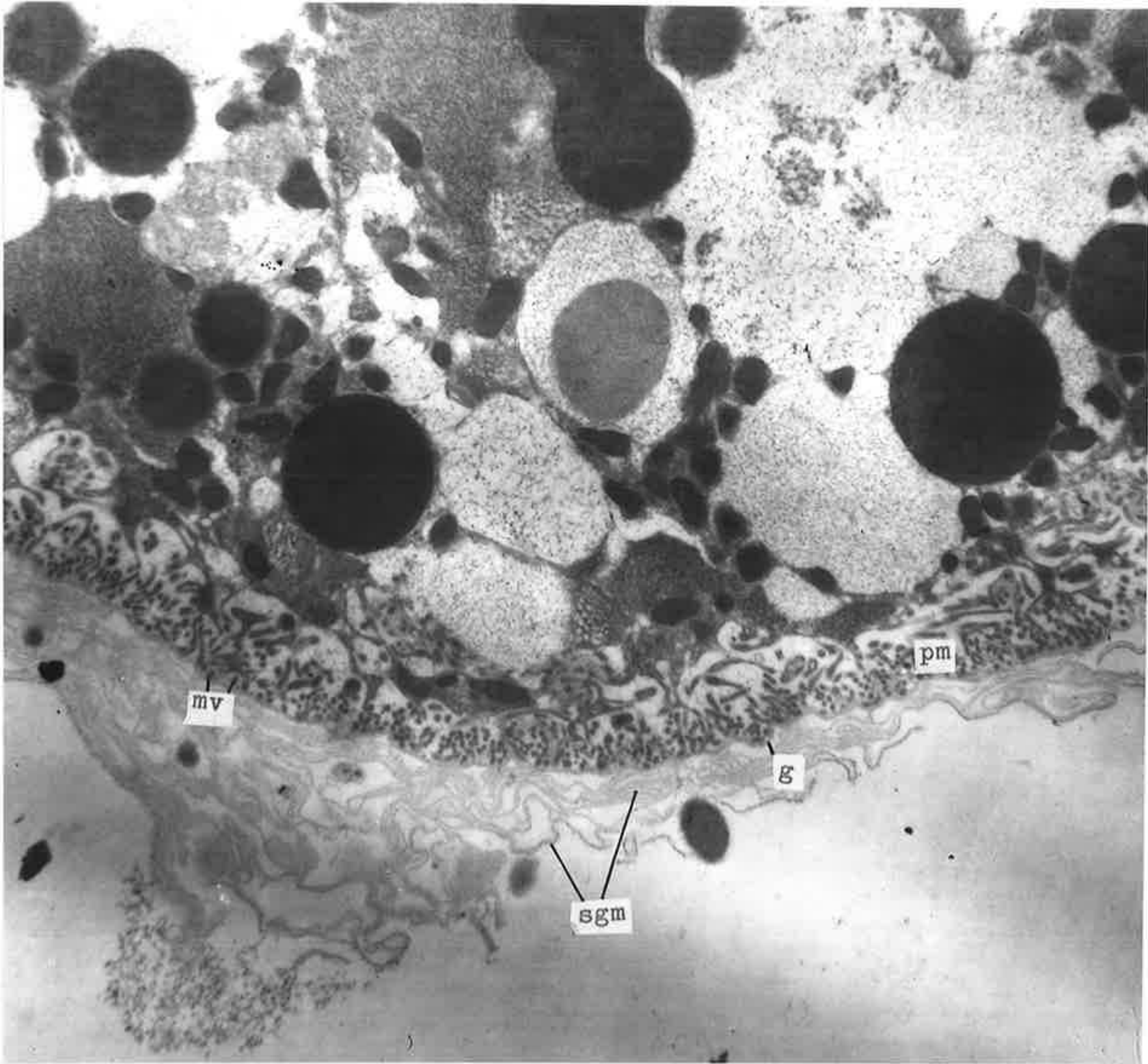


Plate 15. Plasma membrane of the subventral gland, showing microvilli, glycocalyx and strands of granular material.

g, glycocalyx; mv, microvillus; pm, plasma membrane; sgm, strands of granular material.

X 3,000

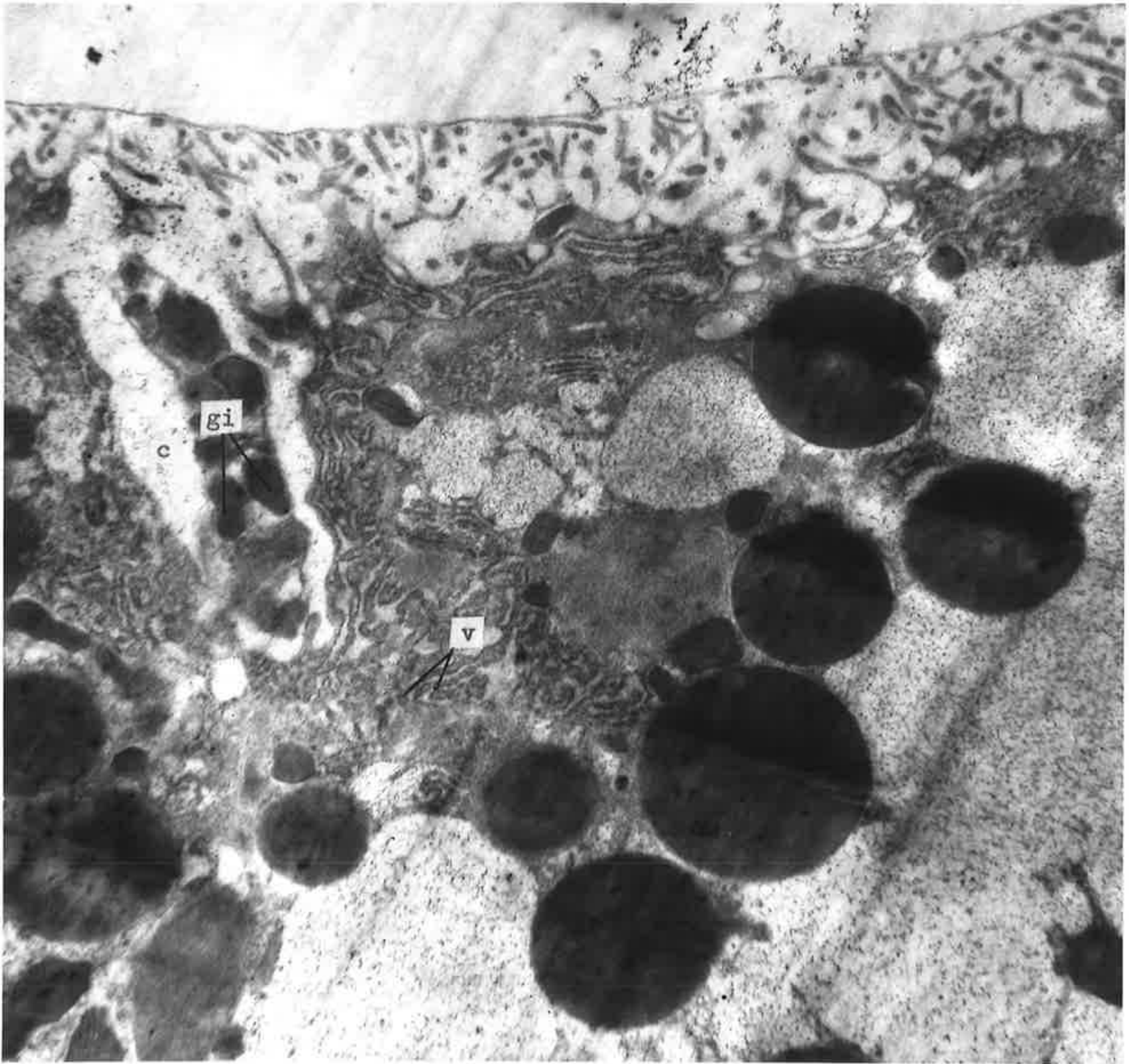


Plate 16. Membrane-bound canals extending from the plasma membrane into the cytoplasm and surrounded by canals and vesicles.

c, canal; gi, granular inclusion; v, vesicle.

X 9,000

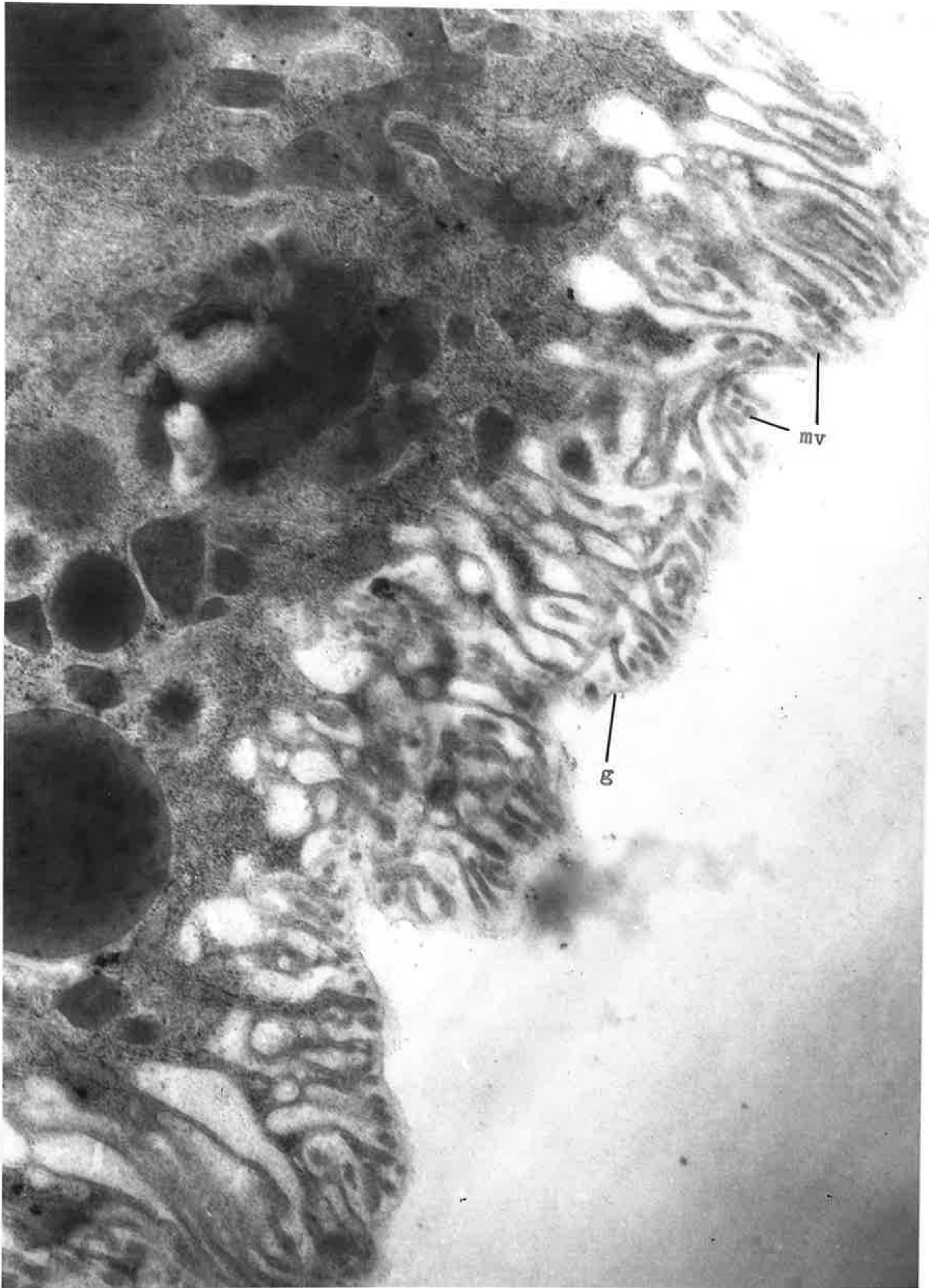


Plate 17. The filamentous external coat or glycocalyx which encloses the entire subventral gland.

g, glycocalyx; mv, microvillus.

4.32 Compartmentation in the Subventral Gland

For convenience in describing its compartmentation, the subventral gland can be divided transversely into four distinct regions. These are shown in Figure 8 and discussed below.

Region I: The posterior region of the gland has a densely granular appearance when viewed through a binocular microscope. Plates 18 and 19 show that this region is dotted with dense secretion granules which are more abundant near the membrane than the centre of the gland. The granules are interspersed with mitochondria, some small pale golgi complexes, a number of irregularly-shaped secretion granules and some tracts of granular endoplasmic reticulum. The cell membrane in Region I is extremely elaborate, containing many microvilli. Strands of filamentous material adhere to the glycocalyx in this region (Plate 15).

Region II: The large irregularly-shaped nucleus (Plate 9) is situated medially in Region II. This region contains all the organelles seen in Region I but with different distributions. Dense secretion granules are fewer although, as before, more common near the cell membrane. Pools of granular material are more abundant and mainly located in the middle of the gland near the nucleus. Mitochondria are also particularly numerous near the nucleus. The granular endoplasmic reticulum is more abundant than in Region I and golgi complexes are larger, more electron dense and more numerous. Large areas of endoplasmic reticulum are seen near the cell membrane and arrays of microfilaments, near the membrane and around the nucleus. The cell membrane is less elaborate than in Region I. No strands of granular material adhere to the glycocalyx; but, small canals extending into the gland from the membrane are common in this region (Plate 16).

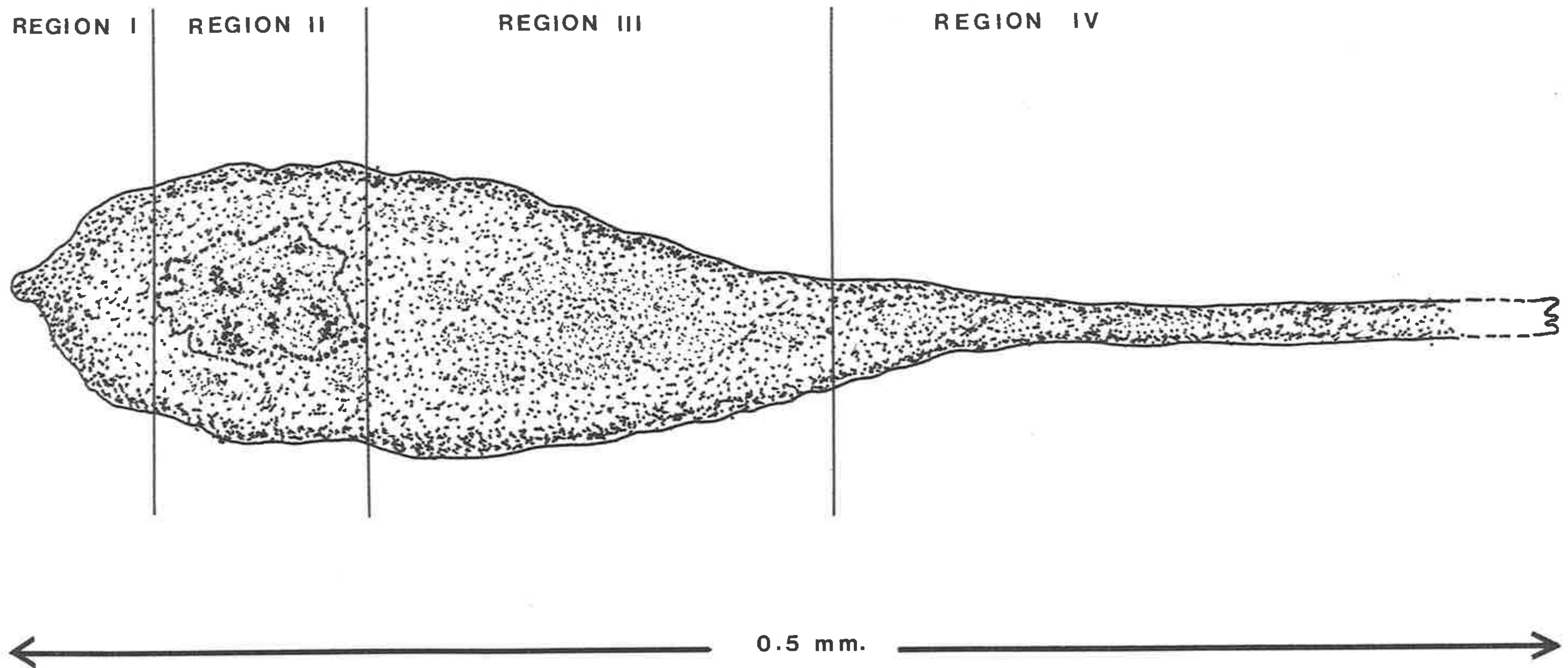


Fig. 8. Sagittal section through the subventral gland of *O. venulosum* showing regions of the gland referred to in the discussion of its compartmentation.

<u>Region III</u>: This region is very similar to Region II. However there is no nucleus present and the middle of the gland is filled by a vast network of pools of granular material (Plate 20). The distribution of the other organelles is the same as in Region II.

<u>Region IV</u>: Region IV is the narrow "neck" of the gland. Pools of granular material are less numerous than in Region III and do not unite to form a network. All the organelles which characterise the subventral gland - secretion granules, mitochondria, golgi complexes, granular endoplasmic reticulum, etc. - are seen along the entire length of this narrow anterior portion of the gland (Plate 21). The membrane is as elaborate as in Region I and, as can be seen in the transverse section from this region (Plate 22), constitutes a great proportion of the gland in this region.

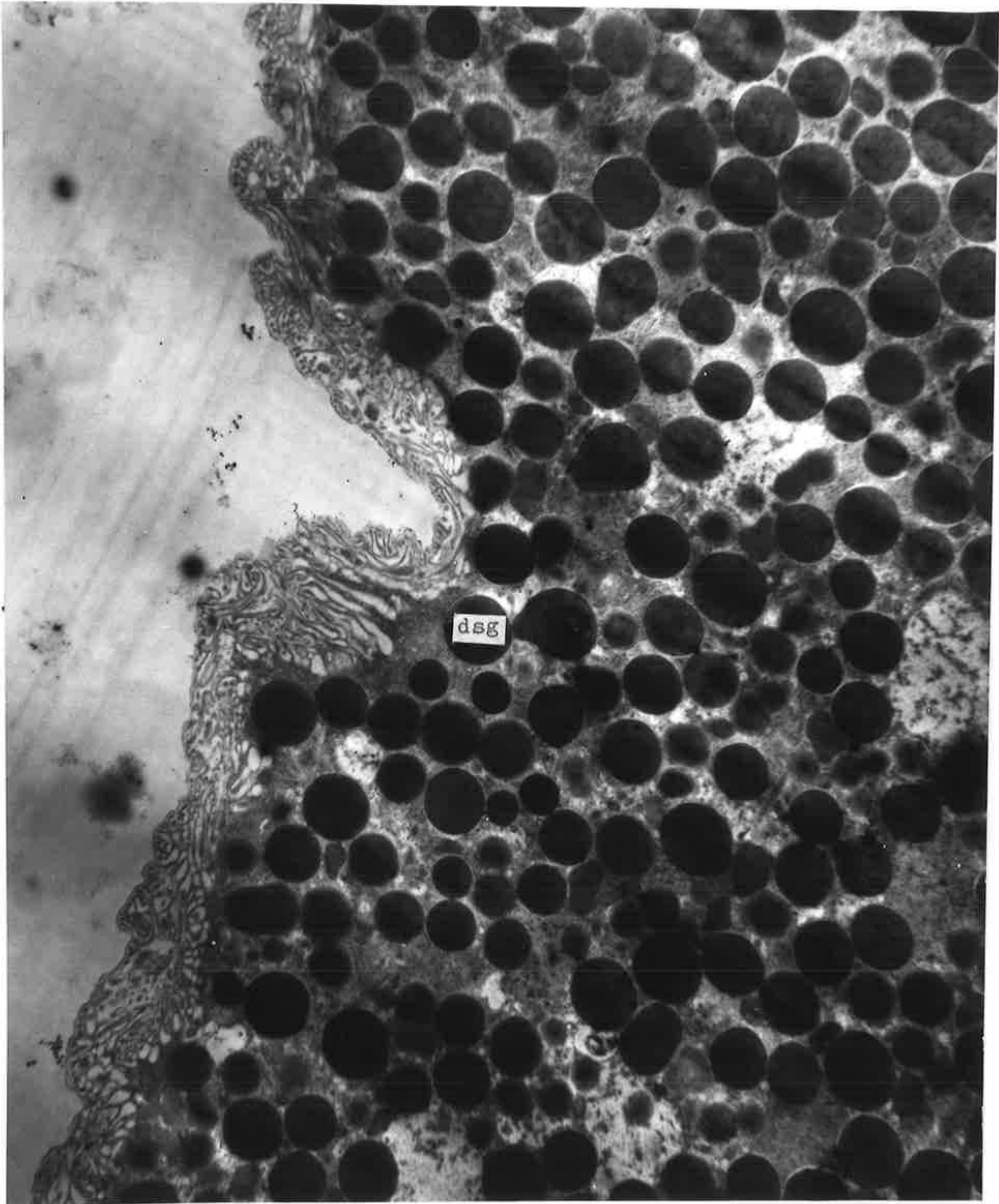


Plate 18. Region I: highly elaborate plasma membrane; many dense secretion granules.

dsg, dense secretion granule.

X 3,000

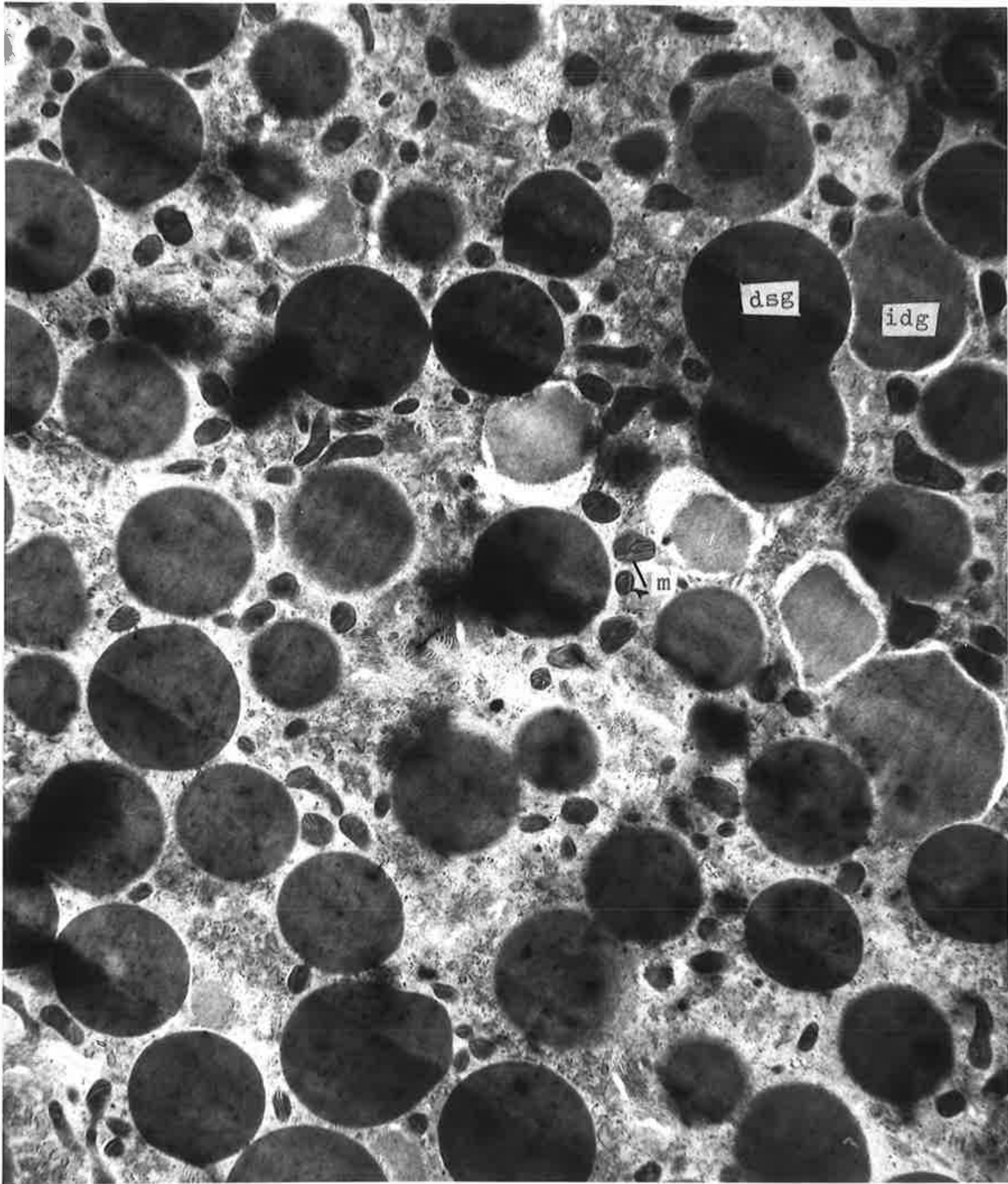


Plate 19. Region I: dense secretion granules, irregular secretion granules, mitochondria.

dsg, dense secretion granules; idg, irregularly-shaped dense granules; m, mitochondrion.

X 3,000

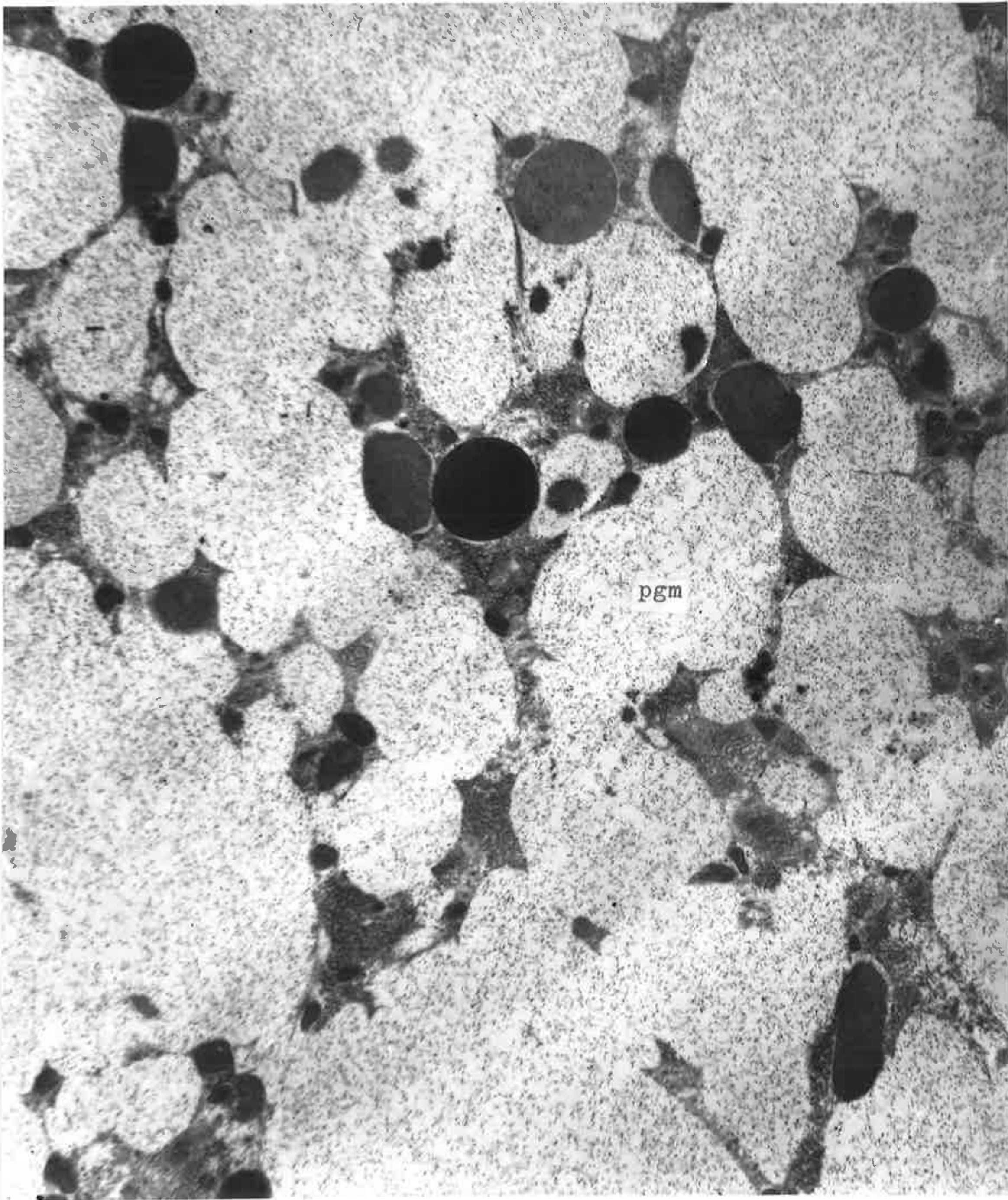


Plate 20. Region III: network of pools of granular material.
pgm, pool of granular material.
X 3,000

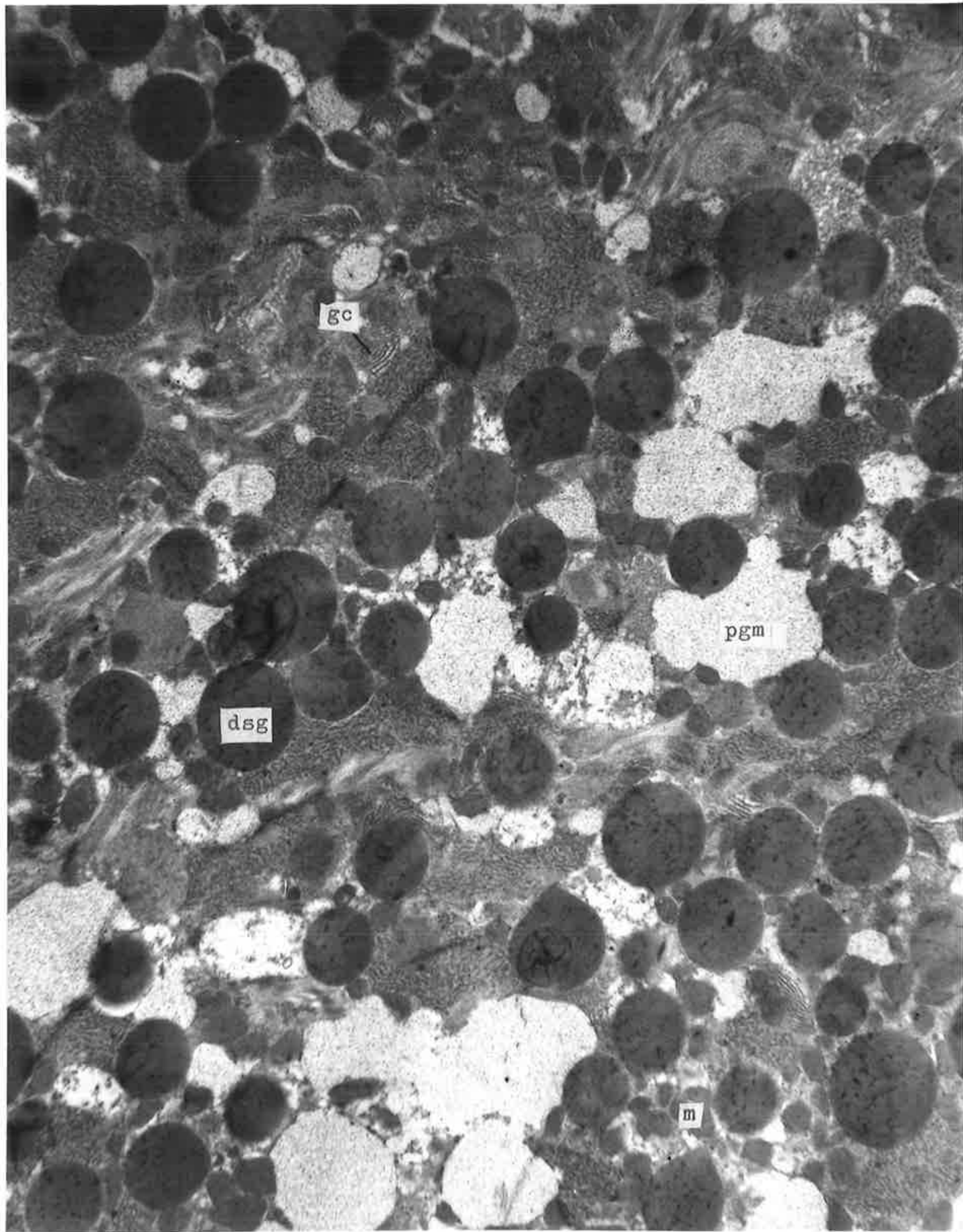


Plate 21. Region IV: note that the fine structure of the narrow anterior neck of the gland is similar to the rest of the gland.

gc, golgi complex; dsg, dense secretion granule; m, mitochondrion; pgm, pool of granular material.

X 3,000

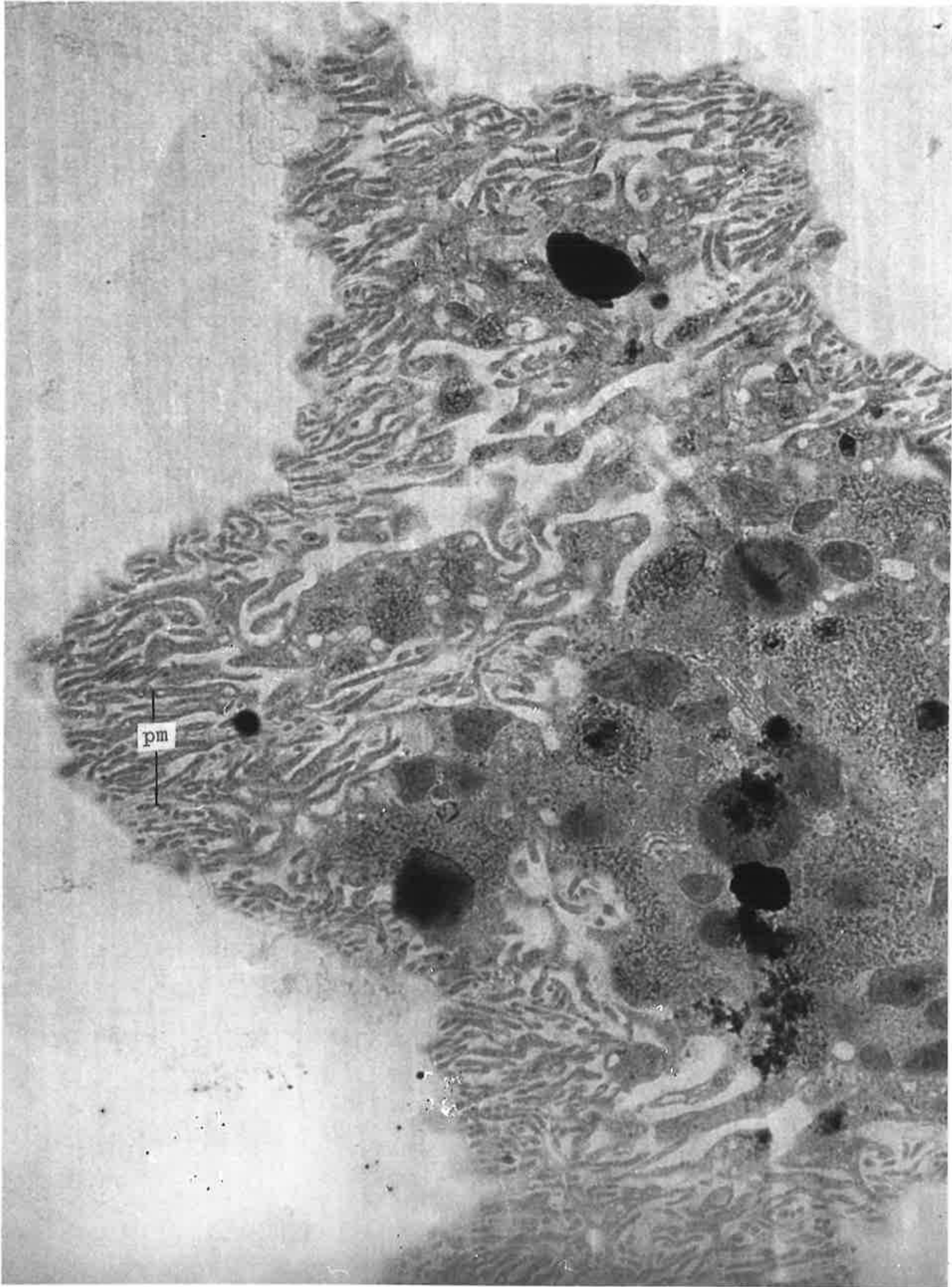


Plate 22. Region IV: very elaborate plasma membrane.
dsg, dense secretion granule; pm, plasma membrane.
X 3,000

4.4 DISCUSSION

The electron micrographs indicate that the subventral gland of *O. venulosum* produces large amounts of secretion throughout the life of the nematode. Examination of the micrographs produced some information on the formation and transport of secretory products within the gland and this suggested a new hypothesis for the function of the gland.

The various secretion granules seen in the subventral gland of *O. venulosum* may represent functionally different classes of secretion granules. However, it seems more likely that they represent stages in the formation of one class of secretion granule which may contain one or more secretory products (Plate 9). Each secretion granule seems to arise as a large pool of granular material within a mass of granular endoplasmic reticulum (Plate 11). This pool condenses to form an irregularly shaped secretion granule (Plate 10 and Fig. 9). Since the dense spherical secretion granules do not appear to be associated with the golgi complexes or the endoplasmic reticulum, it is assumed that these are the end-product of granule formation and arise from the irregularly-shaped secretion granules. The golgi complexes are presumably involved in the formation of granules but their role could not be determined. The mitochondria clustered around the pools of condensing material must supply the energy required for granule formation. Similar processes of secretion granule formation have been postulated for the subventral gland of *E. brevis* (Narang, 1970), *N. brasiliensis* (Lee, 1970) and *P. decipiens* (Davey and Sommerville, 1974).

An understanding of the movement of secretion granules within the subventral glands and a knowledge of their sites of discharge would help in understanding the function of the gland. The gland's relationship with the tubular excretory system (Chapter 3) and conclusions drawn from other studies (see below) suggest that the secretion granules move anteriorly through the subventral gland to discharge their secretory product into the transverse canal or the excretory sinus. However, the distribution of secretion granules in the subventral gland of *O. venulosum* does not suggest a net flow of granules towards the

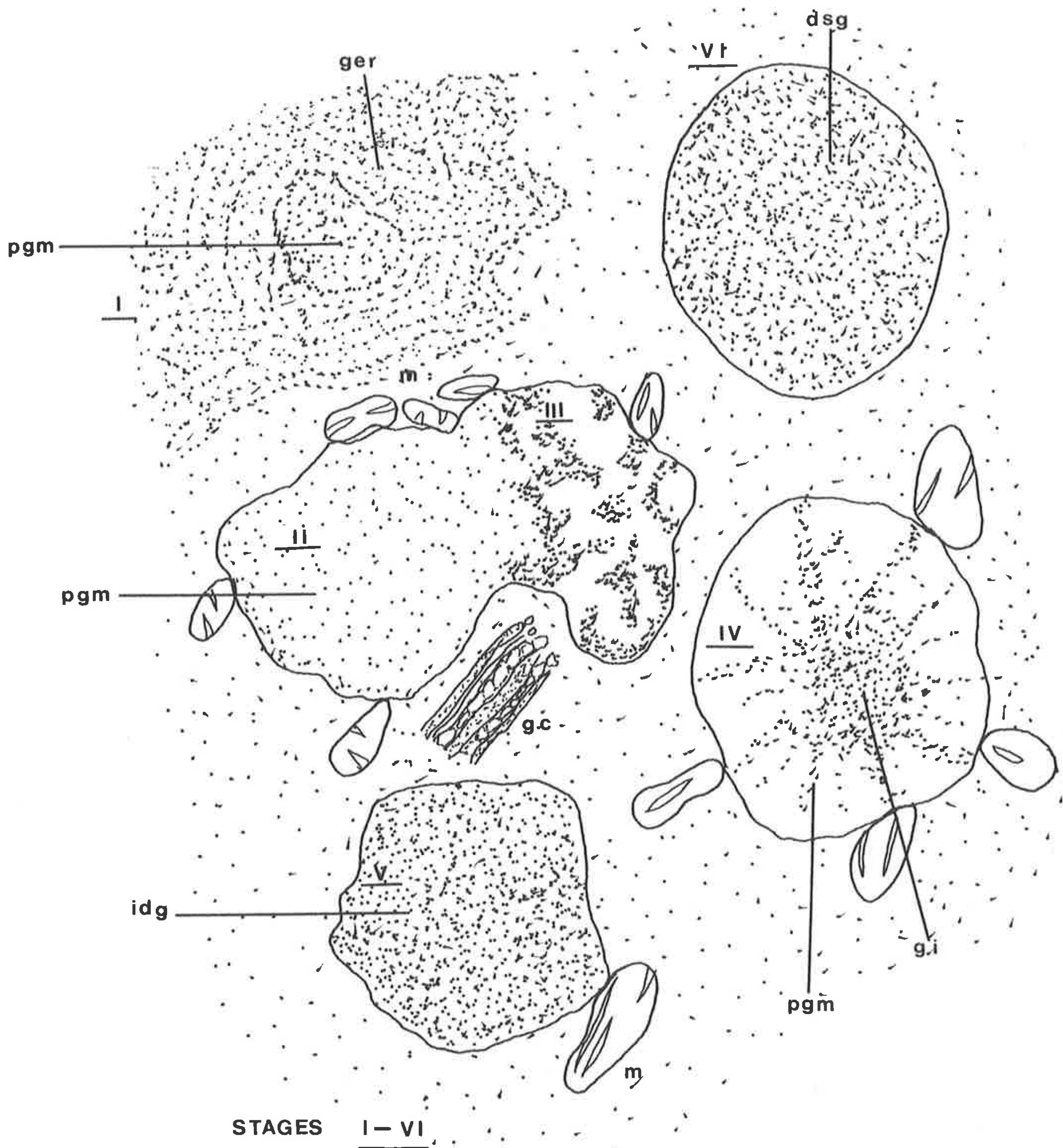


Fig. 9. Formation of secretion granules in the subventral gland of *O. venulosum*.

dsg, dense secretion granule; gc, golgi complex; ger, granular endoplasmic reticulum; gi, granular inclusion; idg, irregularly-shaped dense granule; m, mitochondrion; pgm, pool of granular material.

anterior. On the contrary, it suggests that secretion granules are initially formed in pools in the middle of the gland, particularly in Region III, and then moved towards the membrane of the gland. The concentration of secretion granules near the cell membrane at the posterior tip of the gland is particularly noteworthy. These granules must have moved posteriorly - not anteriorly - from their site of formation and may be discharging their contents across the cell membrane at this point. This hypothesis was supported by observation of isolated glands in BSS on a warm stage under phase contrast. Granular material appeared to accumulate in the medium adjacent to the posterior tip of the gland. Although isolated glands in warm BSS do not function normally (See Chapter 6), it is possible that at least some of the secretory products of the gland, in vivo, are discharged through the cell membrane into the pseudocoelomic fluid.

The structure of the cell membrane indicates that some interaction takes place between the gland and the pseudocoelomic fluid. The presence of microvilli and similar elaborations is usually associated with absorption; but such a surface can also be involved in secretion (Fawcett, 1966). From ultra-structure alone it is not possible to determine the nature of the interaction mediated by the membrane. The strands of granular material adhering to the surface of the posterior end of the gland (Plate 15) may represent secretory product discharged from the granules and still associated with the cell surface when fixed.

The relationship between the gland and the pseudocoelomic fluid is likely to be complicated. The gland may discharge its products into the fluid and also absorb from it precursors required for synthetic activities. The association of endoplasmic reticulum and even pools with the membrane (Plates 7 and 12) suggest that material may be absorbed through the gland's membrane and collected in the endoplasmic reticulum for use in granule formation. The small, irregularly-shaped, dense granules within the "canals" extending from the membrane (Plate 16) may be secretion granules which are discharging their contents through the membrane. Interaction between the gland and fluid

would be expected to be most intense where the membrane is most elaborate, i.e. at the posterior tip of the gland and along its narrow anterior "neck".

Movement of material within the gland is probably achieved through the combined activity of microtubules and filaments (Buckley, 1974). Low fixation temperatures probably destroyed the microtubular matrix which is not visible in micrographs (Murphy and Tilney, 1974). The arrays of microfilaments may be associated with movement; but their arrangement, particularly the presence of striated bundles, suggests a cytoskeletal role (Fawcett, 1966).

Other ultrastructural studies (Table 2) have indicated that all nematode subventral glands are like that of *O. venulosum* in their ultrastructure and compartmentation. However, there are some important differences in the glands that have been examined.

For example, the glands of *B. sergenti* and *P. redivivus* appear to be relatively "innactive" when compared with the glands examined in other species. They contain few secretion granules or golgi complexes, granular endoplasmic reticulum, etc. Other workers have not commented at length on the membrane or compartmentation of the gland. There is evidence that the subventral gland may change ultrastructurally during the life cycle of the nematode. For instance, Davey and Sommerville (1974) have reported ultrastructural changes in the subventral gland of *P. decipiens* associated with ecdysis. It is possible that the ultrastructural differences observed between the subventral glands of different species may be manifestations of the changing role of the gland within the life of the nematode.

So far, workers have examined the function of the gland only in relation to the tubular excretory system. They have assumed that all of the gland's secretion must be released through the excretory pore. The present study on the ultrastructure of the subventral gland in *O. venulosum* has suggested that at least some of the gland's secretory product may pass across the gland's membrane to be utilised in the pseudocoelomic fluid or transported in the fluid to other parts of the worm.

5.1 REVIEW OF EXPERIMENTAL WORK ON THE SUBVENTRAL GLAND

During the last ten years several histochemical and biochemical studies have been made on the subventral glands of three Secernentean orders - the Rhabditida, Strongilida and Ascarida. This limitation is a manifestation of the problems associated with analysis of individual organs within the tiny nematodes which comprise most Secernentean and Adenophorean orders.

Most of the compounds sought and found in subventral glands have been histolytic enzymes; but other substances - proteinase inhibitors, lipids, glycogen - have also been shown to be present in some cases (Table 3). Some of these have been located in the membrane-bound granules of the gland. For example, acetylcholinesterase was seen in all the granules of *T. colubriformis* and in one of the two types of granule present in the glands of *N. brasiliensis* (McLaren *et al.*, 1974). Similarly, aminopeptidase appears to be stored in the granules located in the subventral glands of fourth-stage juveniles of *P. decipiens* (Davey and Sommerville, 1974).

A number of theories have been proposed to account for the compounds found in the subventral glands. Some deal with functions appropriate to hydrolytic enzymes in general; some postulate that particular hydrolytic enzymes or other compounds apparently secreted by the glands, perform unique functions of great importance to the nematode.

The most widely accepted theory concerning hydrolytic enzymes is that these are released through the excretory pore to effect exodigestion (Mueller, 1927; Lee, 1970; Romanowski *et al.*, 1973; McClaren, 1974). Nematodes may extracorporeally digest both food and host tissue which must be penetrated during migration. McClaren *et al.* (1974) have proposed that all the "anterior" glands of the nematode - the oesophageal, subventral and amphidial - may secrete enzymes used in exodigestion. Their findings suggested that each of the glands may secrete different enzymes in different species (Table 4) and that different glands may secrete the same enzyme in an individual nematode.

Table 3: Compounds Located in the Subventral Glands of Various Nematode Species.

Compound	Species	Reference
non-specific esterase	*Phocanema decipiens*	Davey & Kan (1968)
	Nippostrongylus brasiliensis	Lee (1970)
	Stephanurus dentatus	Romanowski, Thompson & Madden (1971)
	Necator americanus	McLaren, Burt & Ogilvie (1968)
non-specific cholinesterase	*N. brasiliensis*	McLaren *et al* (1974)
	Trichostrongylus colubriformis (4th stage)	McLaren *et al* (1974)
acetylcholinesterase	*Oesophagostomum radiatum*	Bremner *et al* (1973)
	N. Americanus	McLaren *et al* (1974)
	N. brasiliensis	McLaren *et al* (1974)
	T. colubriformis	McLaren *et al* (1974)
non-specific aminopeptidase	*S. dentatus*	Romanowski *et al* (1973)
leucine aminopeptidase	*P. decipiens*	Davey & Kan (1968)
	N. brasiliensis	Lee (1970)
	Anisakis larvae	Lee, Chen & Lin, (1973)
protease	*S. dentatus*	Romanowski, Rhoads & Malakatis (1973)
proteinase inhibitors	*S. dentatus*	Rhoads & Romanowski (1974) and Rhoads *et al* (1978)
non-specific hydroytic enzymes	*S. dentatus*	Rhoads & Romanowski (1974)
Glycogen	*N. brasiliensis*	Lee (1970)
	S. dentatus	Romanowski *et al* (1971)
	P. decipiens (4th stage)	Davey & Sommerville (1974)
lipid	*N. brasiliensis*	Lee (1970)
	S. dentatus	Romanowski *et al* (1971)

Table 4.

Enzyme	Species	Anterior Glands		
		Oeso-phageal	Amphi-dial	Sub-ventral
non-specific esterase	*N. americanus*	1	1	0
Acetylcholinesterase	*N. americanus*	1	1	0
	N. brasiliensis	0	0	1
	T. colubriformis (4th stage)	1	0	1
	O. radiatum	0	0	1
	O. venulosum	1	0	0
leucine aminopeptidase	*N. americanus*	1	0	0

Enzymes identified in the anterior glands of nematodes by McClaren *et al.* (1974).

1 indicates that the enzyme was found in the gland
0 indicates that the enzyme was not found.

N.B. *N. americanus* was assayed for all three enzymes. The other species were only assayed for Ach.

Unique functions have been ascribed to two hydrolytic enzymes located in the subventral glands of some species, namely acetylcholinesterase and aminopeptidase. Lee (1970) suggested that acetylcholinesterase (Ach) produced by the subventral glands and released through the excretory pore of adult *N. brasiliensis* may inhibit local nerve endings in the host mucosa and prevent peristaltic dislodgement of the nematodes. Large amounts of Ach have been found in some other nematode species and this enzyme may be functioning as a "biochemical holdfast" in every case (Ogilvie and Jones, 1971; McLaren *et al.*, 1974). Davey and his co-workers (Davey and Sommerville, 1974) have shown in *Phocanema decipiens* that an aminopeptidase appears to be produced and stored in the secretory granules of the subventral gland. Ecdysis in *Phocanema* appears to be effected by hormonal activation of the subventral gland's aminopeptidase and its subsequent release through the excretory pore to act on the sheath.

Various operations have been postulated for other compounds believed to be secreted by the gland. Rhoads and Romanowski (1974), for example, found high specific activity of proteinase inhibitors in the subventral glands of *S. dentatus* and hypothesised that these inhibitors may be secreted to protect the worm from attack by its own or environmental proteinases. Maggenti (1962) observed the production of the gelatinous matrix by the subventral gland in female *Tylenchus semipenetrans* (tylenchids lack a functional rectum, the organ implicated in the production of the gelatinous matrix in some other species -Maggenti and Allen, 1960). In some nematode species the subventral gland is reputed to store energy in the form of glycogen, e.g.*S. dentatus* (Romanowski *et al.*, 1971).

Davey and Sommerville (1974) have speculated that the subventral gland "...may have special functions in the worms, perhaps akin to those of the mammalian liver, or the fat body in insects." The range of compounds thought to be secreted by the gland provides indirect support to the hypothesis that the gland is a key organ of intermediary metabolism, perhaps

secreting different products at different times as the needs of the individual vary.

5.2 INTRODUCTION TO EXPERIMENTS ON *O. VENULOSUM*

In the present study the morphological work on *O. venulosum* had indicated an apparent structural relationship between the gland and the pseudocoelomic fluid. Thus it seemed that a study of the functional nature of this relationship might produce information on the movement of secretory products within the worm, complimenting previous experiments on the release of secreted material into the external milieu. New experimental techniques were sought since the progress of work on the gland and, indeed, on other aspects of nematode physiology seemed to require the development of new methods. Many failures were met searching for new approaches to the gland. Initially attempts were made to apply methods of use in insect physiology to the gland (e.g. ligature, collection of secretion), but even such a large subventral gland proved too small for the application of such manipulative techniques. Eventually two lines of approach were used, viz. radioisotope studies, gel electrophoresis.

It was hoped that radioisotope labelling of live worms in culture combined with scintillation counting and light and electron microscope autoradiography would answer many questions about the movement of material within the gland and between the gland and other compartments in the worm. However, this work was not successful (Chapter 6). Electrophoresis was used to examine the functional relationship between the subventral gland and the pseudocoelomic fluid. This work was more productive and is discussed in Chapter 7.

CHAPTER 6: RADIOTRACER STUDIES

6.1 INTRODUCTION

6.2 MATERIALS AND METHODS

- 6.21 Radioisotope Techniques
- 6.22 Culture Medium
- 6.23 Incubation of Whole Worms
  - 6.231 Control procedures
- 6.24 Incubation of Isolated Glands
  - 6.241 Viability of isolated glands

6.3 RESULTS

- 6.31 Results for Whole Worms
  - 6.311 Results for controls
- 6.32 Results for Isolated Glands
  - 6.321 Viability of glands in medium

6.4 DISCUSSION

6.1 INTRODUCTION

Labelled amino acids were administered to adult *O. venulosum* in culture as a preliminary to investigating the incorporation of amino acids into proteins in the subventral gland. Although a number of different methods were used, all efforts to induce the worms to take up isotope "actively" failed. After abandoning work with whole worms, several experiments were performed to see whether isolated glands in culture would actively take up amino acids and subsequently release labelled material when incubated in balanced salt solution. These experiments were expected to yield information on the nature of the postulated exchange of material between the subventral gland and the pseudocoelomic fluid.

6.2 MATERIALS AND METHODS

6.21 Radioisotope Techniques

Radioisotopes were obtained from the Radiochemical Centre (Amersham). Four different isotopes were used in the studies:

(1) {U-^{14}C} amino acid (hydrolysate) mixture, specific activity 57 mCi/m atom Carbon.

(2) L-{U-^{14}C} leucine, specific activity 324 mCi/mmol.

(3) L-{4,5-^{3}H} leucine, specific activity 53C/mmol.

(4) L-{^{14}C} glucose, specific activity 284 mCi/mmol.

Incubations were performed in 2 ml conical plastic sampling tubes. Packard "Soluene 350" sample solubilizer, Packard "Dimilume" scintillation fluid and Packard glass counting vials were used. Vials were counted in a Packard Tricarb Liquid Scintillation Spectrometer. Optimum counting conditions for both ^{14}C and ^{3}H were established initially and used throughout the study. Washed vials were always counted before use and only vials with negligible activity were used. The "background" level was always seen to be acceptably low before counting of samples proceeded. The degree of quenching which took place under experimental conditions was ascertained and considered not to be significant when comparing the results obtained during this study.

6.22 Culture Medium

Whole worms and isolated subventral glands were cultured in a medium developed by Leland (1963) for maintenance of *Oesophagostomum* spp. The composition of this medium is shown in Table 5 and some notes on the preparation of individual ingredients are given below.

Notes on the preparation of culture medium.

i) Chick embryo extract: prepared according to Weinstein and Jones (1956).

ii) Lamb serum: obtained from Commonwealth Serum Laboratories, Melbourne, Australia.

iii) Sodium caseinate: obtained from Hopkin and Williams, U.K. A 2% solution was prepared (Leland, 1963).

iv) Liver concentrate: obtained from Sigma (cat. no. 202-20). A 2% solution was prepared with KRP.

v) Vitamin mixture: Eagle's minimum essential medium mixture from Commonwealth Serum Laboratories, Melbourne:

Choline chloride	1.0 mg
Folic acid	1.0 mg
l-Inositol	2.0 mg
Nicotinamide	1.0 mg
Calciumpantothenate	1.0 mg
Pyridoxal HCl	1.0 mg
Riboflavin	0.1 mg
Thiamine HCl	1.0 mg

vi) Antiobiotic mixture: prepared from Streptomycin sulphate (Glaxo-Allenbury's); penicillin (Crystaphen, Glaxo-Allenbury's) and mycostatin (E.S. Squibb and Sons, Vic.). 100 ml of medium contained 40 mg streptomycin, 40,000 units penicillin and 10,000 units of mycostatin.

vii) Balanced Salt Solution: Krebs-Ringer phosphate (mammalian), pH 7.3, was used in preparation of chick embryo extract, liver concentrate and final culture medium.

Table 5. Composition of 100 ml of Culture Medium (Leland, 1963).

Ingredient	Volume in ml
Chick embryo extract	50.0
serum from helminth-free lambs	15.0
2% sodium casienate	15.0
2% liver concentrate	5.0
vitamin mixture	5.0
antibiotic mixture	1.0
balanced salt solution (Krebs-Ringer phosphate)	9.0

6.23 Incubation of Whole Worms

For each experiment, a sheep was slaughtered and the caecum was immediately removed, ligatured and sealed in a vacuum flask for transport back to the laboratory. Here the caecum was opened and worms were quickly collected, washed in warm (37°C) BSS and dropped into tubes containing the incubation medium at 37°C. Two worms, one male and one female, were incubated in each tube and 1 ml of medium was used in each case. The time of incubation, incubation medium, isotope and radioactive concentration varied. Worms were incubated for 30 or 60 minutes in Leland's culture medium or in caecal fluid. Four different isotopes - ^{14}C amino acid mixture (57 mCi/m atom Carbon), ^{14}C glucose (284mCi/mmol), ^{3}H-leucine (324 mCi/mmol), ^{3}H-leucine (53C/mmol) - were used and the concentration of isotope in the incubation medium varied. Six different sets of incubation conditions were used and these are detailed in Table 6. Since it was considered necessary to observe active uptake of isotope when examining protein metabolism, control incubations at 4°C were carried out in every case. Individuals of *O. venulosum*, like many other nematodes, are torpid at low temperatures and presumably do not feed. Any uptake of isotope at 4°C would represent passive diffusion of amino acids into the worm.

After incubation each group of worms was removed from the incubation tube, washed in three changes of cold BSS to remove any free isotope attached to the outside of the worms and homogenised in 500 µl cold BSS. Tricholoracetic acid, to make a final concentration of 5%, was mixed with the homogenate and the precipitated protein was spun down to form a pellet by centrifuging for 10 minutes at 750 g. The supernatant was removed and retained, the protein pellet washed twice with cold BSS and then resuspended in 1ml cold BSS. Aliquots, 0.5ml, of the supernatant and pellet fractions of each incubation sample were dropped into counting vials containing 1ml of Soluene. When the samples had been dissolved by shaking for a few minutes, 10ml of Dimilume were pipetted into each vial, the contents thoroughly

Table 6. Incubation conditions to which groups of 2 *O. venulosum* worms were subjected.

Incubation	Incubation Time mins.	Incubation Temperature	Medium	Isotope	Radioactive Concentration
I	30	37°	culture medium	^{14}C amino acid hydrolysate	$0.05\mu Ci/ml$
II	60	"	"	"	"
III	60	"	"	"	$2\mu Ci/ml$
IV	60	"	"	^{3}H-leucine	"
V	60	"	"	^{14}C glucose	"
VI	60	"	caecal fluid	^{3}H-leucine	$5\mu Ci/ml$

mixed and the disintegrations per minute in each vial counted. Figure 10 outlines the treatment given to each sample after incubation.

6.231 Control procedures

As difficulties were encountered with the attempts to label *O. venulosum*, 2 control experiments were performed:

(1) Individuals of *Nematospiroides dubius* were incubated with ^{3}H leucine, in the same way as individuals of *O. venulosum*, to compare the results obtained with those produced by the *O. venulosum* experiments.

(2) A number of individuals of *O. venulosum* were incubated with fluorescent dye and observed to determine whether the worms were in fact feeding in vitro.

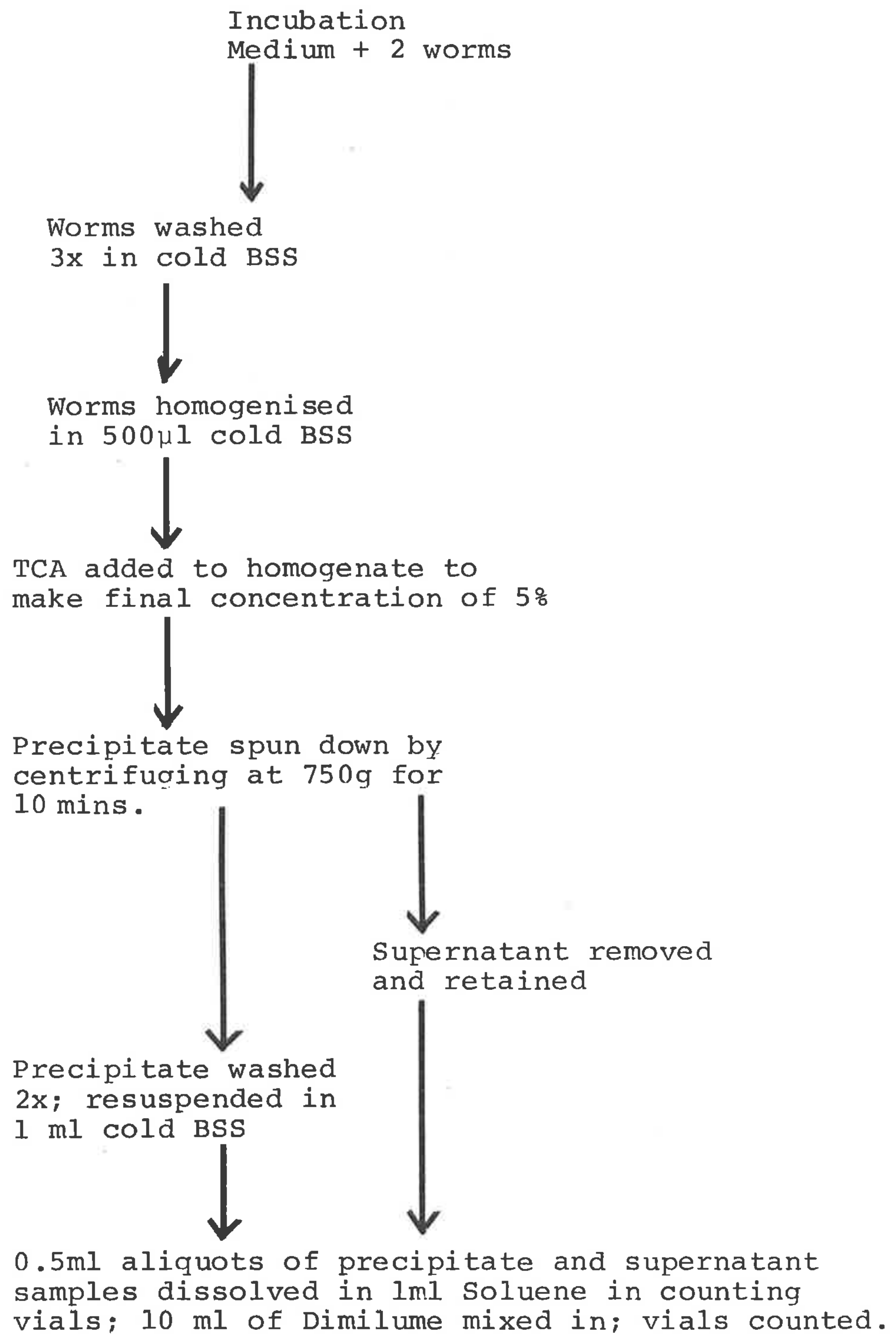
Incubation of *Nematospiroides dubius*

Mice infected for 6 months with *N. dubius* were killed. Their intestines were removed, placed in cold BSS, cut open and undamaged worms were removed. Groups of ten worms, comprising equal numbers of males and females, were rinsed in cold BSS and then incubated in 1ml Leland's culture medium plus 5μCi ^{3}H-leucine. Duplicate incubations were carried out at $4^{\circ}C$ and $37^{\circ}C$. Incubation and subsequent preparation of material for counting were conducted in the same way as for *O. venulosum* in Section 6.22. (N.B. 10 *N. dubius* approximately equals 2 *O. venulosum* in mass.)

Incubation of *O. venulosum* with Fluorescent Dye

A number of *O. venulosum* were incubated in culture medium with 5% serum conjugated with the fluorochrome, lissamine rhodamine B, for 60 minutes at $37^{\circ}C$. Worms were removed at intervals and examined with a fluorescent microscope under ultraviolet light for the characteristic pink-red fluorescence of rhodamine (Sommerville, 1966). The worms were also observed under the binocular microscope to determine whether they were feeding by observation of the mouth and pharynx.

Figure 10. Preparation of Incubation Samples for Counting



6.24 Incubation of Isolated Glands

Worms were collected as in Section 6.22. Washed worms were cut just behind the posterior ends of the subventral glands. The anterior sections of the worms, with subventral glands attached, were dropped into 2ml sampling tubes containing 100 μl of culture medium and 5μCi of ^{3}H-leucine at 37°C. Anterior ends of four worms were incubated in each tube. The incubations were conducted for 5 and 10 minutes. After incubation, the worm sections were quickly removed from the medium and rinsed three times in warm BSS. The subventral glands were dissected away and dropped - in groups of six - into sampling tubes containing 100 μl of BSS at 37°C. After 5 minutes, glands were removed from the BSS and washed three times in warm BSS. The BSS incubate was retained. Each group of 6 glands and each 100 μl of BSS incubate were dropped into separate counting vials containing 1ml of Soluene. After the samples had dissolved, 10 ml of Dimilume were added to the vials. The vials were thoroughly mixed and then counted. Figure 11 shows the scheme for treatment of isolated subventral glands. Identical incubations were made at 4°C to see what effects lowered temperature had on uptake of isotope. Incubations with ten times as much isotope (50μCi ^{14}C-leucine) were also conducted to examine the effect of increased isotope concentration. Washing and dissection were always performed as quickly as possible.

6.241 Viability of isolated glands

To examine the condition of isolated glands during incubation in media, glands were incubated in both culture medium and BSS for 5 and 10 minutes at both 37°C and 4°C (Table 7). After incubation worms were fixed and embedded (Chapter 3), sectioned and examined under the electron microscope.

Table 7. Incubation conditions for testing viability of glands incubated in culture medium and BSS.

Incubation Medium	Incubation Temperature	Incubation Time in Minutes
Culture medium	37°C	5
		10
	4°C	5
		10
BSS	37°C	5
		10
	4°C	5
		10

Figure 11. Scheme for Incubation of Isolated Subventral Glands

4 worm segments, each with subventral glands attached.

Step 1. Incubated in 100 µl culture medium containing 5 Ci ^{3}H - leucine for 5 and 10 mins. at 37°C.

Step 2. Worm segments removed; washed 3x with warm BSS; subventral glands dissected out.

Step 3. 6 glands from each group incubated in 100 µl BSS for further 5 minutes at 37°C.

Step 4. Each group of 6 glands and each 100 µl of BSS incubate dropped into separate vials containing 1 ml Soluene.

Four such experiments were perfomed:

(1) 5µCi ^{3}H-leucine at 37°C

(2) 5µCi ^{3}H-leucine at 4°C

(3) 50µCi ^{14}C-leucine at 37°C

(4) 50µCi ^{14}C-leucine at 4°C.

6.3 RESULTS

6.31 Results for Whole Worms

None of the *O. venulosum* incubations produced a significant number of counts and in no case were the counts obtained for 37°C incubations significantly higher than those obtained for incubations performed at 4°C. Typical counts obtained for a 0.5ml aliquot of each incubation at 37°C and 4°C are shown in Table 8. Variations in cpm between incubations - e.g. between incubations I and VI - were due to differences between the activities of the isotopes used in each case.

6.311 Results for controls

1) Typical counts for 0.5 ml aliquots of *N. dubius* incubations at 37°C and at 4°C are shown in Table 9. It is evident that there is a significant difference between the counts obtained from the 37°C incubation and those measured for the incubation at 4°C. Total counts per minute for the *N. dubius* incubation at 37°C are much higher than the counts measured for the *O. venulosum* incubation under similar conditions (n.b. Incubation VI. Table 7).

2) When *O. venulosum* worms incubated with fluorescent dye, were examined under ultraviolet light, most of them showed no fluorescence from ingested rhodamine. There was very slight fluorescence in the intestines of a few worms. Worms that were examined under the binocular microscope showed no mouth or pharyngeal movement or other evidence of feeding.

6.32 Results for Isolated Glands

Figure 12 shows the counts per minute obtained for the groups of six glands and 100 μl aliquots of BSS processed as in Section 6.24. In glands incubated at 37°C, much higher cpm were recorded for glands incubated for 5 minutes than glands incubated for 10 minutes. In the case of glands incubated at

Table 8. Typical Counts per Minute for 0.5 ml aliquots of *Oesophagostomum venulosum* incubations.

Incubation	Temperature	Counts per Minute For 0.5 ml Aliquot	
		Pellet	Supernatant
I	37°C	27	30
	4°C	29	51
II	37°C	41	132
	4°C	95	116
III	37°C	64	364
	4°C	52	370
IV	37°C	169	449
	4°C	170	400
V	37°C	36	59
	4°C	29	51
VI	37°C	555	398
	4°C	322	548

Table 9. Typical Counts per Minute for 0.5 ml aliquots of *N. dubius* Incubations at 37°C and at 4°C.

Incubation	Counts Per Minute	
	Pellet	Supernatant
37°C	294	2877
4°C	54	970

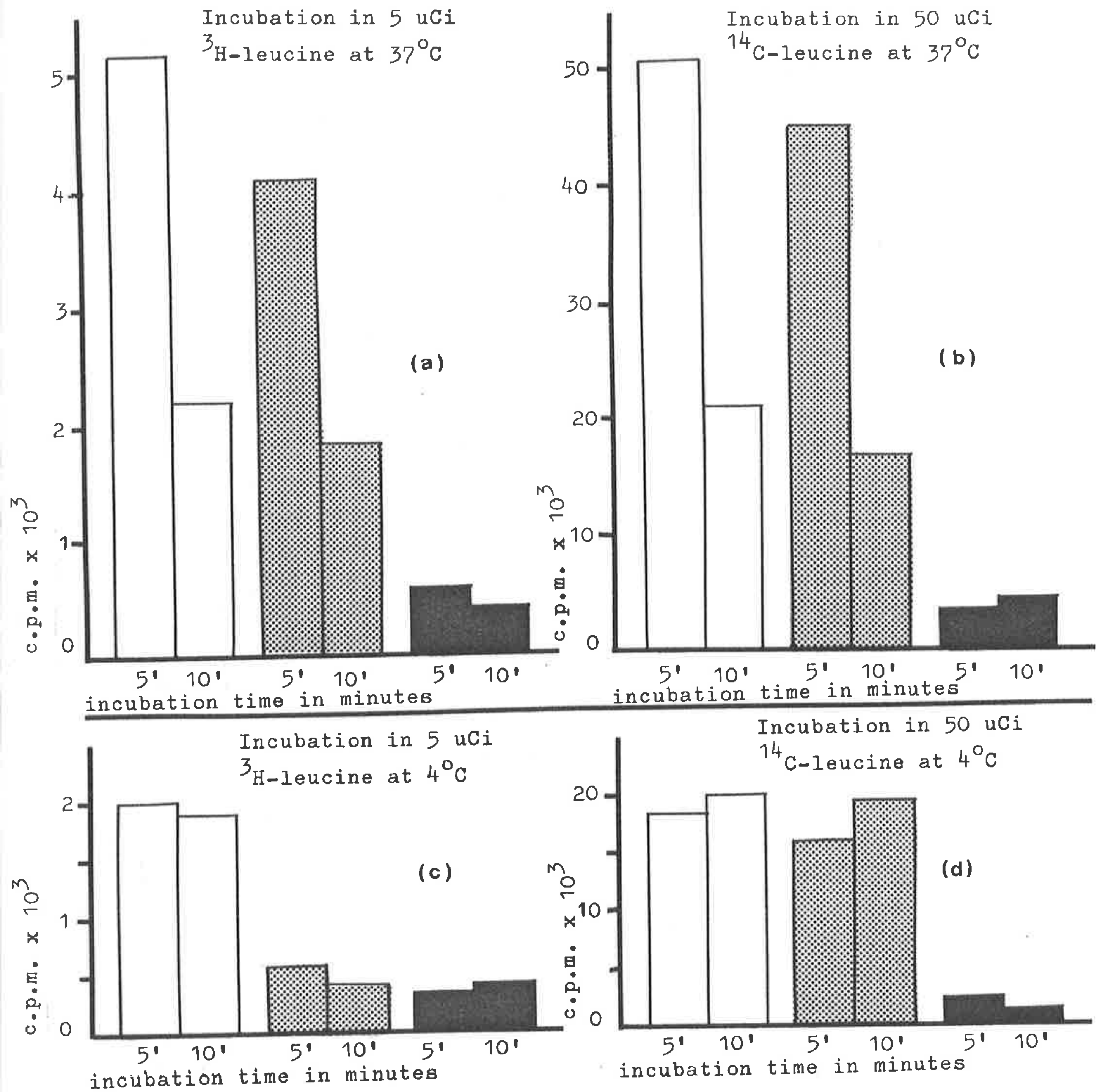


Figure 12. Graphs (a), (b), (c) and (d) show the time of incubation in culture media plotted against the counts per minute (cpm) recorded for glands incubated in 5 μCi ^{3}H-leucine and 50 μCi ^{14}C-leucine. (See Section 6.24)

☐ indicates total cpm for glands plus BSS from each incubation.

▒ indicates cpm for glands alone .

■ indicates cpm for BSS alone .

$4^{o}C$, there was no significant difference between the cpm obtained for glands incubated for 5 minutes and those incubated for 10 minutes. The cpm obtained for both 5 and 10 minute incubations at $4^{o}C$ were close to those obtained after 10 minute incubation at $37^{o}C$. All glands incubated in 50 µCi of ^{14}C-leucine took up ten times as much isotope as glands incubated in 5 µCi ^{3}H-leucine.

6.321 Viability of glands in media

All sections of glands incubated in culture medium and BSS under conditions specified in Table 7 had a similar abnormal appearance. Plates 23 and 24 show the typical fine structure of the incubated glands. The most obviously abnormal features of this ultrastructure are the swollen appearance of all the membranes and the faded secretory granules.

6.4 DISCUSSION

It is evident that *O. venulosum* individuals did not feed in culture under any of the experimental conditions tested and so did not consume significant quantities of labelled amino acids.

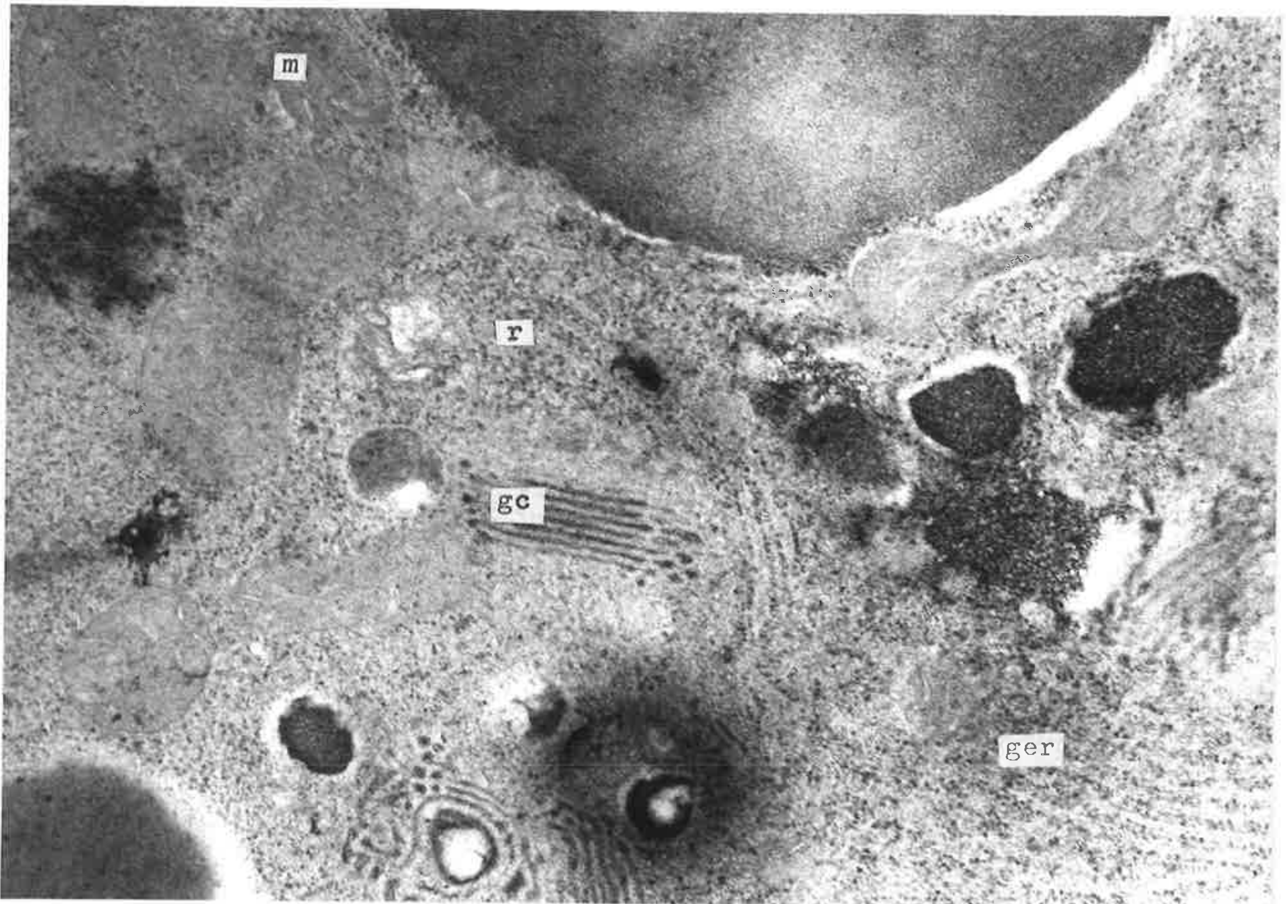
The subventral gland was capable of taking up labelled amino acids from culture medium. The 50% drop in cpm between 5 minute and 10 minute incubations at $37^{o}C$ suggests that isotope taken up by the gland in the first five minutes was then rapidly lost to the culture medium or to the fragments of worm incubated with the glands. Under the experimental conditions used, the glands took up more isotope at $37^{o}C$ than at $4^{o}C$ during the first five minutes but not thereafter. There is no evidence that the glands actually utilised the amino acids and the fact that much of the isotope taken up was rapidly released tends to suggest that they did not.

Uptake "in vitro" does not mean that uptake "in vivo" can also take place. From the electron micrographs of glands incubated in Leland's medium and BSS, it is clear that the glands were not structurally normal during incubation with isotope and could not be expected to function normally. The

Plates 23-24. Typical ultrastructure of all incubated glands. Note the distended membranes and faded secretion granules.

dsg, dense secretion granule; gc, golgi complex; ger, granular endoplasmic reticulum; pgm, pool of granular material; m, mitochondria; r, ribosome.

x 6,000



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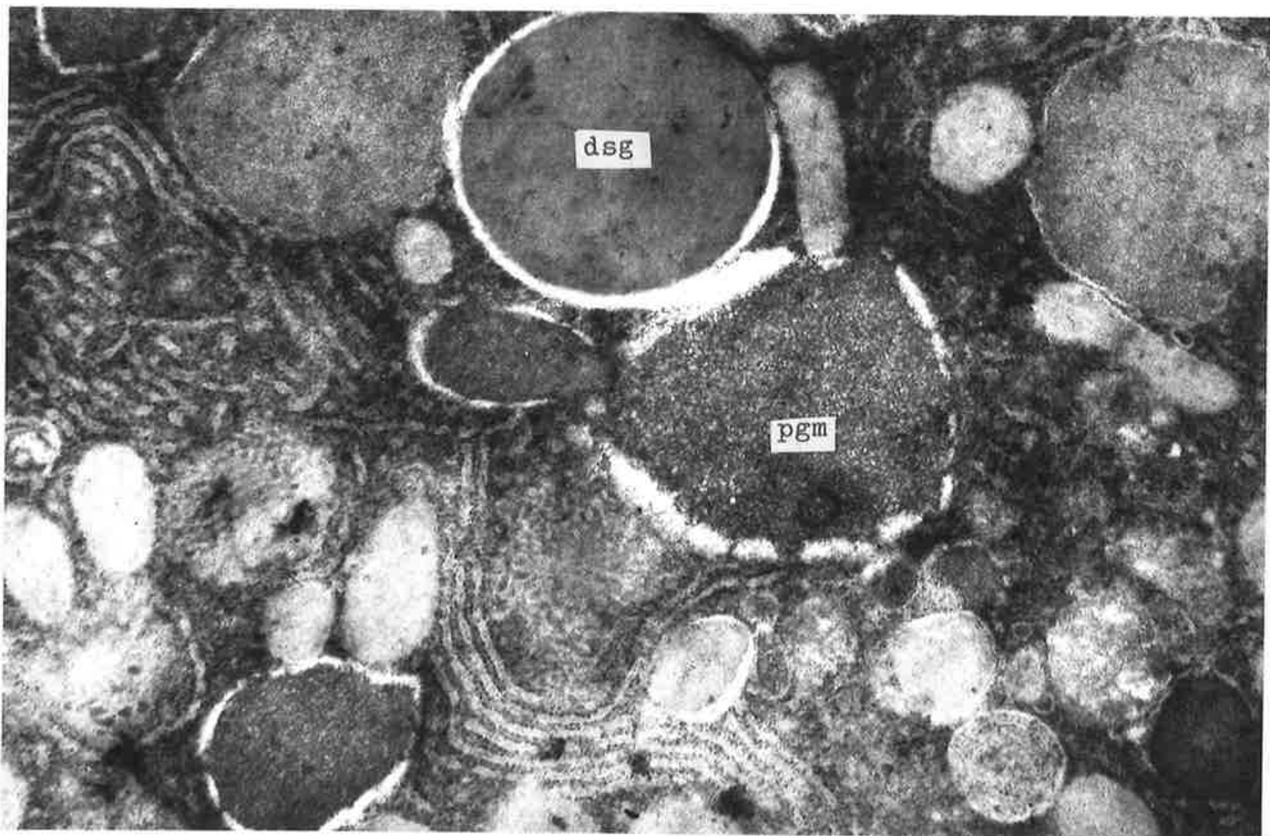

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dramatic drop in uptake of isotope from five to ten minutes and the loss of much of the assimilated isotope are probably manifestations of a "dying" gland. It is unikely that either Leland's medium, which was devised for maintenance of whole worms, or BSS are suitable incubation media for isolated glands and the ultrastructural changes seen in incubated glands may have been produced by an osmotically and ionically unsuitable environment.

It might be possible to devise a system in which isolated subventral glands would function normally and their products be analysed with radiotracer techniques combined with electrophoresis. However the present study could only be rudimentary; limited by the time available and the difficulty of occluding the anterior ends of isolated subventral glands and thus ensuring that material could not leak in and out of the fractured anterior ends of the glands.

CHAPTER 7: ELECTROPHORETIC STUDIES

7.1 INTRODUCTION

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7.1 INTRODUCTION

The proteins present in the subventral gland and the pseudocoelomic fluid were compared electrophoretically to investigate any exchange of protein which might occur between them. A sodium dodecyl suplhate discontinuous polyacrylamide gel system was used and so it was possible to calculate the molecular weights of the major polypeptide chains present in the two compartments (Weber and Osborn, 1969). This is a very widely used technique and its theoretical basis is well understood. Four preparations were subjected to electrophoretic analysis: (1) homogenised subventral glands, (2) pseudocoelomic fluid, (3) the granule fraction of the subventral gland, (4) balanced salt solution (BSS) in which subventral glands had been incubated. Differential centrifugation was used to isolate the secretory granules of the subventral gland (See Fig. 13). It was thought that comparison of the electrophoretic patterns formed by these four samples might show whether there is an exchange of protein between the subventral gland and the pseudocoelomic fluid; whether the secretory granules have any connection with such an exchange; and what proteins, if any, are released by subventral glands incubated in balanced salt solution.

7.2 MATERIALS AND METHODS

7.21 The Electrophoretic System

A sodium dodecyl sulphate (SDS), high pH, discontinuous polyacrylamide gel electrophoretic system was used. This system was a modification of that described by Laemli (1970). The separating gel contained 14% acrylamide, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS; the stacking gel contained 5% acrylamide, 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS; and the electrode buffer contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS (pH 8.3). Gels were polymerised by the addition of tetramethylethylenediamine (TEMED) and ammonium persulphate (APS), and poured into glass moulds (Gradipore). The separating gel was about 60mm high (x 72mm wide x 3mm deep) and the stacking

Fig. 13. Isolation of membrane-bound granules from subventral glands by differential centrifugation (Refer to Appendix I).

i) 60 glands were homogenised in 50 µl KRP at 4°C.

ii) Homogenate plus a further 50 µl KRP, used to rinse the microhomogeniser, were layered over 10 ml of 5% Triton in 1.7 M sucrose/KRP and centrifuged at 110,000 g for 120 mins at 4°C.

iii) Supernatant removed and discarded.

iv) Sediment resuspended in 10 ml KRP and mixed thoroughly.

v) Suspension spun at 3,300 g for 10 mins at 4°C.

vi) Supernatant removed from tube.

vii) Sediment resuspended in 10 µl loading buffer and prepared for electrophoresis.

gel was 10mm high (x 72mm x 3mm deep). A "Gradipore" electrophoresis tank was modified for use with the SDS discontinuous system by blocking the buffer recirculation outlets and the holes connecting the upper and the lower tanks.

7.211 Preparation of samples

Samples were dissolved in a loading buffer containing 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. Generally each sample was mixed with buffer in the ratio 1:1 (volume:volume) and then incubated in a waterbath at 90°C for two minutes. The "unknown" samples were all contained in very small volumes and usually processed in 0.5ml tubes. Samples were loaded by a microsyringe, washed between each load, into the cavities of a Gradipore sample spacer. The unknown samples were usually loaded in 20 μl volumes and the standard solution in 5 μl aliquots. Electrophoresis was performed at a current of 25m Amps for 4 hours - the time required for the blue dye marker to reach the bottom of the separating gel; or for 6 hours - to obtain greater separation of bands for photographic purposes.

7.212 Preparation of standards

To prepare standard solutions, 0.1% cytochrome C (from horse-heart muscle, Sigma); 0.1% bovine serum albumin (Sigma) and 0.1% ovalbumin (Sigma) were dissolved directly in loading buffer and incubated as above (see 7.211). Samples of this standard solution were freshly prepared for each gel and always loaded adjacent to the unknown samples.

7.213 Staining and destaining

The gels were fixed and stained for 12 hours in 0.05% Coomassie brilliant blue R in 25% isopropanol in 10% acetic acid; for 4 hours in 0.005% Coomassie blue in 10% isopropanol in 10% acetic acid; and for 4 hours in 0.005% Coomassie blue in 10% acetic acid (Fairbanks, Steck and Wallach, 1971).

During staining and destaining the gels were shaken constantly. The length of each gel and the distance of dye

migration were measured before staining, and the length of the gel and the distances of migration of the protein bands were measured after destaining. Stained gels were photographed with transmitted light and a yellow filter.

7.214 Calculation of mobility

For gels run for 4 hours, mobility was calculated according to the following formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance of dye migration}}$$

(Weber and Osborn, 1969)

7.22 Preparation of Samples from *O. venulosum*

Four different types of sample were used to compare the protein content of the subventral glands and pseudocoelomic fluid of *O. venulosum* (see 7.1).

7.221 Whole subventral glands

Twenty subventral glands were dissected out of 10 worms (5 male and 5 female), rinsed in cold BSS (the balanced salt solution was KRP), and homogenised in 50 µl of loading buffer. This procedure produced about 100 µl of homogenate (sample: buffer; 1:1).

7.222 Pseudocoelomic fluid

Ten worms were placed in 100 µl of cold BSS in the cavity of a "well" slide. The cuticle of each worm was carefully pierced twice, without perforating the gut, and the worms were all pressed gently with forceps two or three times.

The fluid then surrounding the worms (about 200 µl) was collected with a micropipette and spun in a centrifuge tube at 750 g for 20 minutes. After centrifugation the tube contained a layer of sediment (eggs and other solid particles) and a pink-tinged supernatant (BSS plus pseudocoelomic fluid). The supernatant, about 100 µl, was removed, mixed with 100 µl of loading buffer and prepared for electrophoresis.

7.223 The granule fraction

A granule fraction was prepared from the subventral glands of 30 worms (15 males and 15 females), shown in Figure 13. The resulting fraction, in the form of a sediment on the bottom of a centrifuge tube, was resuspended in 100 µl of loading buffer and prepared for electrophoresis as usual.

7.224 Incubation of subventral glands in BSS

Two groups of 10 subventral glands (each containing 5 glands from male worms and 5 from females) were incubated in 100 µl of BSS for 10 minutes in the tubes of two micro-homogenisers. One group was incubated at $4^{o}C$ and one at $37^{o}C$. After 10 minutes the two tubes were spun at 750g for 5 minutes. Each supernatant (50 µl) was collected and mixed with 50 µl of loading buffer. A further 50 µl of loading buffer was added to the glands remaining in the homogenizers and these were homogenized. Further processing of the BSS supernatant and the subventral gland homogenate was as usual.

7.3 RESULTS

Preparation of each of the four samples of *O. venulosum* tissue for electrophoresis was repeated two to eight times and duplicate samples were run on each gel. The electrophoretic patterns obtained for each type of sample were displayed photographically.

7.31 Calculation of Molecular Weight

Electrophoretic mobility of a polypeptide chain is directly related to the logarithm of its molecular weight. With the SDS discontinuous gel system, molecular weights of unknown polypeptide chains can be determined with an accuracy of 10% (Weber and Osborn, 1969). For each gel, the molecular weights of the three standards were plotted against the logarithms of their mobilities (calculated as in 7.214) and a line of best fit was drawn through the three points. The molecular weights of the unknown polypeptide chains represented by bands on the gels could be determined from the standard curves.

An example of a standard curve is shown in Figure 14. This particular curve was drawn from the mean mobilities of standards run on seven separate gels. The mobilities are given in Table 10.

The molecular weights of the polypeptide chains represented in the electrophoretic patterns, obtained for each of the *O. venulosum* tissue samples, were determined independently for each gel. The values quoted hereafter for these molecular weights are mean values calculated from a number of gels in each case.

7.32 Electrophoretic Patterns

Typical electrophoretic patterns formed by SDS polyacrylamide electrophoresis of the four types of *O. venulosum* tissue preparations are shown in Plates 27, 28, 29 and 30. The logarithmic scales incorporated into each figure indicate the molecular weights of the polypeptide chains represented by the stained bands on the gels. Comparison of the four electrophoretic patterns shows various instances of similarities between bands which may be significant.

7.321 Comparison of electrophoretic patterns formed by subventral gland and pseudocoelomic fluid.

A comparison of the electrophoretic patterns obtained for the subventral glands and the pseudocoelomic fluid shows that a number of polypeptide chains of identical mobility appear to be present on both patterns (Plate 31). The molecular weights determined for four bands are given in Table 11. These bands are numbered on Plate 31 and can be described as follows:-

Band 1: located just above BSA; sometimes stains as densely as bands 2, 3 and 4 but is sometimes lighter.

Band 2: just below ovalbumin.

Band 3: just above band 4.

Band 4: approximately the same position as cytochrome C.

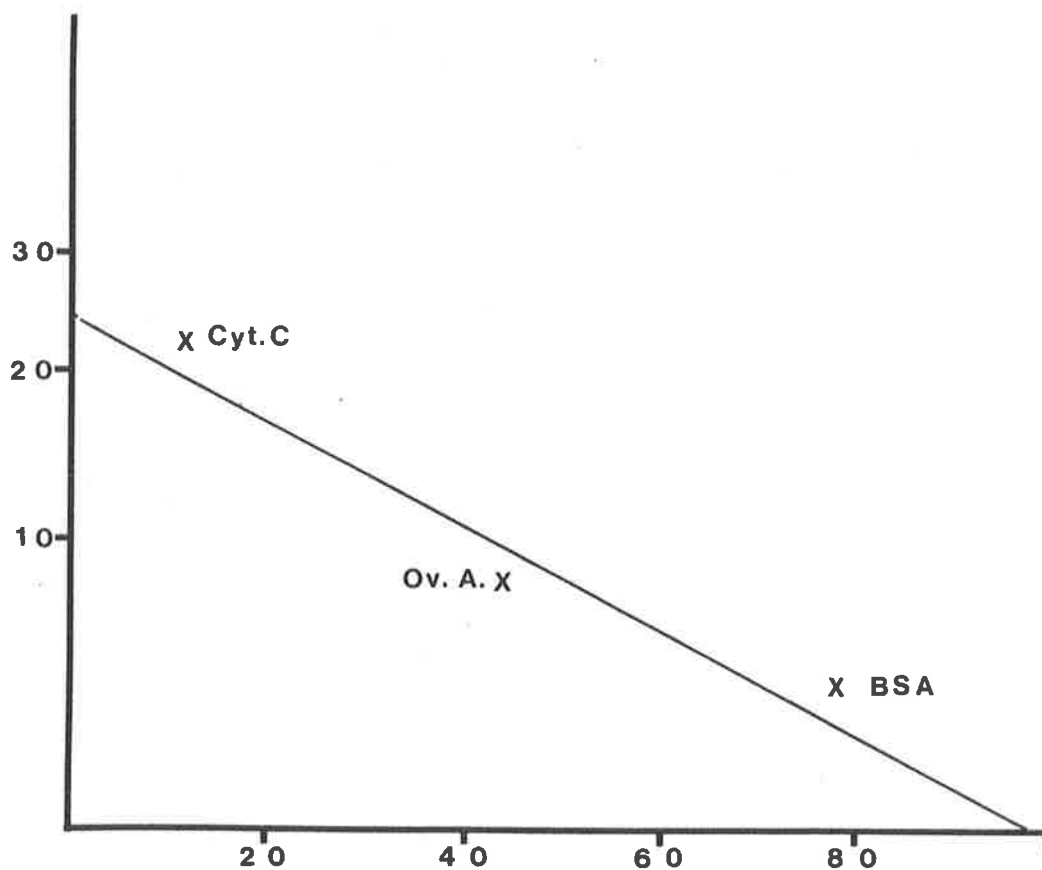

Fig. 14 Typical standard curve for a gel run for four hours. Molecular weights of cytochrome C-13,400 (Atlas and Farber, 1956); BSA-68,000 (Castellino and Barker, 1968); ovalbumin - 44,000 (Castellino and Barker, 1968).

Table 10. Mean mobility for each of the standards as used to draw the typical standard curve shown in Figure 14.

	$\bar{x}$ Mobility in mm	S
BSA	4.3	0.8
ovalbumin	8.3	0.7
cytochrome C	24.3	1.8

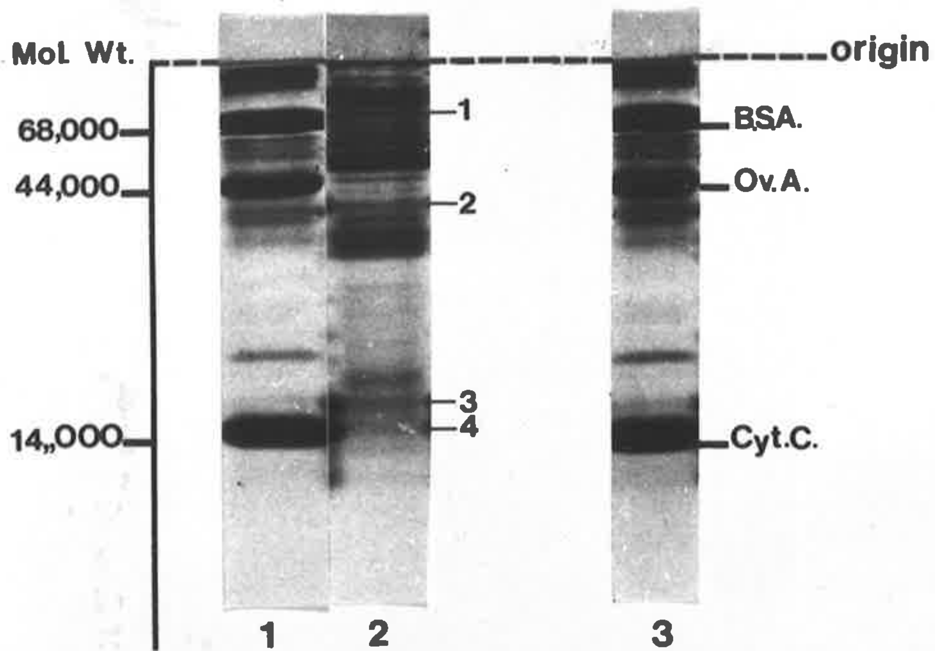

Plate 27. Polyacrylamide gel electrophoretic pattern for homogenates of whole subventral glands. Slots 1 and 3 represent 5 µl volumes of standard solution. Slot 2 is the pattern formed by 20 µl of subventral gland homogenate. The log scale indicates molecular weight represented by a given distance from the origin.

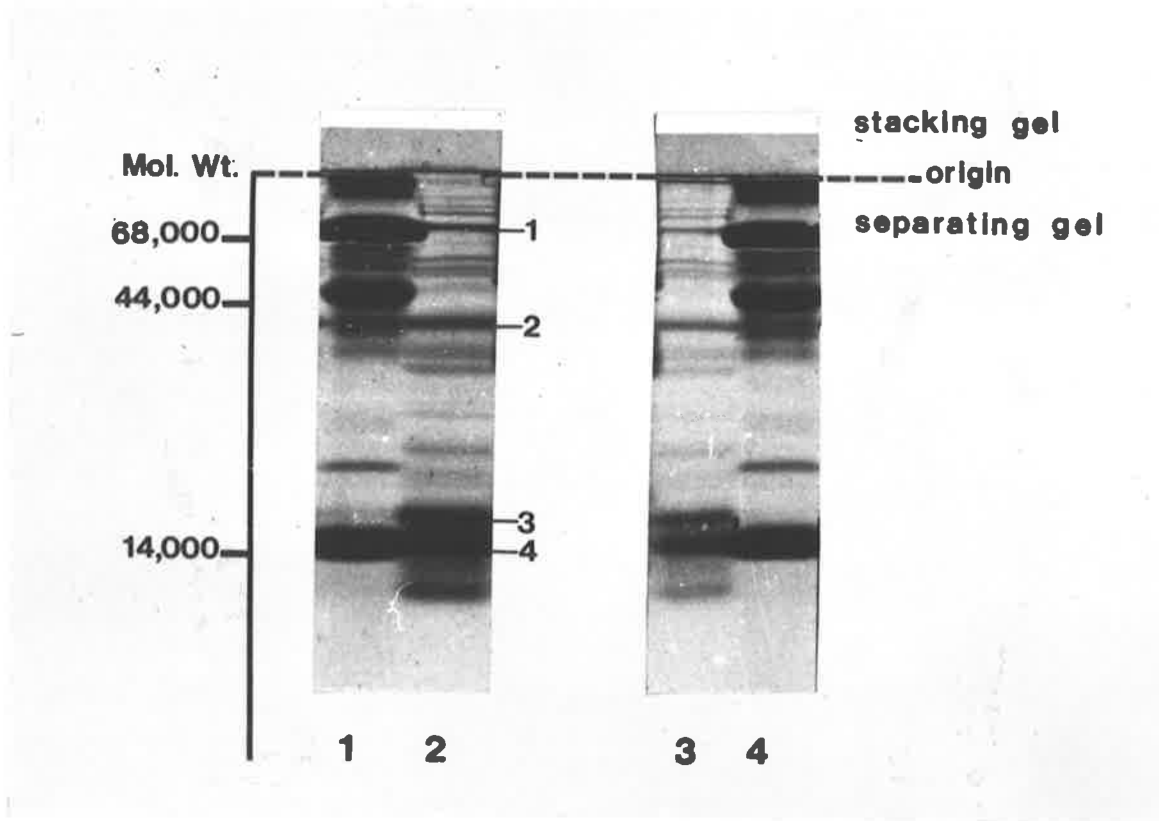

Plate 28. Polyacrylamide gel electrophoretic pattern for pseudocoelomic fluid extract. Slots 2 and 3 represent 20 µl volumes of pseudocoelomic fluid. Slots 1 and 4 represent 5 µl volumes of standard solution. The log scale indicates the molecular weight represented by a given distance from the origin.

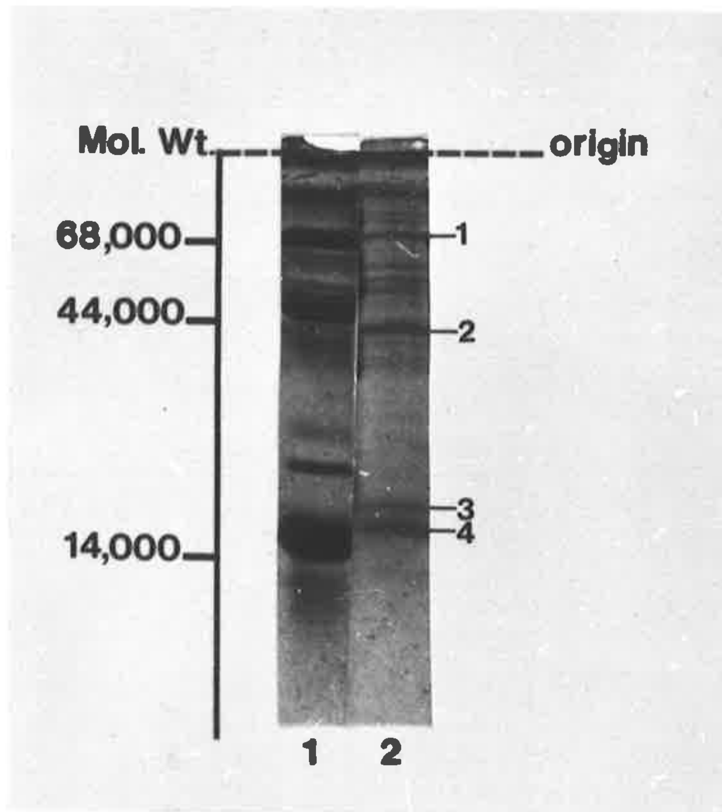

Plate 29. Electrophoretic pattern formed by 20 µl of BSS after incubation with 10 subventral glands per 100 µl BSS for 10 mins. There was no difference between the patterns obtained for incubations at 37°C and at 4°C. The log scale indicates molecular weight. Slot 1 represents 5 µl of standard solution; slot 2 represents 20 µl of BSS.

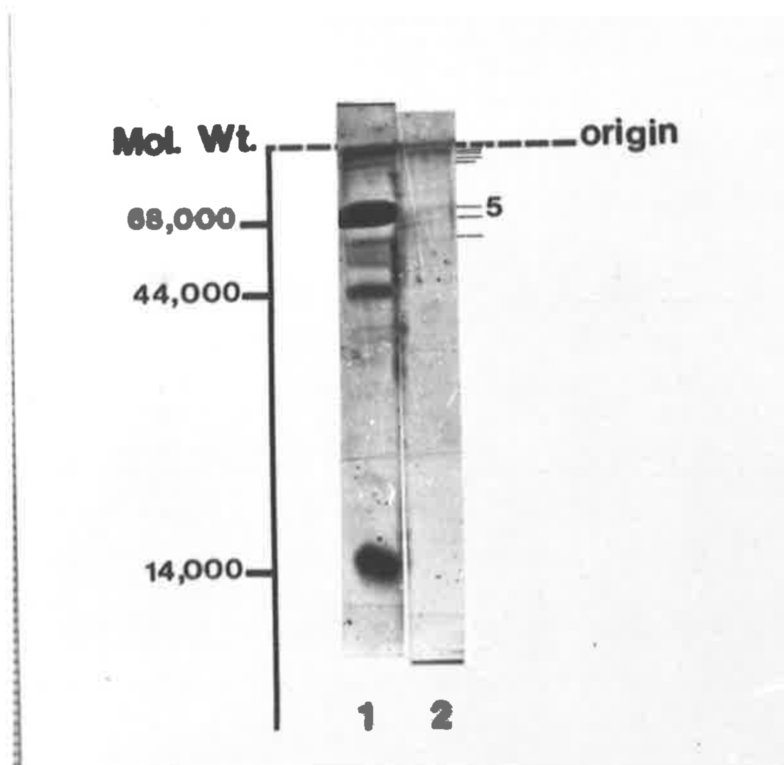

Plate 30. Electrophoretic pattern formed by granule fraction of subventral gland. Slot 1 represents 5µl standard solution; slot 2 represents 20 µl of granule fraction preparation. Log scale indicates molecular weight. The five numbered bands are referred to in Section 7.321.

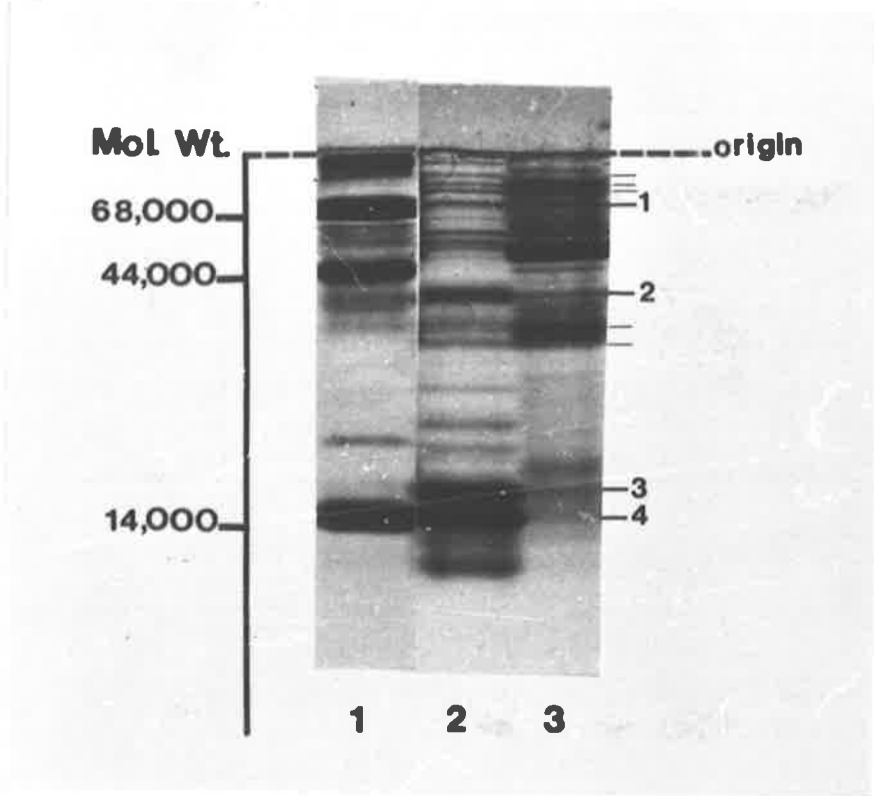

Plate 31. Comparison of electrophoretic patterns obtained for pseudocoelomic fluid (slot 2) and subventral gland (slot 3). Slot 1 represents standard solution. Bands which appear on both patterns are marked with a dash. The four numbered bands are those which appear to be most distinct in the pseudocoelomic fluid and which also appear in the gland pattern.

Table 11. Mean molecular weights calculated for the polypeptide chains represented by bands common to the subventral gland and the pseudocoelomic fluid electrophoretic patterns.

Band	Molecular Weight	Standard Deviation
1	70,000	1,897
2	43,000	1,369
3	14,000	1,851
4	9,600	748

These four bands are the most distinct bands on the pseucodoelomic fluid pattern (bands 2, 3 and 4 are always the densest staining bands on the pattern). All four bands are also present on the subventral gland pattern.

7.322 Comparison of pattern formed by BSS incubate with results obtained for subventral gland and pseudocoelomic fluid.

Plate 32 shows the electrophoretic pattern formed by the BSS incubate juxtaposed with those formed by the subventral gland and the pseudocoelomic fluid. It is evident that the pattern formed by the balanced salt solutions appears to be identical to that formed by the pseudocoelomic fluid. The four bands (bands 1, 2, 3 and 4) are the most distinct bands on the BSS pattern, as they were on the pseudocoelomic fluid pattern. The mean molecular weights calculated for these four bands on the BSS pattern are given in Table 12.

7.323 Comparison of pattern formed by granule fraction with the other results.

As shown in Plate 29 the electrophoretic pattern formed by the granule fraction of the subventral gland shows a number of bands representing polypeptide chains of high molecular weight. When this pattern is compared with the patterns obtained for the subventral gland and the pseudo-coelomic fluid (Plate 33) it appears that band 5 of the granule fraction corresponds to band 1 of the gland and pseudocoelomic fluid patterns. The molecular weight represented by this band is 69,000 as determined from granule fraction electrophoretic patterns.

7.4 DISCUSSION

7.41 Reproducibility of Results

The SDS polyacrylamide gel system used gave consistent and reproducible results. This is demonstrated by the low standard error of the mean mobility figures used to draw the typical standard curve in Section 7.31. It was not considered

Table 12. Mean molecular weights calculated for polypeptide chains represented by bands 1,2, 3 and 4 from BSS incubate data.

Band	Molecular Weight	Standard Deviation
1	68,000	-
2	44,000	-
3	15,000	-
4	13,000	-

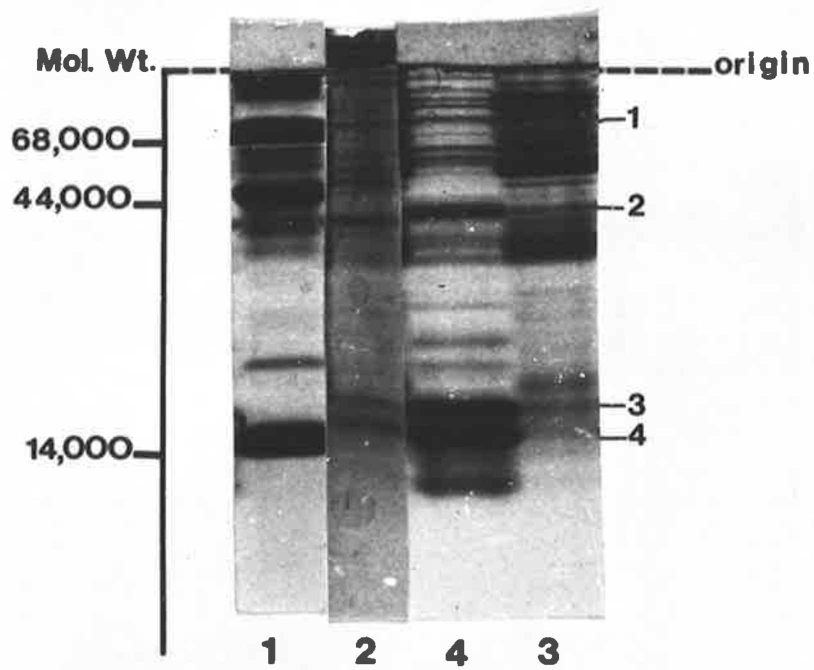

Plate 32. Electrophoretic pattern formed by BSS incubate (slot 2) compared with those formed by subventral gland (slot 3) and pseudocoelomic fluid (slot 4). Slot 1 represents standard solution. Bands 1, 2, 3 and 4 (see Section 7.322) are marked.

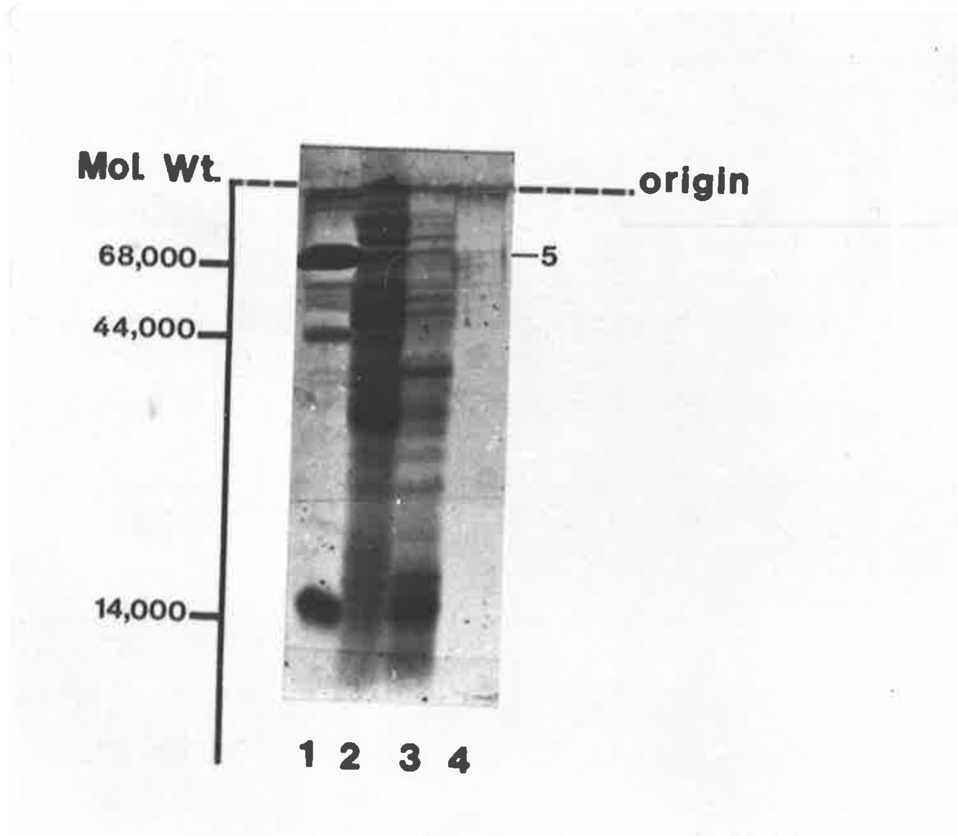

Plate 33. Pattern formed by granule fraction (slot 4) compared with those formed by the pseudocoelomic fluid (slot 3) and the subventral gland (slot 2). Slot 1 represents standard solution. Band 5 (see Section 7.323) is marked.

appropriate to undertake a thorough investigation of the validity of the results obtained in the present study. However if this work was to be continued several control measures should be taken to ensure that the final measurements of molecular weight are as accurate as possible:

(1) The number and range of standards should be increased to cope with the large number and range of proteins present in the subventral gland and pseudocoelomic fluid.

(2) Patterson and Knight (1975) have shown that "...for the Tris-glycine buffer system, a linear relationship between log MW and migration distances holds only for a certain MW range for a given gel concentration."

(See Table 13) To obtain accurate estimates of molecular weight for polypeptide chains it is necessary to run them on a number of gels of different concentrations. This is particularly necessary when a large number of polypeptide chains are being examined, as in the present study.

The gel concentration used in the present study appeared to give less consistent mobilities for polypeptides of low molecular weight, e.g. 15,000. For example the mobility of cytochrome C was less consistent than that of ovalbumin and BSA, as seen in Section 7.31.

(3) It has been shown that some proteins do not migrate according to molecular weight in SDS gels (Banker and Cotman, 1972; Camacho, Carrascosa, Vinuela and Salas, 1975). These discrepancies are not always predictable. Electrophoresis using two different SDS gel systems will usually show whether any anomalous behaviour is taking place.

7.42 Discussion of Results

Comparison of the electrophoretic patterns formed by the four *O. venulosum* samples reveals a number of biochemical similarities which may represent functional relationships

between the subventral gland, its membrane bound secretory granules and the pseudocoelomic fluid.

At least eleven polypeptides appear to be present in both the subventral gland and the pseudocoelomic fluid. These include four polypeptides which appear to be the most highly concentrated constituents of the pseudocoelomic fluid. The molecular weights of these four polypeptides were calculated to be 70,000, 43,000, 14,000 and 9,600 respectively. As seen in Chapter 4, the ultrastructure of the membranous interface between the subventral gland and the pseudocoelomic fluid, suggests that material is being exchanged between these two compartments. So it is possible that at least some of the eleven polypeptides mentioned above do actually pass from the pseudocoelomic fluid into the gland or vice versa.

The electrophoretic pattern formed by BSS in which glands had previously been incubated is remarkably similar to the pattern obtained for the pseudocoelomic fluid extract. Almost all the same bands are present and with the same relative concentrations. N.B. the concentration of stain on a band indicates the relative concentration of polypeptide in a sample (Maizel, 1971). Here, too, the four most prominent bands are those four bands already seen to be present in the subventral gland and the pseudocoelomic fluid. The values calculated for the molecular weights represented by these four bands on the basis of BSS sample mobility do not differ significantly from the values determined from pseudocoelomic fluid data (Table 14). The differences between the molecular weights of bands 1, 2 and 3 are as expected since the determination of molecular weight by SDS electrophoresis is only accurate to within 10% (Weber and Osborn, 1969). There is a greater discrepancy between the two values obtained for band 4. But this is probably insignificant, considering the unsuitability of the gel system used for very low weight polypeptides (see 7.41). Electrophoresis of BSS and pseudocoelomic fluid samples on gels containing a higher concentration of acylamide should produce closer agreement in values for band 4. So it seems, on the basis of position in the electrophoretic patterns

Table 13. Range of molecular weights for which the relationship - log MW$\propto$migration - holds for gels containing different concentrations of acrylamide.
From Patterson and Knight, 1975.

Gel Concentration % Acrylamide	Range of Molecular Weights for which log MW$\propto$Migration ($\times 10^3$)
5%	300-150
8%	150-60
12.5%	60-20
16%	40

Table 14. Values calculated for the molecular weights of the 4 polypeptides present in the subventral gland, pseudocoelomic fluid and BSS samples.

Band	Mol.Weight According to Pseudocoelomic Fluid measurements	Mol. Weight according to BSS measurements	Mol. Weight according to fraction measurements
1	70,000	68,000	69,000
2	43,000	44,000	-
3	14,000	15,000	-
4	9,600	13,000	-

and of molecular weight determination, that the polypeptides represented by bands 1,2,3 and 4 may be present, in the subventral gland, pseudocoelomic fluid and BSS incubate. Balanced salt solution is an unsuitable medium for maintenance of subventral glands and the organelles concerned with secretion appear faded and drained after incubation in it (see Chapter 6). This suggests that the material which passes into BSS when glands are incubated in it is composed largely of the contents of the secretion granules which comprise most of the gland's volume. The fact that glands incubated in BSS will release the very polypeptides which have been located in both the subventral gland and the pseudocoelomic fluid, suggests that these polypeptides could move between the gland and the pseudocoelomic fluid in vivo.

The granule fraction sample was shown to contain a number of high molecular weight polypeptides. Several of these appear to correspond to polypeptides present in both the subventral gland and the pseudocoelomic fluid. Band 1 (of the subventral gland and pseudocoelomic fluid) appears to be an important constituent of the granule fraction and has a molecular weight of 69,000, as calculated from granule fraction data. Even allowing for contamination, the appearance in the granule fraction of polypeptides which are also present in the subventral glands and the pseudocoelomic fluid, indicates that these particular high weight polypeptides are either products of the pseudocoelomic fluid which are taken up by the gland and packaged into membrane-bound compartments, or secretory products of the gland, packaged into secretory granules and released into the pseudocoelomic fluid. The latter possibility seems the more likely - when the highly active secretory nature of the gland is considered.

So, in summary, the results obtained by SDS electrophoresis suggest that a number of polypeptides of various weights move between the gland and the pseudocoelomic fluid. At least some of these could represent secretory products of the gland which are released into the fluid. It is possible that low molecular weight polypeptides are transported into the gland from the

pseudocoelomic fluid to be used in its secretory and other functions. Some of the gland's secretory products - high molecular weight polypeptides - may be released into the pseudocoelomic fluid for use in that matrix or for transportation to other parts of the nematode.

CHAPTER 8: GENERAL DISCUSSION

8.1 REVIEW OF THE PRESENT STUDY

8.2 FUNCTION OF THE SUBVENTRAL GLAND

8.1 REVIEW OF THE PRESENT STUDY

In the present study, new techniques have been applied to the subventral gland in an attempt to compliment previous work and to develop an integrative analysis of the results that have been obtained. This seems essential if generalisations are to be made about the function of the gland rather than particular statements relevant to one species.

The range of nematode species employed as experimental animals in physiological work has been limited, as have the techniques used to examine them. A few species - *A. lumbricoides, N. brasiliensis, C. elegans* - have been "good" laboratory animals. However more can be learnt about the biology of the nematodes as a group, if a wider range of species is used in experiments (almost no physiological work has been done with the small Adenophorean nematodes). Such considerations led to persistence with work on *O. venulosum* even when, as in the case of the radiotracer experiments, the techniques could have been more profitably applied to another animal. It seemed more important to devise new techniques which could be used with this animal, extending the range of "model" nematodes, than to re-examine species already well known.

The light and electron microscope studies confirmed the findings of previous workers with other nematodes. Two important features of the fine structure of the subventral gland in *O. venulosum* have not been reported previously: (1) the ultrastructure of the plasma membrane of the gland indicates that material might pass between the gland and the pseudocoelomic fluid; (2) the intracellular distribution of the secretion granules suggests that at least some of these may release their products through the plasma membrane into the pseudocoelom - particularly in the posterior region of the gland.

The hypothesis that the subventral glands produce secretory product for release into the pseudocoelomic fluid was a new idea and seemed worth testing. The gel electrophoresis work showed that the gland manufactures large amounts of protein,

much of which is stored in secretion granules. The very active gland can take up labelled amino acids in vitro. Many proteins are present in the gland and some of these may be present in the pseudocoelomic fluid, transported from the gland to the fluid.

Work with *O. venulosum* if a suitable medium could be developed, or with some other species may eventually lead to a more precise understanding of the relationship between the gland and the pseudocoelomic fluid.

8.2 FUNCTION OF THE SUBVENTRAL GLAND

In Chapter 5 a number of hypotheses on the function of the gland were outlined. All of these can be criticised:

(1) Exodigestion

An exodigestive function has been ascribed to the pharyngeal and amphidial glands as well as the subventral glands. It seems doubtful that all of these glands perform the same function and the importance of exodigestion for nematodes, with their well developed gut, is unknown. Moreover, the excretory pore is often not strategically placed as an outlet for exodigestive enzymes.

(2) Tissue Penetration

The role of the subventral glands in tissue penetration is also suspect. Subventral glands often, as in *S. dentatus*, grow markedly as the worm assumes a parasitic existence. However, the correlation that would be expected between the size of the subventral glands and the extent of migration undergone by the nematode, does not occur. For example, *N. brasiliensis* and *N. dubius* have subventral glands of similar size. *N. brasiliensis*, a skin penetrating nematode, migrates for quite long distances within its host, whereas *N. dubius* is simply ingested and undergoes no migration.

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(3) Biochemical Holdfast

It was proposed by Lee (1970) that "nests" of *N. brasiliensis* individuals living together in the crypts of the gut mucosa could protect themselves against dislodging peristalsys by releasing acetylcholinesterase. However, not all *N. brasiliensis* live in crypts and some occur as individuals rather than "nests". It is unlikely that the Ach released by one worm would be effective in halting spasms. Many nematodes, occupying very different niches, secrete Ach in large volumes (Bremmer, Ogilvie, Keith and Berrie; 1973). It seems more likely that the enzyme is secreted for a quite different purpose.

(4) Ecdysis

It was tentatively suggested by Rogers and Sommerville (1960) that the enzymes effecting ecdysis are released through the excretory pore and therefore, presumably, manufactured in the subventral gland. This theory has been supported by Davey and Kan (1968). However, the original work of Rogers and Sommerville has never been repeated. In addition, it should not be assumed that all substances released from the excretory pore, originate in the subventral glands. The tubular excretory system and all structures associated with it, including the hypodermis, also has access to this route to the exterior.

(5) Proteinase Inhibitors; and (6) Gelatinous Matrix

The work which suggests that these materials might be produced in the subventral gland was concerned, in each case, with only one species of nematode and has not been substantiated.

Davey and Sommerville (1974) proposed that the subventral gland might be akin to the mammalian liver or the insect fat body. I would expand this hypothesis to suggest that the nematode subventral gland is an organ of intermediary metabolism

producing proteins to fulfil general and specialised needs of each nematode species. The structure and function of the gland vary from species to species and change during the development of individual nematodes; probably according to a specific program of differentiation. These postulates are supported by the ultrastructural and experimental studies that have been performed on *O. venulosum* and other nematodes.

Ultrastructural investigations of nematode subventral glands suggest that the structure and, so, the function of these secretory organs vary as the individual nematode develops. The most dramatic instance of such a change was seen in *P. decipiens* where distinct ultrastructural changes in the subventral gland were observed to accompany ecdysis (Davey and Sommerville, 1974). In *S. dentatus* the subventral gland grows rapidly and becomes associated with the tubular excretory system after the third moult (Waddell, 1968), the stage at which this nematode becomes parasitic. Changes in the subventral gland would be expected to be more marked in those nematodes like *S. dentatus* which experience great changes in environment during their life cycles. The difference in "activity" suggested by the ultrastructure of *B. sergenti* and *P. redivivus*, on the one hand, and nematodes like *O. venulosum* and *N. brasiliensis*, on the other (see Chapter 5) may be an expression of the subventral gland's capacity to perform different functions in different species.

Several routes are available to the subventral gland for the release of its secretions and all of these routes may be used for dispersal of subventral gland secretions throughout the nematode. Through its association with the tubular excretory system, the gland can potentially release its secretions into the hypodermis or through the excretory pore to the exterior. The present study has shown that the gland may release secretory product into the pseudocoelomic fluid for use there or transport to other parts of the worm.

The association of a gland with a tubular excretory system may be more universal amongst the nematodes than has been thought. Narang's work on *Enoplus brevis* (Narang, 1970), as the only ultrastructural study of a subventral gland in an Adenophorean

nematode is very important. This work suggests that the anterior portion of the subventral gland in *E. brevis* may be an analogue of the tubular excretory system of the Secernentean nematodes. This anterior portion is embedded in the hypodermis, surrounded by "capitate tubules" like those usually seen around the lateral canals, and contains a lumen lined by the tissues of the sinus and hypodermis. This observation arouses conjecture that "glandular" excretory systems in general may be associated with abbreviated tubular systems and that glandular tissue may be attached to "tubular" systems where it has not previously been observed. There have been cases where the application of more sophisticated technology to observation of nematode excretory systems has revealed structures not seen before. For example Narang (1972) has described the subventral gland of *P. redivivus*, whereas earlier workers believed that the Cephaloboid excretory system did not include a subventral gland (Mueller, 1927). Other references could be cited.

In Chapter 6 the secretory products which have been identified in various species of nematode were discussed. These were mainly hydrolytic enzymes. However it need not be assumed that the gland secretes only hydrolytic enzymes since these enzymes have been identified in the gland because they were deliberately sought there in order to test various hypotheses. It is evident that an individual subventral gland can secrete different products at different times (see above) and more than one product at the same time. For example McLaren, Burt and Ogilvie (1974) reported that only one of the two classes of secretion granule in *N. brasiliensis* contained acetylcholinesterase, and that in *T. colubriformis* the same secretion granules apparently contain several enzymes. The present study has suggested that the subventral gland may secrete a number of substances which are released into the pseudocoelomic fluid. The results discussed in Chapter 9 permit speculation that all major constituents of the pseudocoelomic fluid may be produced in the subvental gland. The polypeptide, represented by band 2 of the pseudocoelomic fluid and subventral gland electrophoretic patterns, has approximately the same molecular weight as the haemoglobin purified from *Ascaris* pseudocoelomic

fluid by Wittenberg, Okazaki and Wittenberg (1965). This polypeptide might be a haemoglobin produced in the gland for transport to the pseudocoelomic fluid.

Whatever functions are finally ascribed to the subventral gland, it is certain that its role is important to the nematode. The relationship between the gland and the fluid will surely prove to be significant, as indeed will the connections between the gland and the tubular excretory system. It is to be hoped that the technical barriers preventing our understanding these and other problems in nematode physiology will soon be overcome.

APPENDIX I: ISOLATION OF SECRETORY GRANULES

A technique for fractionation of subventral glands and isolation of the secretory granules was developed. This facilitated further biochemical analysis of the granules (See Chapter 8). The method for isolation of the granules involved three stages which are outlined below.

Stage One: Preparation of Homogenates

Subventral glands were carefully dissected out of live worms kept in cold Krebs-Ringer Phosphate (KRP) over ice. Any attached pseudocoelomocytes were removed and the glands were rinsed in cold KRP and then dropped into a microhomogeniser, containing 50 µl of cold KRP and kept over ice. Homogenates were prepared from 60 glands which were collected as quickly as possible. Care was taken that the glands be obtained from equal numbers of male and female worms. The glands were homogenised by 10-12 clockwise strokes of the pestle.

Stage Two: Removal of Cell Membranes

The plasma and other cell membranes were separated from the rest of the homogenate by high speed centrifugation over a sucrose barrier, using a modification of the method of Harlow, Tolstosher and Wells (1972). The homogenate (about 50 µl) was carefully layered over 10 ml of 5% Triton in 1.7 M sucrose/KRP in a 16.5 ml MSE polypropylene centrifuge tube. The homogenate/sucrose interface was stirred gently with a Pasteur pipette before spinning in a MSE High Speed 25 centrifuge at 110,000 g for 120 minutes at 4°C. After centrifugation, the supernatant was discarded.

Stage Three: Isolation of Granules

The sediment was resuspended in 10ml of cold KRP and spun at 3,300 g for 10 minutes at 4°C. Differential centrifugation and subsequent electron microscope analysis of pelleted sediments were used to establish that 3,300 g was a suitable centrifugal force for sedimentation of a large

sample of secretory granules. The procedures used for differential centrifugation and electron microscopy of the pellets are summarised in Figure 15.

The fractionation method described above allowed the isolation of a fraction of the gland which was predominantly composed of secretory granules. Although centrifugation at 3,300 g produced the largest collection of granules there was some contamination with other material, as can be seen in Plate 34.

Fig. 15 Differential Centriguation and Electron Microscopy of Subventral Gland Fractions.

i) 60 glands homogenised in KRP at 4°C.

ii) 200 µl homogenate layered over 10ml of 5% Triton in 1.5 M sucrose/KRP and centrifuged at 110,000 g for 120 minutes at 4°C.

iii) supernatant removed and discarded.

iv) sediment resuspended in 10 ml KRP and mixed thoroughly.

v) ten 1ml aliquots taken and each made up to 10ml with KRP.

vi) the aliquots were spun at ten different centrifugal forces for ten minutes at 4°C:

aliquot	1	2	3	4	5	6	7	8	9	10
g	2,300	3,300	4,000	4,750	5,250	5,750	6,250	6,750	7,000	7,500

vii) supernatant removed from each of the ten tubes.

viii) each sediment resuspended in 16ml KRP and spun at 110,000g for 10 minutes to form a compact pellet.

ix) supernatant removed and pellets fixed by overlaying with 4% formaldehyde in KRP for 120 minutes, washed (3 x 10 minutes) in fresh KRP, postfixed in Millonig's buffered osmium tetroxide for 45 minutes.

x) pellets washed for 5 minutes with KRP dehydrated in ethanol (2 x 10 min. in 30%, 50%, 70%, 80% and 90%; 4 x 10 min. in 100%) and then lifted out of the centrifuge tube on the end of a tungsten needle (Ernster, Sickevitz and Palade, 1962) and ilfiltrated overnight with Spurr's epoxy resin (epoxy:100% ETOH, 1:1).

xi) further infiltration, embedding and polymerization were effected as for whole glands (See Chapter 3); ultrathin sections were cut, mounted, stained and examined as in Chapter 3.

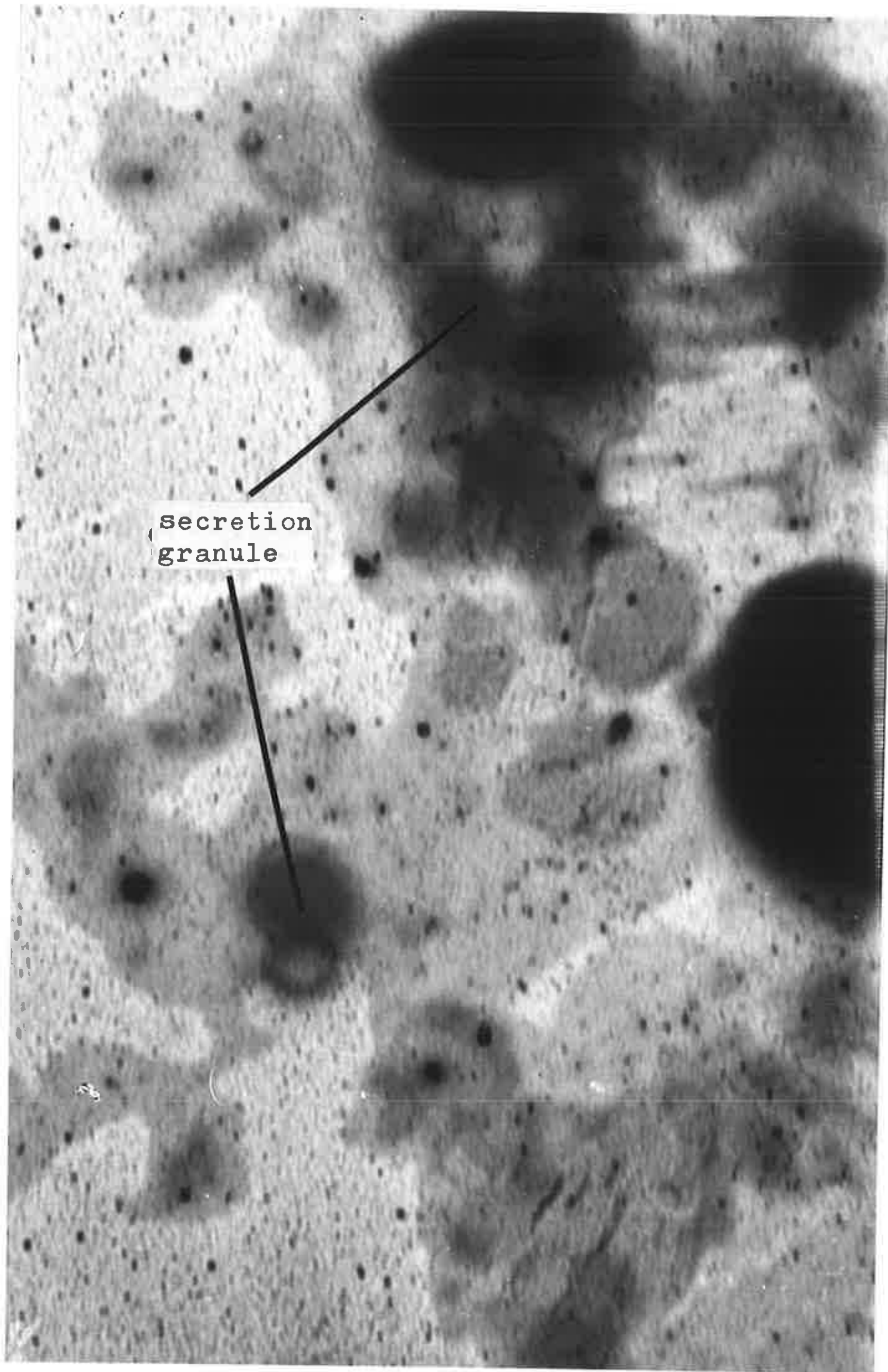

Plate 34. Micrograph of part of a thin section from a pellet of sediment recovered after spinning at 3,000 g. Membrane-bound granules are present but it is not possible to identify these with granules seen in sections from whole glands.

X 3,000

BIBLIOGRAPHY

ATLAS, S.M. and FARBER, E. 1956. "On the molecular weight of cytochrome c from mammalian heart muscle" *Journal of Biological Chemistry*, 219, 31-37.

BANKER, G.A. and COTMAN, C.W. 1972. "Measurement of free electrolytic mobility and retardation coefficient of protein-sodium dodecyl sulfate complexes by gel electrophoresis. A method to validate molecular weight estimates" *Journal of Biological Chemistry*, 247, 5856-5861.

BATES, W.K. and McALLISTER, D.F. 1974. "Some methods for nonlinear regression using desk-top calculators with an application to the Lowry Protein method" *Analytical Biochemistry*, 59, 190-199.

BIRD, A.F., 1971. *The Structure of Nematodes*, Academic Press, U.S.A.

BOGHEN, A.D. and DAVEY, K.G. 1975. "A pseudocoelomocyte in the parasitic nematode *Phocanema decipiens*" *Transactions of the American Microscopical Society*, 94, 203-210.

BREMNER, K.C., OGILVIE, B.M., KEITH, R.K. and BERRIE, D.A. 1973. "Acetylcholinesterase secretion by parasitic nematodes - III *Oesophagostomum spp.*" *International Journal of Parasitology*, 3, 609-618.

BUCKLEY, I.K. 1974. "Subcellular motility: a correlated light and electron microscope study using cultured cells" *Tissue and Cell*, 6, 1-20.

CAMACHO, A., CARRASCOSA, J.L., VINEULA, E. and SALAS, M. 1975. "Discrepancy in the mobility of a protein of Phage ø29 in two different SDS polyacrylamide-gel systems" *Analytical Biochemistry*, 69, 395-400.

CASTELLINO, F.J. and BARKER, R. 1968. "Examination of the dissociation of multichain proteins in guanidine hydrochloride by membrane osmometry" *Biochemistry*, 1, 2207-2217.

CHITWOOD, B.G. and CHITWOOD, M.B. 1950. *An introduction to Nematodology*, Monumental Printing Co., U.S.A.

COBB, N.A. 1950. "Nematodes and their relationships" United States Department of Agriculture Yearbook : 1914, published 1915; reference from Chitwood and Chitwood.

DE CONNINK, L. 1965. "Généralitiés" in *Traite de Zoologie*, Tomes III and IV, ed. Grassé, P.P., Masson et Cie, France.

CROLL, N.A. and MATTHEWS, B.E. 1977. *Biology of Nematodes*, Blackie and Sons, U.K.

CROFTON, H.D. 1966. *Nematodes*, Hutchinson and Co., U.K.

DASH, K. 1973. A personal communication.

DAVEY, K.G. and KAN, S.P. 1968. "Molting in a parasitic nematode, *Phocanema decipiens*. IV ecdysis and its control" *Canadian Journal of Zoology*, 46, 893-

DAVEY, K.G. and SOMMERVILLE, R.I. 1974. "Molting in a parasitic nematode, *Phocanema decipiens*. VII the mode of action of the ecdysial hormone" *International Journal of Parasitology*, 4, 241-259.

DIKMANS, G. and ANDREWS, J.S. 1933. "A comparative morphological study of the infective larvae of the common nematodes parasitic in the alimentary tract of sheep" *Transactions of the American Microscopical Society*, 52, 1-25.

DULLEY, J.R. and GRIEVE, P.A. 1975. "A simple technique for eliminating interference by detergents in the Lowry method of protein determination" *Analytical Biochemistry*, 64, 136-141.

ERNSTER, L., SIEKEVITZ, P. and PALADE, G.E. 1962. "Enzyme-structure relationships in the endoplasmic reticulum of rat liver" *Journal of Cell Biology*, 15, 541.

EVANS, A.A. and FISHER, J.M. 1970. "The excretory systems of three *Ditylenchus* spp." *Journal of Nematodology*, 2, 260-264.

FAIRBANKS, G., STECK, T.L. and WALLACH, D.F.H. 1971. "Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane" *Biochemistry*, 10, 2606-2617.

FAWCETT, D.W. 1966. *An Atlas of Fine Structure*, W.B. Saunders and Co., U.S.A.

GEBAUER, O. 1932. "Zur Kenntnis der Parasiten fauna der Gemse" *Zeitschrift fur Parasitenkunde*, 4, 162-1966.

GOLDBERG, A. 1951. "Life history of *Oesophagostomum venulosum*, a nematode parasite of sheep and goats" *Proceedings of the Helminthological Society of Washington*, 18, 36-47.

GOODEY, T. 1924. "Oesophagostomes of goats, sheep and cattle" *Journal of Helminthology*, 2, 97-100.

HARLOW, R., TOLSTOSHEV, P. and WELLS, J.R.E. 1972. "Membrane-a major source of chromatin-associated RNA and non-histone proteins in avian erythroid cells" *Cell Differentiation*, 2, 341-349.

HARRIS, J.E. and CROFTON, H.D. 1957. "Structure and function in the nematodes: internal pressure and cuticular structure in *Ascaris*" *Journal of Experimental Biology*, 34, 116-130.

HARTREE, E.F. 1972. "Determination of protein: a modification of the Lowry method that gives a linear photometric response" *Analytical Biochemistry*, 48, 422-427.

HÖRCHNER, F. 1969. "Immunologische Untersunchgen an *Chabertia ovina* - bzw *Oesophagostomum venulosum* - befallenen Schafen" *Zeitschrift fur Parasitenkunde*, 31, 6-7, abstracted in *Helminthological Abstracts*, 1969. 38, 3348.

HYMAN, L.H. 1951. *The Invertebrates* : Acanthocephala, Aschelminthes and Entroprocta : The Pseudocoelomate Bilatera Vol. III. McGraw-Hill Book Co., U.S.A.

JAMIESON, J.D. 1972. "Transport and discharge of exportable proteins in exocrine cells : in vitro studies" *Current Topics in Membranes and Transport*, 3, 273-338.

KANAGASUNTHERAM, R., MULKIT SINGH, BENG-CHUAN HO and HENG-LEONG CHAN, 1974. "Some ultrastructural observations on the microfilaria of *Breinlia sergenti* - the excretory complex, rectal cells and anal vesicle" *International Journal of Parasitology*, 4, 7-15.

KATES, K.C. and SHORB, D.A. 1943. "Identification of eggs of nematodes parasitic in domestic sheep" *American Journal of Veterinary Research*, 4, 54-60.

KELETI, G. and LEDERER, W.H. 1974. *Handbook of Micromethods for the Biological Sciences*, Van Nostrand Reinhold Co., U.S.A.

LAEMLI, U.K. 1970. "Cleavage of structural proteins during assembly of the head of bacteriophage T4" *Nature*, 227, 680-685.

LEE, D.L. 1965. *The Physiology of Nematodes*, Oliver and Boyd Ltd., U.K.

LEE, D.L. 1970. "The fine structure of the excretory system in adult *Nippostrongylus brasiliensis* (Nematoda) and a suggested function for the 'excretory glands'" *Tissue and Cell*, 2, 225-231.

LEE, Hong-Fang, I-Li Chen and Ruey-Ping Lin, 1973. "Ultrastucture of the excretory system of *Anisakis* larva (Nematoda: Anisakidae)" *The Journal of Parasitology*, 59, 289-298.

LELAND, S.A. 1963. "Studies on the in vitro growth of parasitic nematodes. I. Complete or partial parasitic development of some gastrointestinal nematodes of sheep and cattle" *The Journal of Parasitology*, 49, 600-611.

LOCKE, M. and COLLINS, J.V. 1968. "Protein uptake into multivesicular bodies and storage granules in the fat body of an insect" *Journal of Cell Biology*, 36, 453-488.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., and RANDALL, R.J. 1951. "Protein measurement with the folin phenol reagent" *Journal of Biological Chemistry*, 193, 265-275.

MAGGENTI, A.R., 1962. "Production of the gelatinous matrix and its taxonomic significance in *Tylenchulus* (Nematoda" Tylenchulinae)" *Proceedings of the Helminthological Society of Washington*, 29, 139.

MAGGENTI, A.R. and ALLEN, M.W. 1960. "The origin of the gelatinous matrix in *Meliodogyne*" *Proceedings of the Helminthological Society of Washington*, 27, 4-10.

MAIZEL, J.V. 1971. "Polyacrylamide gel electrophoresis of viral proteins" in *Methods in Virology*, Academic Press, U.S.A., ed. K. Maramorosch and H. Kaprowski.

MC LAREN, D.J. 1974. "The anterior glands of *Necator americanus* (Nematoda : Strongyloidea) - I Ultrastructural Studies" *International Journal of Parasitology*, 4, 25-37.

MC LAREN, D.J., BURT, J.S. and OGILVIE, B.M. 1974. "The anterior glands of adult *Necator americanus* (Nematoda : Strongyloidea) - II cytochemical and functional studies" *International Journal of Parasitology*, 4, 39-46.

MUELLER, J.S. 1929. "The excretory system of *Anisakis simplex*" *Zeitschrift fur Zellforschung und mikroskopische Anatomie*, 8, 361-403.

MURPHY, D.B. and TILNEY, L.G. 1974. "The role of microtubules in the movement of pigment granules in teleost melanphores" *Journal of Cell Biology*, 61, 757-779.

NARANG, H.K. 1970. "The excretory system of nematodes : structure and ultrastructure of the excretory system of *Enoplus brevis*" *Nematologica*, 16, 517-522.

NARANG, H.K. 1972. "The excretory system of nematodes" structure and ultrastructure of the excretory system of *Panagrellus redivivus*, *Ditylenchus myceliophagus* with some observations on *D. dipsaci* and *Jeterpdera rostochiensis*" *Parasitology*, 64, 253-268.

NICHOLAS, W.D. 1975. *The Biology of Free-living Nematodes*, Clarendon Press, U.K.

OGILVIE, B.M. and JONES, V.E. 1971. "*Nippostrongylus brasiliensis:* a review of immunity and the host/parasite relationship in the rat" *Experimental Parasitology*, 29, 138-177.

PATTERSON, R. and KNIGHT, C.A. 1975. "Protein synthesis in tobacco protoplasts infected with tobacco mosaic virus" *Virology*, 64, 10-22.

PEASE, D.C. 1964. *Histological Techniques for Electron Microscopy*, 2nd ed., Academic Press, U.S.A.

PEREGRINE, P. 1973. "Observations on arcade cells and pseudocoelomocytes in some oxyuroid nematodes parasitic in cockroaches" *Parasitology*, 66, 165-179.

RHOADS, M.L. and ROMANOWSKI, R.D. 1974. "The secretory nature of the excretory gland cells of *Stephanurus dentatus* III proteinase inhibitors" *Experimental Parasitology*, 35, 363-368.

RHOADS, M.L., ROMANOWSKI, R.D., DOHERTY, R.F. and STEWART, K.K. 1978. "Proteinase inhibitors from the excretory gland cells of *Stephanurus dentatus*" *The Journal of Biological Chemistry*, 253, 1639-1642.

ROBERTS, F.H.S. and O'SULLIVAN, P.J. 1950. "Methods for egg counts and larval cultures for strongyles infecting the gastro-intestinal tract of cattle" *Australian Journal of Agricultural Research*, 1, 99-102.

ROGERS, W.P. and SOMMERVILLE, R.I. 1960. "The physiology of the second ecdysis of parasitic nematodes" *Parasitology*, 50, 329-348.

ROMANOWSKI, R.D., THOMPSON, D.E. and MADDEN, P.A. 1971. "The secretory nature of the excretory gland cells of *Stephanurus dentatus* I Morphology and histochemistry" *Proceedings of the Helminthological Society of Washington*, 38, 143-149.

ROMANOWSKI, R.D., RHOADS, M.L. and MALAKATIS, G.M. 1973. "The secretory nature of the excretory gland cells of *Stephanurus dentatus* II presence of hydrolytic enzymes" *Experimental Parasitology*, 34, 40-43.

SEDDON, H.R. 1969. *Diseases of Domestic Animals in Australia*, Part I Helminth Infestations, revised by H.E. Albiston, Commonwealth of Australia : Department of Health. Service Publications (Veterinary Hygiene) No.5 Australia.

SMITH, L. 1965. "The excretory system of *Panagrellus redivivus* (T. Goodey, 1945)" *Comparative Biochemistry and Physiology*, 15, 89-92.

SOMMERVILLE, R.I. 1966. "The development of *Haemonchus contortus* to the fourth stage in vitro" *Journal of Parasitology*, 52, 127-136.

STEEDMAN, H.F. 1960. *Section Cutting in Microscopy*, Blackwell Scientific, U.K.

WADDELL, A.H. 1968. "The excretory system of the kidney worm *Stephanurus dentatus* (Nematoda)" *Parasitology*, 58, 907-919.

WEBER, K. and OSBORNE, M. 1969. "The reliability of molecular weight determinations by dodecyl sulfate - polyacrylamide gel electrophoresis" *Journal of Biological Chemistry*, 244, 4406-4412.

WEINSTEIN, P.P. 1960. "Excretory mechanisms and excretory products of nematodes : an appraisal" in *Host Influence on Parasite Physiology*, ed. L.A. Stauber, Rutgers University Press, U.S.A.

WEINSTEIN, P.P. and JONES, M.F. 1956. "The in vitro cultivation of *Nippstrongylus muris* to the adult stage". *Journal of Parasitology*, 42, 215-236.

WETZEL, R. Von and Marholott, D., 1955. "Zur Differential diagnose derdritten larven von *Chabertia ovina* und *Oesophagostomum venulosum*" *Deutsch Tierarztiche-Wochenschrift*, 62, 429-430.

WITTENBERG, B.A., OKAZAKI, T. and WITTENBERG, J.B. 1965. "The hemoglobin of *Ascaris* perienteric fluid I purification and spectra" *Biochimica et Biophysica Acta*, III, 485-495.