# FUNCTIONAL MORPHOLOGY OF THE MIDGUT AND AN UNDESCRIBED ORGAN IN THE HETEROTARDIGRADE ECHINISCUS VIRIDISSIMUS PETERFI, 1956

A Thesis

by

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ABSTRACT

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This study was undertaken to examine adaptations of the heterotardigrade, Echinsiscus viridissimus, to the problems of osmoregulation and excretion posed by its terrestrial environment. It provides a description of the presumed osmoregulatory and excretory tissues, the midgut and a newly discovered organ, based on cytochemistry, comparative morphology, computer assisted reconstruction and stereology.

The midgut appears to be a relatively straight sac with a large lumen. The only structures possibly involved in osmoregulation found were deep apical septate desmosome junctions and dilated baso-lateral spaces on both the dorsal and ventral surfaces. Although the midgut cells do not exhibit a typical morphology for osmoregulation, they do display prominent electron dense inclusions that may indicate an excretory function. These inclusions appear to



be enclosed in a system of interconnected canals. Accumulations of electron dense material were also found free in the body cavity. Cytochemical and stereological tests were conducted to assess similarities between the midgut and body cavity inclusions. All tests suggest they are not related.

During the course of a three-dimensional reconstruction of the midgut two organs were discovered. They are identical and occur serially in two segments corresponding with the second and third pairs of legs. The organs lie between the ventral midgut and the underlying epidermis. They are made up of three cells of two different types, two small lateral cells and a much larger medial cell. The lateral cells have few organelles and possess a convoluted plasmalemma with an elevated basement membrane. Lateral cells also contain ducts that continue past the junction of the lateral and medial cells and proceed in a deep cleft in the medial cell to the endocuticle. Projections of the lateral cells follow the ducts in the medial cell and both generate a single loop as they course through medial cell. Although the lateral cell projections terminate between epidermal cells, the ducts continue into the endocuticle where they end blindly just ventral to the dorsal plates. The ducts are surrounded by a layer of saccules formed by invaginations of the medial

cell plasmalemma. These saccules, which behave differently under different fixation methods, together with high density of mitochondria and deep basal infolds suggest that this organ may be the primary osmoregulatory tissue. The thickened basement membrane of the lateral cells is continuously secreted into the body cavity producing an accumulation of free basement membrane. In close association with these accumulations are clumps of body cavity material which appear to be modified by the secreted basement membrane. Since similar electron dense material is also found in the infolds of the medial cell, the ducts in the medial cell and the endocuticle, the organ appears to be excretory as well as osmoregulatory.



ACKNOWLEDGEMENTS

I would like to express my appreciation to several people for their contributions to me during this project.

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The Graduate School provided the funds necessary to build the cryofixation device used in the project. Without this funding it would have been impossible to have obtained these results.

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## INTRODUCTION

The Tardigrada is a phylum of microscopic, bilaterally symmetrical invertebrates. They are commonly called "water bears" because of their body shape and their four pairs of claw bearing legs which often move deliberately reminding Huxley in 1869 of an imagined ponderous gait of a bear (Pennak, '78). The animals possess a cuticular surface which is flattened ventrally and convex dorsally. The body is divided into five segments. Although microscopic they possess several complete organ systems. The nervous system is made up of an anterior brain with circumesophageal connectives, paired eyes and a segmented double ventral nerve cord joined at five ganglia associated with the head and each set of legs. A terminal mouth, buccal tube, salivary glands, stylets, pharynx, esophagus, midgut, hindgut and subterminal cloaca complete the digestive system. Although in eutardigrades the excretory system consists of Malpighian tubules and a modified hindgut (Dewel and Dewel, '79), no excretory system has been reported for the heterotardigrades. Movement is effected by muscle cells which extend from insertion points on the cuticle in various arrays throughout the body cavity. The

body cavity is also characterized by the presence of free floating, lipid-rich, body cavity cells. Lying along the inner cuticular surface is a layer of epidermal cells which enclose the body cavity and secrete and maintain cuticle. Tardigrades include many cosmopolitan groups including Arctic and Antarctic to tropical forms which live in a variety of habitats ranging from fresh water to marine and including semiterrestrial forms living in moss communities. In the latter case, since their activity depends upon the presence of a film of water surrounding the body, they are all essentially aquatic. Some are able to undergo anhydrobiosis which enables them to survive extended periods of dessication (Crowe and Madin, '71).

There are two major classes within the phylum; the Eutardigrada and the Heterotardigrada. Systematically, affinities can be shown with several phyla including the Gastrotricha, Rotifera, Nematoda, Annelida, Onychophora, and Arthropoda (Riggin, '62). The eutardigrades are unarmored (i.e. the cuticle is not in the form of plates), live primarily in fresh water or terrestrial habitats and are either herbivorous or predaceous. The heterotardigrades are armored (i.e. the cuticle is divided into individual plates) and are found in marine, freshwater and terrestrial habitats. This group is considered exclusively herbivorous, feeding primarily on moss and algal cells (Kristensen, '87, Nelson and Higgins, '90).



Echiniscus viridissimus is in the Echiniscidae family group of terrestrial heterotardigrades that have been hypothesized by Thulin ('28) and Marcus ('29) to have arisen directly from a marine ancestor (order, Arthrotardigrada within the class Heterotardigrada). Since they are usually found in moss or lichen habitats, they are thought to have coevolved with the lower plants (Kristensen, '87). This genus is the most successful within the phylum in terms of species number (Kristensen, '87). E. viridissimus is found living in moss cushions of Grimmia alpicola, a community which includes various organisms including rotifers, ciliates, nematodes, and a eutardigrade, Milnesium tardigradum (Dewel and Dewel, '87). The family is mainly herbivorous possessing stylets which pierce the cell wall and a muscular pharynx which sucks out the cellular fluids of the moss. Their ability to undergo cryptobiosis (reduced metabolism when faced with environmental extremes) has been hypothesized by Pilato ('79) to be the reason for their success. This phenomenon allows the animals to survive periodic dessication (anhydrobiosis).

The adapatations of tardigrades to a terrestrial habitat experiencing periods of hydration and dessication poses two extremes, that of being surrounded by hypotonic water constantly entering by osmosis and that of the absence of water. The ability of an organism to survive in this niche requires adaptations for the removal of excess

water, retention of salts, and the conservation of water prior to anhydrobiosis. Understanding these adaptations and how they relate to excretion will help elucidate the radiation of terrestrial heterotardigrades. Echiniscids have become successful in this niche, and an investigation was undertaken to examine cell/tissue/organ specializations for excretion and osmoregulation in one species of the family. Through electron microscopy, cytochemistry, three-dimensional reconstruction, and stereology (three-dimensional morphometric analysis) a study of the midgut, reported to function in excretion and osmoregulation, and a recently discovered organ (Dewel, et al., '89) could give insights into the way these problems have been solved.

## MATERIALS AND METHODS

The Heterotardigrade Echiniscus viridissimus was collected from the moss Grimmia alpicola found on mortar substrates of rock walls located on the campuses of Appalachian State University, Boone, North Carolina and East Tennessee State University, Johnson City, Tennessee. Tardigrades were extracted by soaking the moss in a closed vial of distilled water for at least 20 minutes and then vigorously shaking it several times and decanting the water into a petri dish. Specimens were isolated with an Irwin loop using a Wild dissecting microscope and placed in separate drops of distilled water in a petri dish. Both standard chemical fixation techniques and cryofixation were used to prepare the specimens for electron microscopy.

### CHEMICAL FIXATION

Specimens of E. viridissimus were transferred using an Irwin loop to fixative (prefix) containing one part 25% glutaraldehyde, 5 parts 0.2M PIPES buffer, 4 parts 4% osmium tetroxide making the final solution concentrations 2.5% glutaraldehyde, 0.1M PIPES buffer and 1.6% osmium tetroxide. After 25 seconds the animals were cut transversely in half, transferred within 60 seconds to fresh prefix and refrigerated at 4°C. for 1 hour. After rinsing in 0.1M PIPES buffer animals were further preserved in a second fixative (postfix) of 2.0% osmium tetroxide in

0.1M PIPES buffer, rinsed in distilled water, and en bloc stained in 1.0% uranyl acetate in distilled water. Dehydration was accomplished using a graded acetone series; 30%, 50%, 70%, 95%, and 3 rinses at 100% followed by embeddment in Spurr's epoxy resin (Spurr, '69). Since this species has a dense outer cuticle resistant to penetration by the fixative this method has a low success rate so an alternative method was utilized allowing comparison between the two techniques.

CRYOFIXATION

Animals were collected as before and placed on a copper grid which was blotted lightly from the side away from the specimen and immediately plunged into liquid propane maintained at a temperature of -180 to -186$^{o}$C. The cryofixation device used was one similar to that designed by Howard and O'Donnell ('87) and was built by R. A. Dewel, W. C. Dewel and the author. A one liter Dewar flask held a liquid nitrogen bath used to cool a copper cup placed within it supported by a copper pipe with sidearms. The propane was dispensed into the cup from a tank of propane gas. A thermocouple was placed in the cup to monitor the temperature and an air driven stirrer was also inserted to keep the propane in a liquid state and to increase the rate of freezing. During the freezing process the animals generally remained attached to the grid. The specimens were then transferred to a gelatin capsule immersed in liquid nitrogen. After all animals were pooled



in the gelatin capsule it was transferred to a freeze substitution fluid consisting of 2% osmium tetroxide in absolute acetone (-80 to -92$^{o}$C.) and maintained at a temperature of -85$^{o}$C. for a period of 2-3 days in an ultra low temperature freezer (Revco Industries). Specimens were then brought to room temperature over a period of 6 hours, rinsed in fresh acetone and infiltrated with Spurr's epoxy resin, embedded and sectioned using both glass and diamond knives. Sections were placed on slotted, formvar coated grids and stained according to standard procedures for transmission electron microscopy.

RECONSTRUCTION

Serial thin sections (70nm) of the entire organ were photographed at 3000X using a Philips 201 transmission electron microscope. Electron microscope negatives were serially mounted in a standard Durst photographic enlarger and imaged at an additional magnification of 2.5X onto plain sheets of paper. Major sturctures and contours of the organ were traced on the paper with a pen and then entered into a computer by placing the tracings, in order, on a digitizing tablet and retracing using an electronic stylus (digitizing was done at the Regional Resource Center, MICROMED, Bowman-Gray School of Medicine, Winston Salem, North Carolina). Once entered, the collection of two-dimensional data sets were merged on the computer screen utilizing HVEM 3-D software obtained from J. C. Kinnamon, Boulder, Colorado. Three-dimensional stereopairs

were obtained by photographing the image on the CRT screen twice with a separation angle of 5 degrees.

INTERMEDIATE VOLTAGE ELECTRON MICROSCOPE

Specimens were fixed, stained and embedded according to the procedure listed above for cryofixation. Thick sections (1 um) were placed on formvar coated slotted grids and photographed at 300 kV using the Intermediate Voltage Electron Microscope located at Bowman Gray School of Medicine, Winston Salem, North Carolina.

STEREOLOGY

Stereological methods were used to make unbiased estimates of the volume of the animal, midgut and body cavity inclusions and the volume and number of midgut inclusions. Ten animals were randomly selected from a pool that had been cryofixed in isopentane chilled with liquid nitorgen, cryosubstituted in osmium tetroxide and acetone at -85$^{o}$C for three days and embedded in epoxy resin. Serial longitudinal sections, 0.75um in thickness, were dried onto slides, stained and photographed using Technical Pan film. Projected images magnified 8X were counted and from this a section interval, k, was determined. Using a random numbers table, the starting section was determined and sampling was done systematically from this point (Gundersen, '87). The volumes, V(ref), were determined using Cavalieri's principle in which serial sections of known thickness, t, and a set interval were chosen throughout the animal. A tesselation grid was placed



randomly over the sections and the points, Pn, hitting the structure being measured were counted (each point having a determined area a(p)) (Pakkenberg and Gundersen, '88).

$$Ea = a(p)\ (Pn)$$

$$V(ref) = t(k\ (Ea))$$

The disector, a stereological tool for sampling and counting three-dimensional objects regardless of their size and shape, (Sterio, '84, Gundersen, '86) was used to determine the number of midgut inclusions, N. The section interval, k, was determined based on the size of the animal. Echiniscus specimens were sectioned, separated by a known distance of 0.75um, h. On one of the sections the sets of profiles through the inclusions were sampled over a known area, a, which in this case was the entire animal. All the inclusions sampled in this way were looked for in the second section and those not present in this "look-up" section are counted, Q-.

$$N = (EQ-)\ (k)$$

The two requirements for this procedure, the ability to unambiguously identify an item from the set(s) of profiles by one or more sections through it and h be small enough that no particles are left undetected in the sections were met (Gundersen, '86).

CYTOCHEMISTRY

Several cytochemical tests were conducted to determine the composition of the midgut and body cavity inclusions. Some of the tests required removal of the epoxy resin which

was accomplished by an 11 minute immersion of the slides in ethanol saturated with KOH (Hayat, '81).

Lipids (Wigglesworth, '81)

E. viridissimus were cut in a 0.34M sucrose solution and simultaneously fixed with 1.6% osmium tetroxide and 0.25% glutaraldehyde in the 0.1M PIPES buffer. They were dehydrated using a graded ethanol series to 70% and then incubated in 10% farnesol in 70% ethanol for one hour. The treated animals were then rehydrated, postfixed in 2% osmium tetroxide before dehydration in acetone, embedded in epoxy resin and sectioned at 70nm for electron microscopy. Farnesol is a terpene containing three double bonds. It is able to incorporate into lipid molecules in a process called partitioning. As a consequence of farnesol incorporation the osmium tetroxide which reacts with double bonds will stain lipids more effectively.

Masked Lipids (Wigglesworth, '81)

Commonly much of the lipid in the cell is "bound" or masked by proteins which prevents staining. Since thymol has been proven effective in unmasking lipids, animals were treated to the same prefix listed above and then treated with 0.1% thymol in 0.34M sucrose overnight before placing them in the farnesol treatment.

Polyphenols (Locke and Krishnan, '71).

Organisms were treated with the same prefix and washed in 5% sodium metabisulfite for 10 minutes to block aldehyde groups from fixation. They were rinsed in distilled water



and incubated in a solution of 0.4% silver nitrate, 2% hexamethylenetetramine, and 0.2% sodium borate in distilled water at 60°C. for 30 minutes in the dark. Treated animals were then rinsed in distilled water and placed in 3% sodium thiosulfate in ethanol to remove any unreduced silver salts.

Another test for polyphenols was conducted using a ferric choloride reagent as described by Shriner, et al. ('80). Living and cryofixed animals in acetone were placed on a glass slide in a drop of pure chloroform, free of any ethanol, and squashed. A drop of 1% anhydrous ferric chloride reagent followed by a drop of reagent grade pyridine was added under the cover slip. This test renders a dramatic color change in the presence of phenolics.

Proteoglycans

Specimens were placed in a 1:1 mixture of 2% paraformaldehyde and 0.2M cacodylate buffer, cut transversely after 30 seconds and fixed for an additional 30 minutes. After rinsing once in cacodylate buffer, they were incubated in cationized ferritin in cacodylate buffer for 38 minutes and then rinsed again in buffer. Cationic ferritin binds to negatively charged proteoglycans in the basement membrane. The specimens were then fixed in a 3:5:2 mixture of 1% glutaraldehyde, 0.1M cacodylate buffer with sucrose and distilled water for 30 minutes. After this second prefixation, they were postfixed in a 1:1 mixture of 2% oxmium tetroxide and 0.2M cacodylate for one

hour. Dehydration and embeddment were as described previously.

## RESULTS

### EXTERNAL MORPHOLOGY

A scanning electron micrograph (fig. 1A) reveals many external features of Echiniscus viridissimus including the prominent thickened dorsal plates formed of exocuticle. This characteristic of the family Echiniscidae is absent in most marine tardigrades and all other limnic and terrestrial tardigrades. The plates are characterized by pits and bumps used as a taxonomic tool for species identification. In living specimens of this echiniscid species, a green coloration is particularly evident in molted cuticle. The body is made up of a head segment and four trunk segments. Externally, the head segment has long and short cirri functioning as tactile sense organs, papillate clavae functioning as chemoreceptors and a terminal mouth (Kristensen and Higgins, '84) (fig. 1A). Each of the four body segments has a pair of appendages ending in claws. Depressions between the body plates are characterized by the presence of numerous bacteria (fig. 1A) which are able to flourish on its body surface.

### INTERNAL MORPHOLOGY

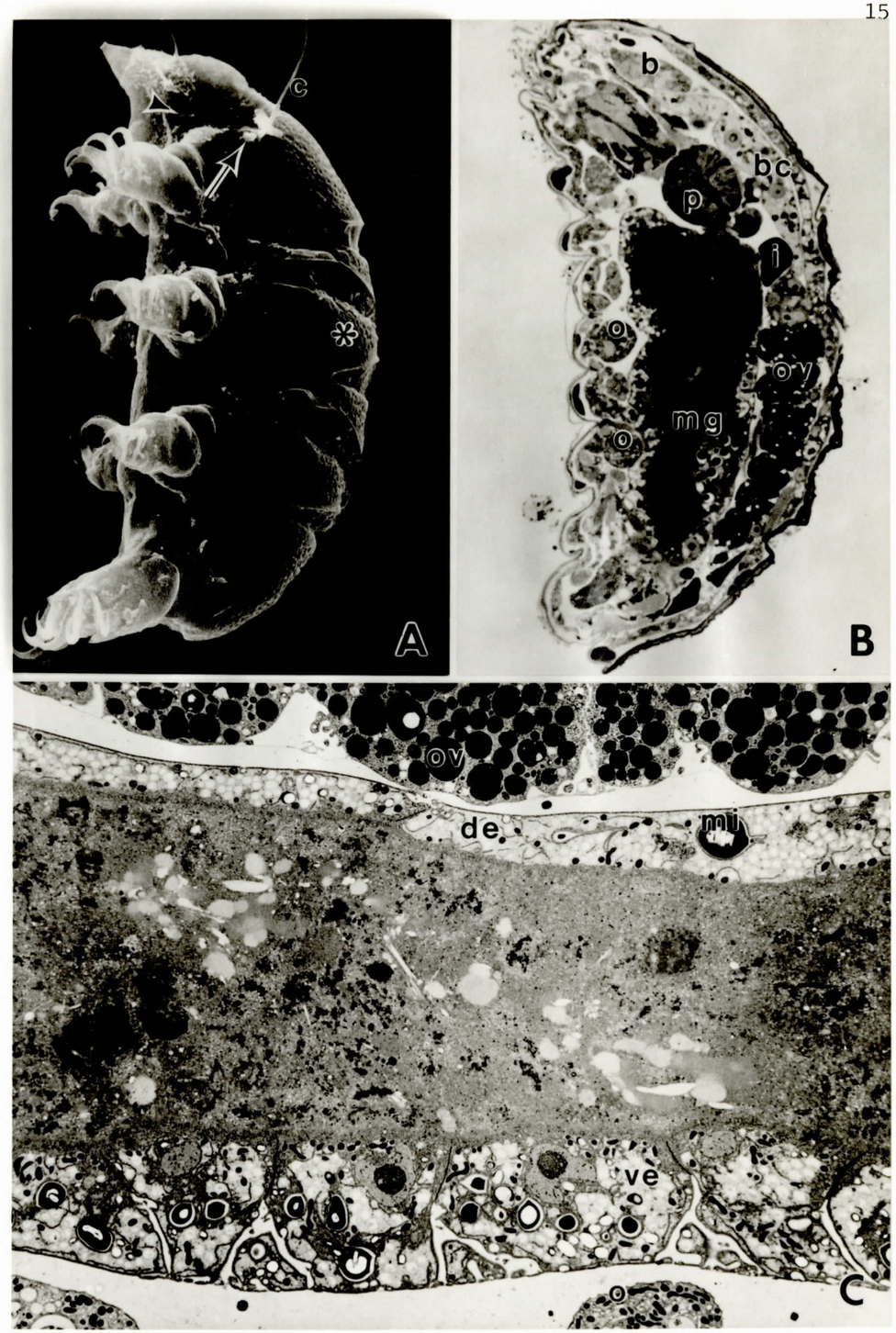
A midsagittal light micrograph and transmission electron micrograph reveal the major internal structures of the Bauplan of E. viridissimus (fig. 1B). A muscular,

FIGURE 1

A. SEM of the external surface with long and short cirri (c), papillate clavae (arrow), appendages ending in claws, a terminal mouth and bacteria indicated by an arrowhead. Note the dorsal plates (*). (600X)

B. Light, midsaggital section showing dorsal brain (b), pharynx (p), midgut (mg), body cavity cells (bc), ovary (ov), body cavity inclusions (i) and organ (o). (2,800X)

C. Midsaggital TEM through the midgut demonstrating differences between dorsal epithelium (de) and ventral epithelium (ve), midgut inclusions (mi), ovary (ov) and organs (o). (2,800X)



triradiate pharynx posterior to the buccal cavity acts to draw fluids from the plant cells. A short esophagus leads into a long midgut which is generally packed with ingested material. Within the body cavity are body cavity cells which are lipid rich and may also be absorbing and digesting foreign materials within the body cavity. A large dorsal brain is located above the mouth tube. Corresponding to each body segment are ganglia located ventrally between each pair of legs. A large dorsal ovary lies between the midgut and epidermis.

MIDGUT

The midgut is proportionately large (fig. 1B); in general it is a relatively straight sac with a large lumen composed of a single layer of epithelial cells. The dorsal epithelium is squamous and much thinner than the ventral columnar cells (fig. 1C). All cells are connected by deep apical continuous septate junctions and basal gap junctions. These junctions bracket dilated intercellular spaces (fig. 3B). Other than these specializations, the cells contain no typical elaborations for osmoregulation such as basal infoldings, apical microvilli and numerous mitochondria. However, they do display prominent electron dense inclusions (MGI). In addition to these, a second distinctive inclusion that has an electron dense coat around the membrane and an electron lucent center, is found in some animals (fig. 3A). The apical surface of the midgut has irregular lobed projections which are unlike

FIGURE 2

A. Cryofixed electron micrograph through a portion of the midgut showing collapsed canals (arrowhead) surrounding midgut inclusions (mi) which are not fixed in the centers. The canals are lined on the cytoplasmic face with endoplasmic reticulum which appear as an electron dense layer surrounding the canals. (8,800X)

B. Cryofixed electron micrograph of a midgut inclusion with internal concentric concretions (cc) in the center. Note the rough endoplasmic reticulum (arrowheads). (22,500X)

C. Cryofixed electron micrograph showing strands of extracellular material that line the canals (arrowhead) enclosing the midgut inclusions. (50,000X)

D. Cryofixed micrograph of the apical surface of the midgut showing lobed projections (p), midgut lumen (l), midgut inclusion (mi), extracellular strands of the canals (arrowhead) and endoplasmic reticulum both lining the cytoplasmic face of the canals and extending into the lobed projections. (75,000X)



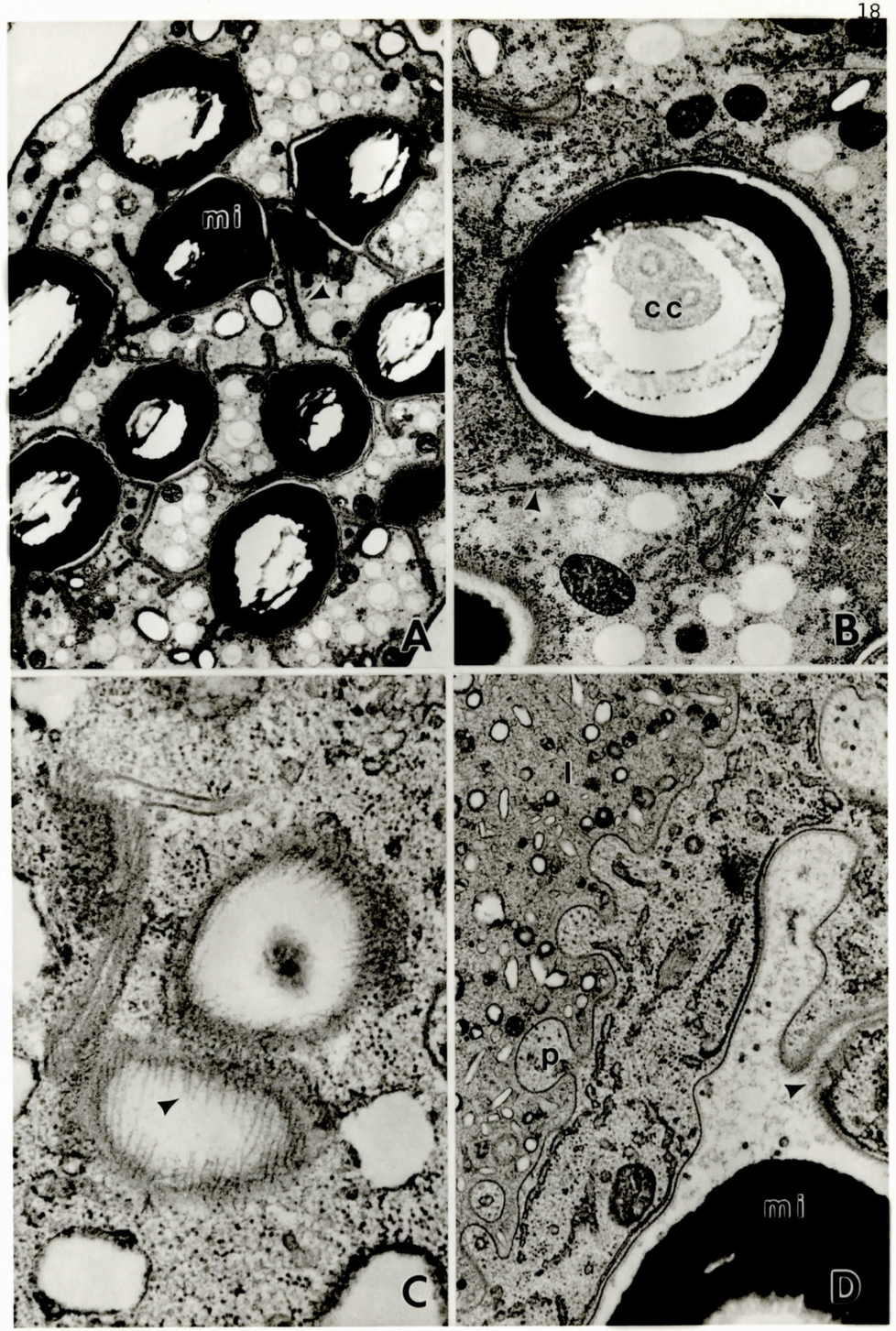


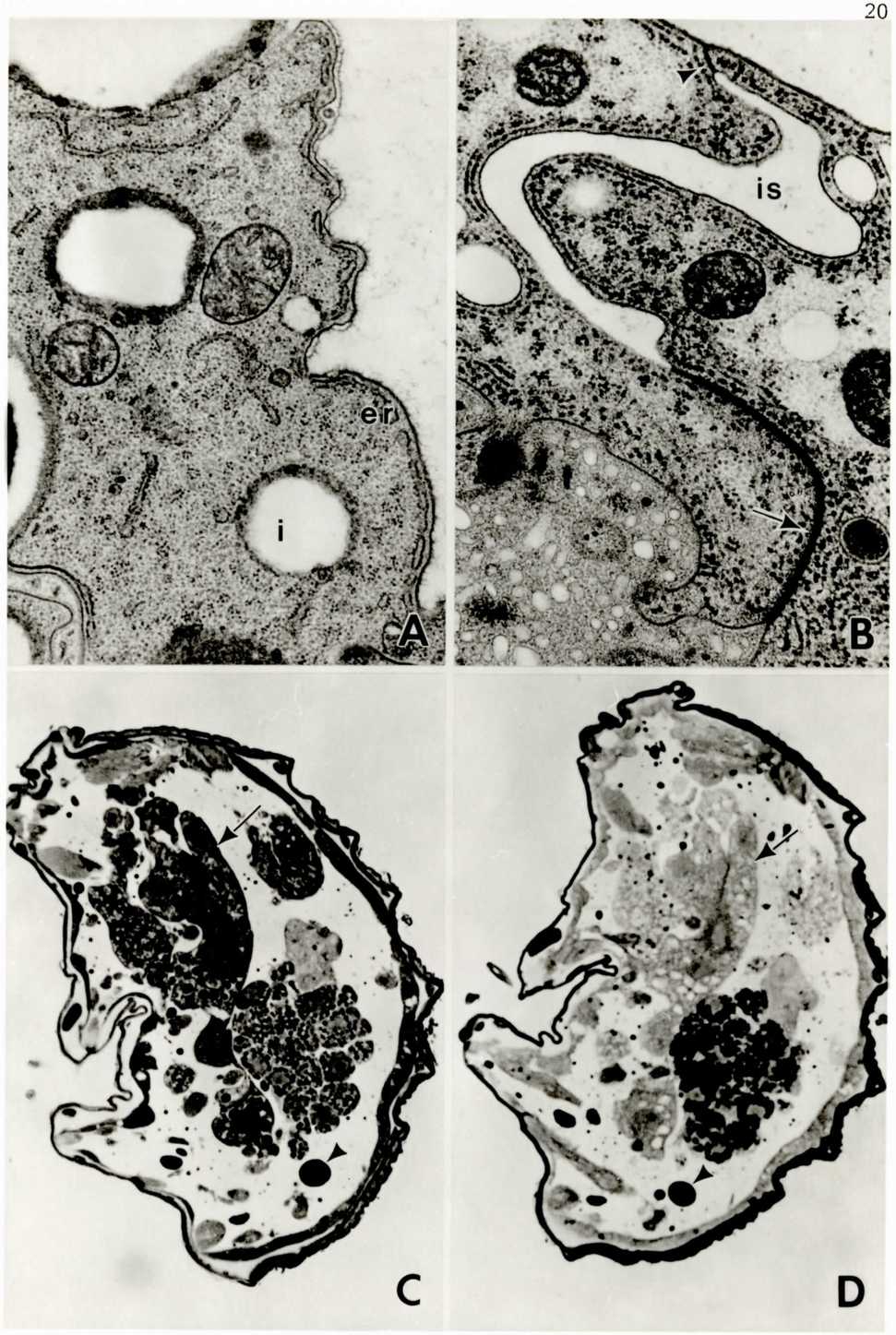
FIGURE 3

A. The basal surface of midgut lined with endoplasmic reticulum (er) also shows another type of inclusion contained in the midgut (i). (36,000X)

B. Junction between midgut cells is made up of an apical continuous junction (arrow), an intercellular space (is), and a basal gap junction (arrowhead) each of these is lined with endoplasmic reticulum. (48,000X)

C. Untreated section with both midgut inclusions (arrow) and body cavity inclusions (arrowheads). (3,300X)

D. Section treated with KOH saturated with ethanol to remove the plastic. Midgut inclusions have been removed (arrow) while body cavity inclusions remained (arrowhead). (3,300X)



typical microvilli (fig. 2D). An electron lucent zone lies beneath the apical surface between the projections and large aggregations of free, densely packed ribosomes. Some RER and ribosomes are found in the projections and the apical surface is coated with a glycocalyx (fig. 2D). Endoplasmic reticulum lines the basal (fig. 3A), apical (fig. 2D), and lateral surfaces (fig. 3B) throughout the midgut.

The MGI appear to be enclosed in a system of interconnected vacuoles or canals. In chemically fixed specimens the vacuoles are more swollen or dilated than those in cryofixed animals (fig. 2A). Traditional chemical fixation reveals concentric concretions in the center of the inclusions (fig. 2B) but cryofixation generally does not preserve that region (fig. 2A). The enclosing membrane has an extracellular coat (fig. 2C) which consists of parallel strands of extracellular coat that appear thicker and more electron dense than a glycocalyx but discontinuous and thinner than a sheet of basement membrane. It is made up of separated, parallel strands of unknown origin (fig. 2C). The interconnected canals are lined on their cytoplasmic face with rough endoplasmic reticulum (fig. 2A).

Material of a similar electron density to MGI is also found in the body cavity (fig. 1B, 3A). These inclusions (BCI) are found in a variety of sizes and shapes. In addition to being electron dense, they are frequently



heterogeneous with a finely fibrillar substructure. Since living specimens contain orange pigmented droplets within the body cavity, these probably correspond to this carotenoid-like material. Although there is no evidence that the MGI are being exocytosed from the midgut to the body cavity, cytochemical tests were performed to determine if there is any relationship between these two inclusions.

CYTOCHEMISTRY

The farnesol test for lipids and the thymol/farnesol test for masked lipids labelled lipid droplets throughout the animal but labelled neither MGI nor BCI. The midgut inclusions of both living and fixed specimens readily dissolve in strong base. When 1 um sections were treated with EtOH saturated with KOH the midgut inclusions were completely removed but the body cavity inclusions were left apparently unaffected and when live animals were treated with 1N NaOH the midgut inclusions were lost and the pigmented body cavity inclusions remained (fig. 3C & D). Since phenolics are in high concentration in moss cells and polyphenols are found in insect cuticles, tests for these were considered important. Although the central region of MGI, the cuticle and the contents of the midgut all tested positively and the BCI was lightly labelled, the electron dense material in the endocuticle was negative and some unexpected sites such as yolk in the ovary were positive. Thus the tests need to be repeated under more controlled conditions.

STEREOLOGY

The disector, a stereological technique, was used to give an unbiased estimate of the number of midgut inclusions (MGI). It and volume determinations of the MGI, BCI, midgut and total organism were used to determine if there were any relationships between the two types of inclusions (Tables 1, 2, & 3). Correlations were done testing for a significant relationship between the midgut inclusion volume and number and body cavity inclusion volume. The volume of the midgut inclusions and body cavity inclusions give the three dimensional space each of these occupies and can be used to indicate if a change in one is correlated with a change in the other. Additionally, since the number of midgut inclusions can be determined accurately, this number also can indicate whether there is a significant correlation between it and the volume of BCI. A negative correlation in these cases would suggest that the MGI is being moved en masse into the body cavity. When these are plotted, a positive correlation was found at the 0.05 (r=.64) and 0.01 (r=.74) levels. However, since the size of the animal would bias the results toward a positive correlation (larger animals would have proportionately larger number and volume of MGI and BCI), the body volume factor was removed. This new plot had an r=0.05. Thus a routine arcsin transformation was performed on the data which rendered an r=0.00 indicating no correlation between the two.

TABLE 1

Number of points counted in certain structures of Echiniscus viridissimus.

| Animal # | W | N | Mt | L | I | BCI |
|---|---|---|---|---|---|---|
| 1 | 480 | 39 | 275 | 118 | 372 | 691 |
| 2 | 522 | 75 | 328 | 408 | 509 | 202 |
| 3 | 287 | 59 | 211 | 223 | 1004 | 1726 |
| 4 | 322 | 39 | 232 | 111 | 409 | 328 |
| 5 | 286 | 45 | 148 | 119 | 304 | 272 |
| 6 | 598 | 82 | 367 | 141 | 1473 | 769 |
| 7 | 450 | 30 | 220 | 104 | 222 | 159 |
| 8 | 290 | 36 | 133 | 116 | 674 | 290 |
| 9 | 427 | 47 | 160 | 112 | 491 | 275 |
| 10 | 324 | 49 | 262 | 299 | 558 | 1621 |

W = whole animal
N = number of midgut inclusions
Mt = total midgut
L = lumen of midgut
I = midgut inclusions
BCI = body cavity inclusions

TABLE 2

Area calculated ($u^2$) for each structure analyzed using Cavalieri's Principle

| Animal # | W | Mt | L | I | BCI |
|---|---|---|---|---|---|
| 1 | 120,960 | 17,319 | 7,432 | 234 | 435 |
| 2 | 690,606 | 108,486 | 34,272 | 1,589 | 3,995 |
| 3 | 72,324 | 13,293 | 3,568 | 632 | 1,087 |
| 4 | 81,144 | 14,616 | 6,993 | 258 | 207 |
| 5 | 72,072 | 9,324 | 1,904 | 191 | 171 |
| 6 | 150,696 | 23,121 | 8,883 | 928 | 484 |
| 7 | 113,400 | 13,860 | 6,552 | 140 | 100 |
| 8 | 73,080 | 8,379 | 1,856 | 425 | 183 |
| 9 | 107,604 | 10,080 | 1,792 | 309 | 173 |
| 10 | 81,648 | 16,506 | 4,784 | 351 | 1,021 |

W = area of whole animal
Mt = area of entire midgut
L = area of the midgut lumen
I - area of the midgut inclusions
BCI = area of the body cavity inclusions



TABLE 3

Volume calculated ($u^3$) for each structure analyzed utilizing Cavalieri's Principle

| Animal # | W | Mt | L | I | BCI |
|---|---|---|---|---|---|
| 1 | 816,480 | 116,907 | 50,164 | 1,581 | 2,938 |
| 2 | 690,606 | 108,486 | 34,272 | 1,589 | 3,995 |
| 3 | 542,430 | 99,697 | 26,760 | 4,744 | 8,155 |
| 4 | 426,006 | 76,734 | 36,713 | 1,353 | 1,085 |
| 5 | 378,378 | 48,951 | 9,996 | 1,005 | 900 |
| 6 | 904,176 | 138,726 | 53,298 | 4,872 | 2,907 |
| 7 | 765,450 | 93,555 | 44,226 | 944 | 676 |
| 8 | 511,560 | 58,653 | 12,992 | 2,972 | 1,279 |
| 9 | 403,515 | 37,800 | 6,720 | 1,160 | 650 |
| 10 | 816,480 | 165,060 | 47,840 | 3,515 | 10,212 |

W = volume of whole animal
Mt = volume of entire midgut
L = volume of midgut lumen
I = volume of midgut inclusions
BCI = volume of body cavity inclusions

THREE-DIMENSIONAL RECONSTRUCTION

The three-dimensional reconstruction revealed the spatial relationship of the midgut, midgut inclusions, and body cavity inclusions. The midgut makes up a very large part of the total animal. The midgut inclusions appear to be different sizes depending on their location. Ventral midgut inclusions are smaller than those located dorsally. The body cavity inclusions are spread throughout the body cavity with some clumping around the ovary. During the course of tracing the micrographs used for the reconstruction, a structure was noticed which lay very close to the midgut and was initially mistaken for part of the midgut. After investigation of the structure, we were able to determine that it is a previously undescribed organ (Dewel, et al., '89).

ORGAN

A light and electron microscopic study was done to determine the structure of the undescribed organ. It occurs serially in two segments, one between the second pair of legs and another between the third. Lying ventrally beneath the midgut, the organs are very close to both the midgut and the underlying epithelium (fig. 1B). Each consists of three cells of two different cell types, two lateral and one medial (fig. 4A). A distinct nucleus can be seen in both cell types (fig. 4B & 5A). The lateral cells are much smaller than the medial cell and are not well endowed with organelles such as rough endoplasmic





reticulum, Golgi, and mitochondria. However, these cells possess a convoluted plasma membrane that is coated on both sides with electron dense material. The basement membrane next to this membrane is thickened and elevated (fig. 4C). In addition, each lateral cell contains a duct that continues into the medial cell (fig. 4A). The medial cell is characterized by deep infoldings of the plasma membrane (fig. 4B), numerous mitochondria and lipid droplets (fig. 5A).

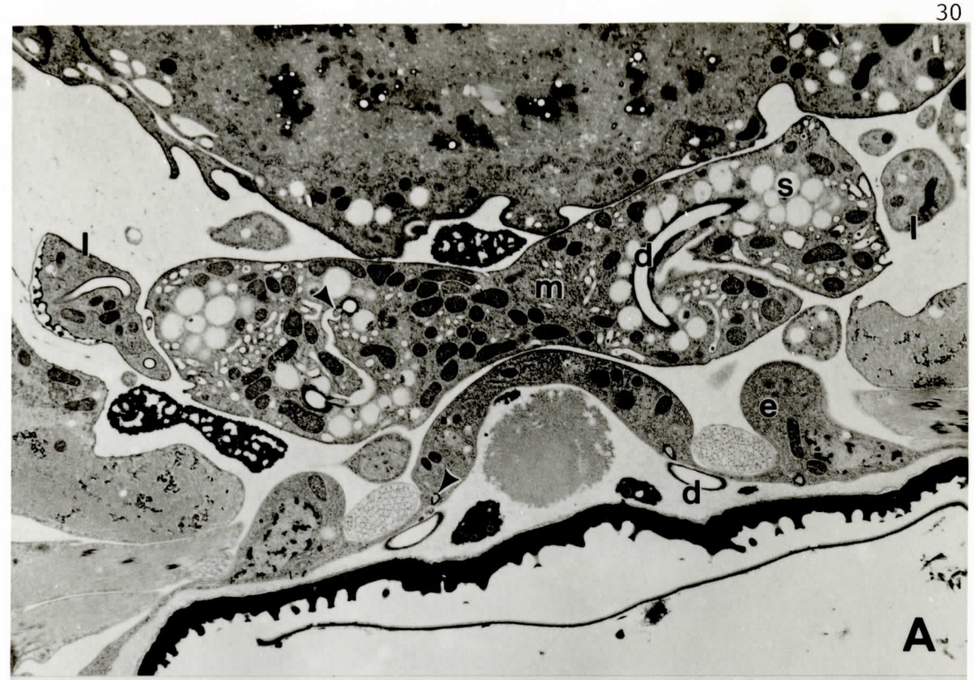
The duct in each of the lateral cells originates as an infolding of the distal plasma membrane (fig. 6A). Like the plasma membrane associated with the elevated basement membrane, the duct membrane is coated at the opening and at the junction with the medial cell with electron dense material. From the junction (fig. 6B & 6C), a finger-like projection of the lateral cells follows a cleft in the medial cell (fig. 5B & 6D). It junctions to the medial cell by "tight" zonulae adherens and lies at the base of the cuticle lined duct in the medial cell. Together the complex of lateral cell projection and duct proceed in the cleft to the epidermis (fig. 4A, 5C, 6D, 7B). The three dimensional reconstruction of the organ revealed that each duct and lateral cell projection forms a loop within the medial cell (fig. 9C) The medial cell then releases the duct into the endocuticle (fig. 4A). No openings to the outside of the animal which correspond to these ducts were found.

FIGURE 4

A. Medial, cross-section of the ventral organ showing lateral cells (l), medial cells (m), ducts in medial cell (d), lateral cell extension (arrowheads) leading all the way to the ventral epidermis (e), saccules (s) and the duct in the endocuticle (d). (6,000X)

B. Lateral cell with nucleus (n) and infoldings of the medial cell containing electron dense material (arrow). The lateral cell has an elevated basement membrane (arrowheads). Located in the same area are fragments of body cavity inclusions (bi) and strands of basement membrane (bm). (13,600X)

C. Lateral cell with a folded plasma membrane, elevated basement membrane (bm), basement membrane strands in the body cavity (s) and the noncuticular duct of the lateral cell (d). (13,300X)



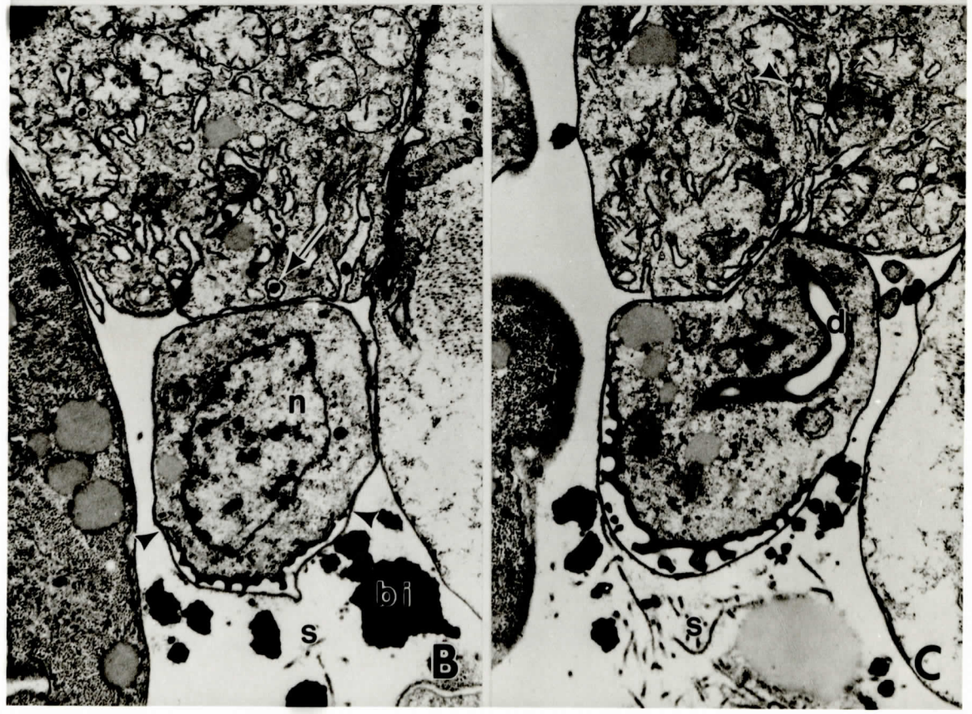



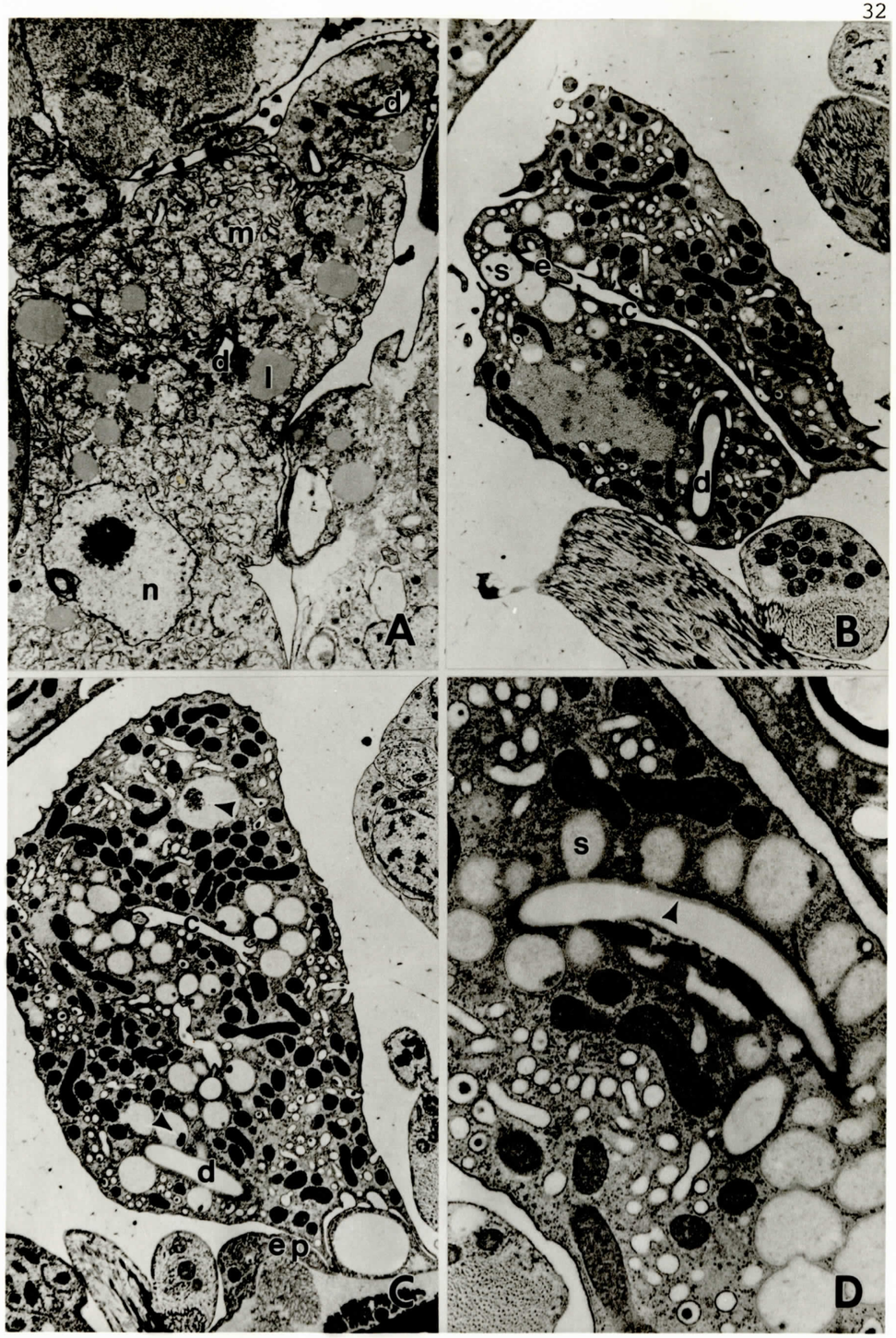
FIGURE 5

A. Medial cell with nucleus (n), lipid droplets (l) and mitochondria (m). Note the duct (d) in the lateral and medial cells. (7,000X)

B. Medial cell with a deep cleft (c) which is followed by an extension of the lateral cell (e) and the duct of the medial cell (d) surrounded by a rosette of saccules (s). (7,000X)

C. Medial cell with duct (d). Note how the lateral cell extension follows the cleft (c), and the saccules some of which contain electron dense material (arrowheads). The medial cell junctions with the ventral epidermis (ep). (6,700X)

D. Higher magnification of duct with a homogeneous cuticle-like lining (arrowhead) which is surrounded by dilations in the membrane(s) that connect to and surround the duct. Note the dilations contain a flocculant material. (22,500X)



The basement membrane of the lateral cells is thickened and elevated (fig. 9B). It forms projections which trail into the body cavity as string-like arrays. This is very evident in the intermediate voltage electron micrograph (fig. 9B). The basement membrane arrays appear associated with fragmented body cavity material found in this area (fig. 4C, 9B). Similar material is found in the medial cell infoldings (fig. 4B), the saccules surrounding the cuticle lined duct and the duct itself (fig. 5C). The endocuticle often has large accumulations of a substance of similar appearance (fig. 7A). Of interest the exocuticle and mesocuticle contain canals which have electron dense particles (fig. 7C).

The cytochemical test utilizing cationic ferritin produces interesting results. Structures of the lateral organ that were labelled include the outside and infolds of the medial cell and both attached and elevated portions of the basement membrane of the lateral cell. The "free" basement membrane associated with the lateral cell was also heavily labelled. Interestingly, the body cavity inclusions were not labelled; however, labelled strands of what may be basement membrane can be seen very closeto these structures. Ferritin labeling was also found in various parts of the cuticle including endocuticle, spaces between mesocuticle and exocuticle (fig. 8A-D).

The medial cell duct is surrounded by a layer of saccules or dilated invaginations of the medial cell plasma

FIGURE 6

A. High magnification of lateral cell basement membrane (bm) and duct of lateral cell (d). Note the inner coat of plasma membrane (small arrowhead); outer coat of plasma membrane (large arrowhead). (67,500X)

B. Lateral-medial cell junction showing duct in medial cell (d) and the lateral cell (l). (52,500X)

C. Animal chemically fixed. The lateral cell duct (d), lateral cell (l) and the cuticle like duct of the medial cell (cu) are noted. (15,100X)

D. High magnification of a chemically fixed animal. Note the collapsed saccules (s), lateral cell extension (l), cleft (c), cuticle like lining (cu) and tight zonulae adherens (arrowhead). (87,700)

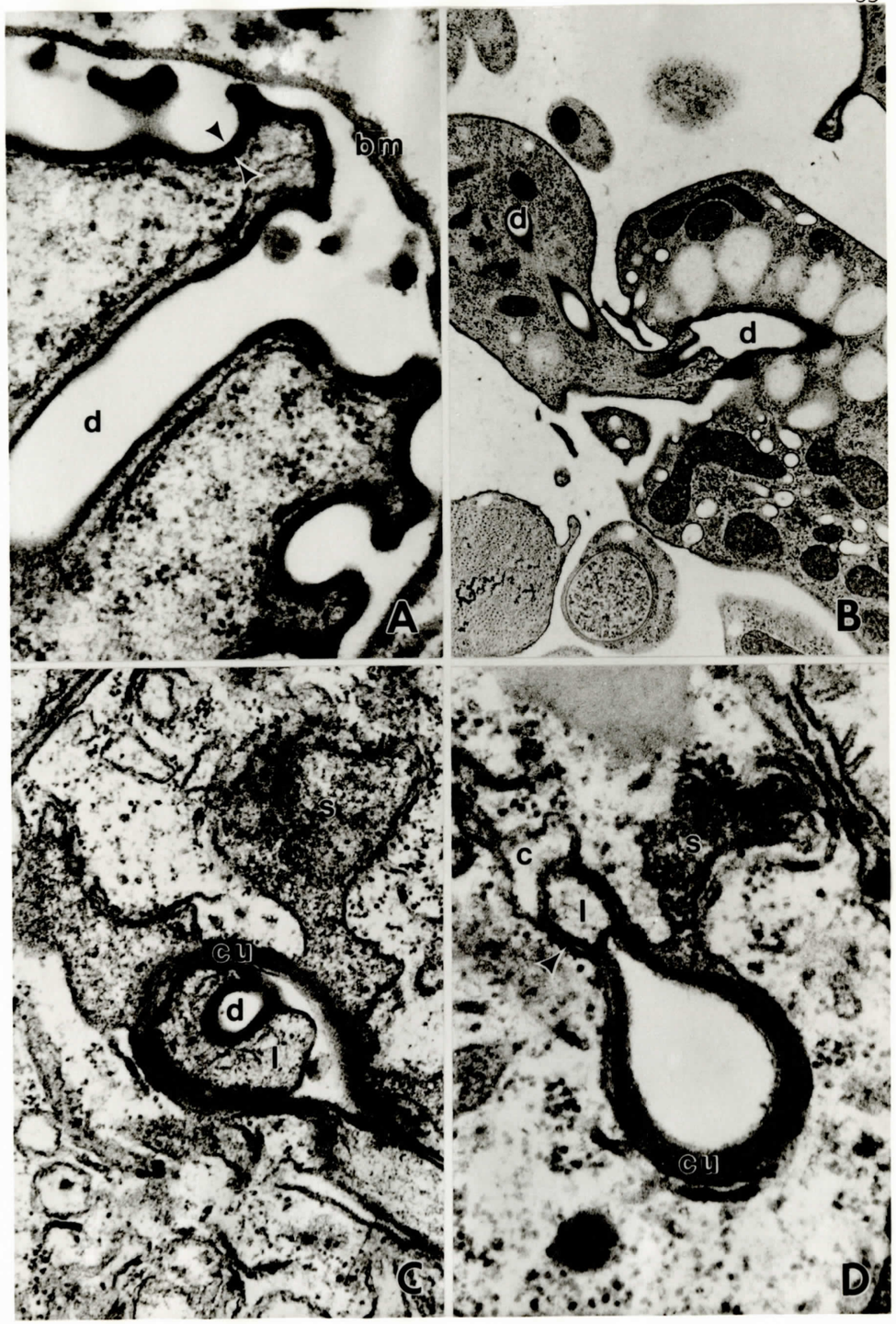



FIGURE 7

A. Electron dense material in body cavity (bi) and in the endocuticle (en) electron dense masses (*) can be seen. Note the electron dense material in the duct (d). (6,600X)

B. Duct in endocuticle (d) and lateral cell extension (l) between epithelial cells. (57,000X)

C. The mesocuticle (m) and exocuticle (ex). Note the pores (arrow) and lacunae (*) containing electron dense material (arrowhead). (57,000X)

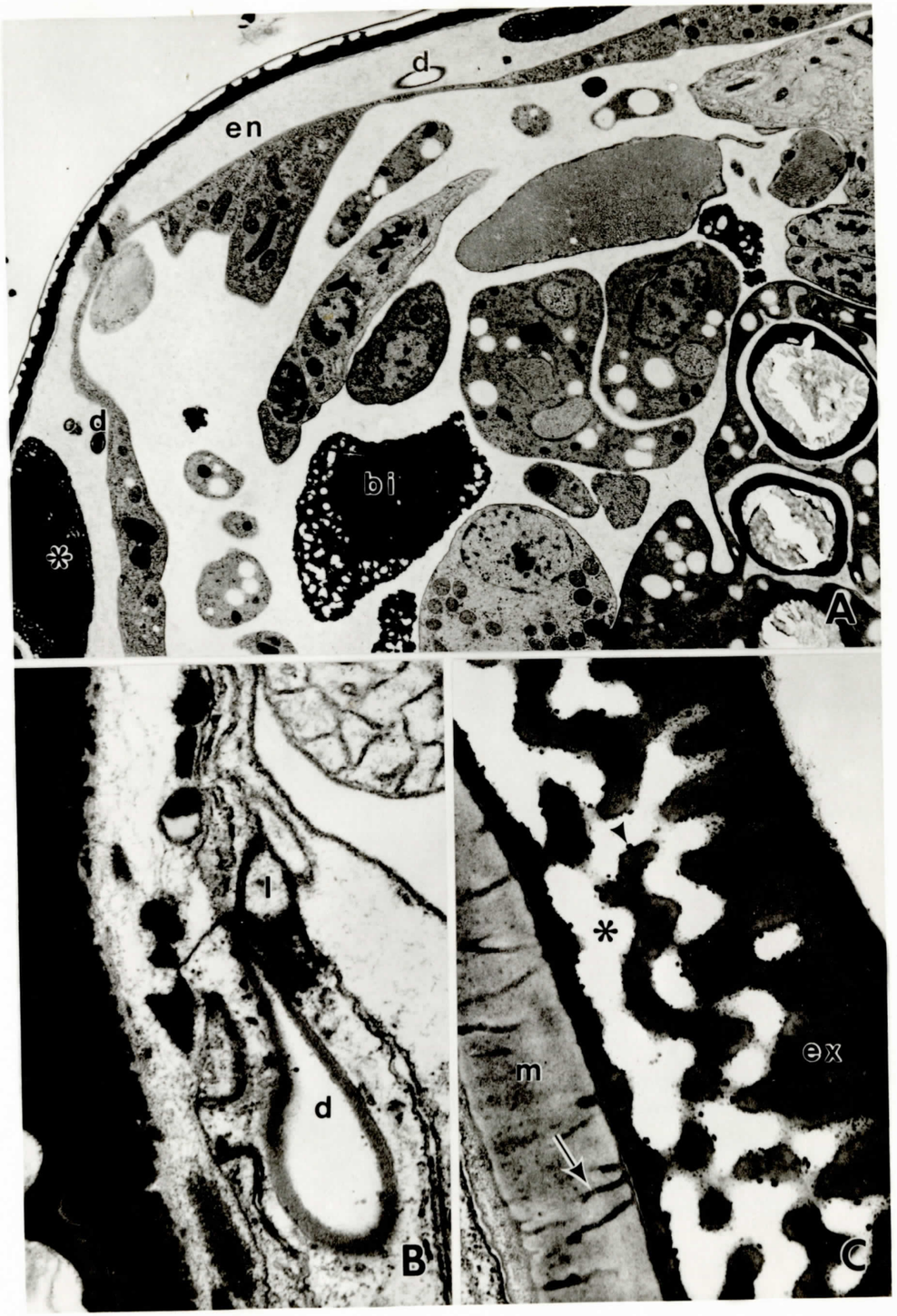



FIGURE 8

A. Ferritin labeled basement membrane strands (arrowhead) in close relation with the body cavity material (bi). (73,500X)

B. Ferritin labeled basement membrane strands in body cavity (arrowhead) and the lateral cell basement membrane is also labeled (*). (70,500X)

C. Ferritin labeled infoldings of the medial cells basement membrane (arrow). (73,500X)

D. Ferritin labeled dense material in the endocuticle (en) and exocuticle. (63,000X)

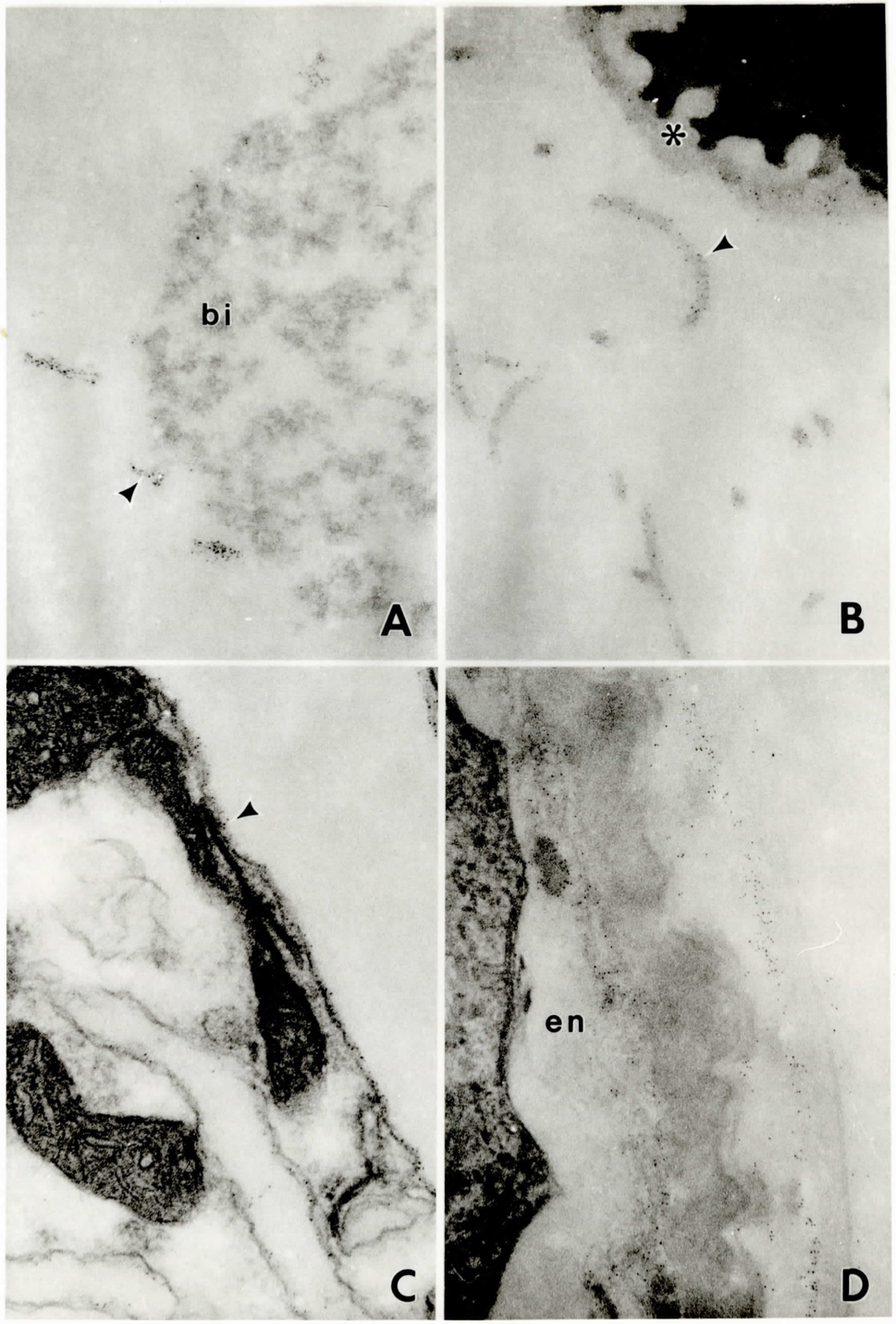
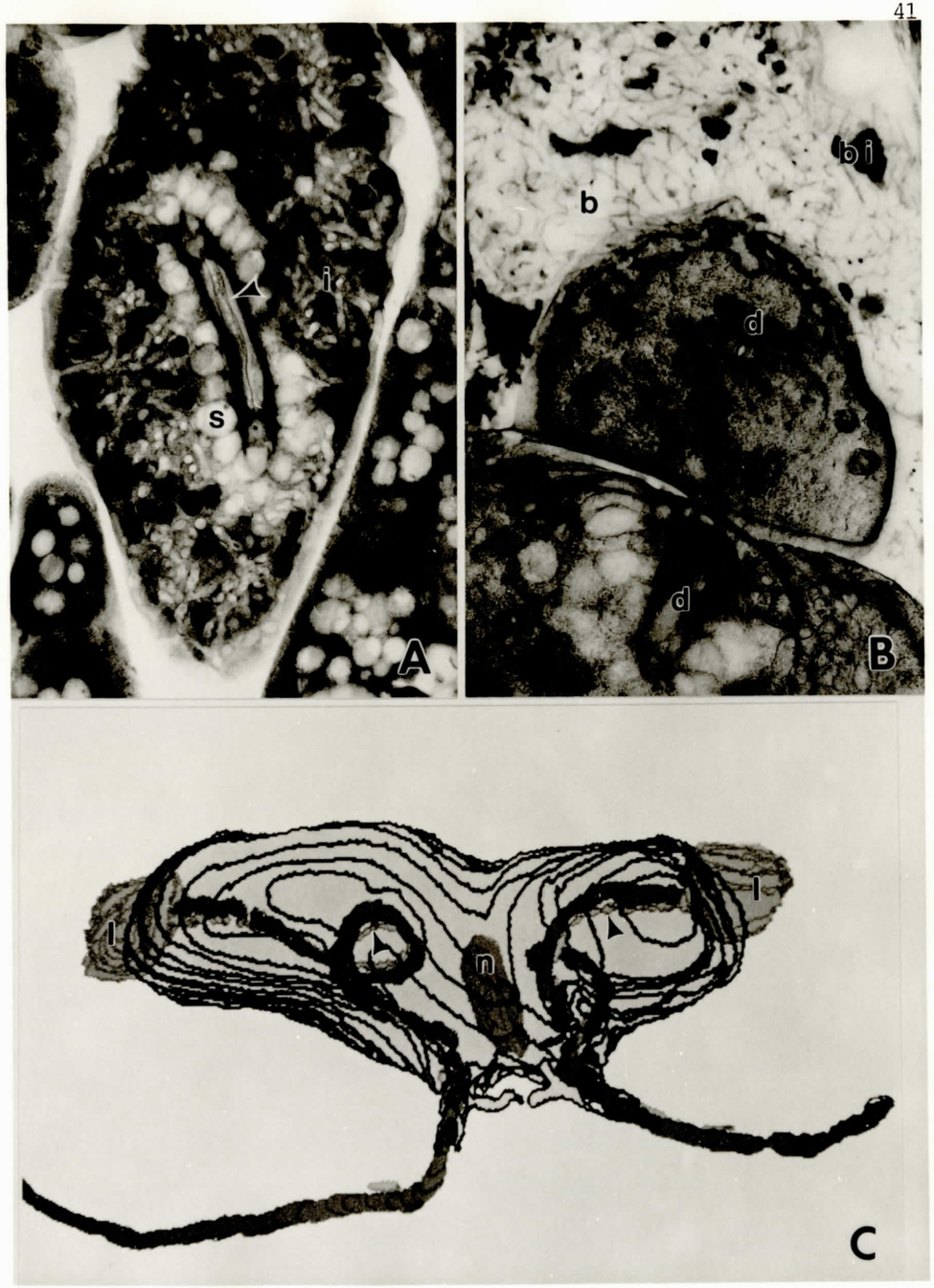



FIGURE 9

A. IVEM of medial cell with duct followed by the lateral cell extension (arrowhead), saccules (s), and infolds (i). (9,520X)

B. IVEM of the lateral cell basement membrane showing strands in the body cavity (b), fragmented body cavity inclusion material (bi). Note the convoluted surface of the lateral cell and the duct (d) in lateral and medial cells. (13,300)

C. Three-Dimensional reconstruction of organ with lateral cell (l), ducts and nucleus (n) of medial cell and the lateral cell extensions (arrowheads).






membrane. Characteristically the "saccules" contain a homogenous flocculent material and some have electron dense inclusions. Chemical fixation produces saccules that are collapsed (fig. 6C, D) and have no uniform shape, but when the animals are cryofixed the saccules appear uniformly spherical and swollen (fig. 5B, C, D).

## DISCUSSION

Echinsicids have successfully adapted to freshwater (hyposmotic) habitats that experience periodic drying. As herbivores they have also adapted to the problem of excreting plant toxins, minerals or excess plant metabolites that cannot be used. However, they have no reported excretory/osmoregulatory organs as do members of the Eutardigrada. Although Marcus ('29) and Ramazotti ('72) have postulated that the midgut is serving in an excretory/osmoregulatory capacity in heterotardigrades, it is unclear how it is carrying out these functions based on our findings (Dewel, et al., '88).

Osmoregulatory tissues possess certain structural characteristics that allow for net transport of minerals, water and nitrogenous wastes across cells. Folding of the cell surface is a common ultrastructural feature of transporting epithelia. In addition the cells are joined by "tight" junctions of various types to prevent small molecules from following diffusion gradients between compartments established by the epithelium. Characteristically, the cytoplasm is packed with mitochondria, a feature expected in cells carrying out oxidative phosphorylation for active transport. However, in E. virdissimus the midgut is a relatively straight sac





with little evidence of basal infoldings or high mitochondrial density. Although there are deep septate desmosomes, the "tight" junction of most invertebrates, and dialated intercellular spaces, the other specializations are absent.

Nevertheless, the midgut does contain electron dense inclusions (MGI) enclosed in a vacuole or sac of interconnecting channels which may be accumulated waste products. The center of the inclusions usually have concentric concretions similar to those mineral deposits found in the midgut cells of some insects (Ludwig and Alberti, '88). In addition to these midgut inclusions there are electron dense inclusions in the body cavity (BCI). Both midgut and body cavity inclusions stain similarily with standard electron microscopical techniques. In view of this it was thought that the two were related in some way.

An early working hypothesis proposed that the midgut was depositing waste material from the gut lumen into the body cavity (Dewel, et al., '88). However, there was no indication of exocytosis at the basal surface of the midgut. Further, correlative tests indicated that the midgut and body cavity inclusions are not related. A negative correlation would have been expected if the midgut inclusions are being moved en masse into the body cavity. However, this was not the case. The body cavity inclusion volume neither decreased or increased with midgut inclusion

number or volume when the body volume factor was removed. In addition cytochemical tests have shown that the two types of inclusions behave differently when treated with a strong base that removed midgut inclusions but not body cavity inclusions. This along with the results of the farnesol and thymol-farnesol treatments for lipids and the silver hexamine reaction for polyphenols suggest that the midgut and body cavity inclusions are different chemically and probably not related. Therefore, the question of the origin of the BCI remains unanswered.

Ramazzotti ('72) lists four possible methods of excretion within the phylum Tardigrada. They include: 1) Malpighian tubules, 2) deposition of excretory products into the cuticle prior to shedding, 3) midgut excretion and 4) salivary gland excretion at ecdysis. Echiniscus viridissimus does not have Malpighian tubules. Additionally, there is no clear evidence supporting salivary gland excretion (Dewel and Dewel, personal communication). However, it does appear that the body cavity inclusions could be excreted into the endocuticle by the organ and shed during ecdysis. Although it appears that the BCI is being "excreted", the opening of the ducts into the endocuticle rather than the outside is puzzling. The BCI may actually be necessary for the proper functioning of the cuticle, for instance, slowing the rate of evaporative water loss as the animal enters anhydrobiosis. Finally, the midgut has what appears to be



excretory granules, but the origin and fate of these bodies are presently unknown.

Excretion and osmoregulation are almost invariably linked structurally and functionally in protists and metazoans. These forms have developed five major types of excretory/osmoregulatory structures. The type utilized is usually related to body size and organization. The smallest organisms (with a mean diffusion path from center to surface of less than 0.5 mm) excrete by simple diffusion across the cell membrane. Freshwater protozoans have developed a contractile vacuole which moves osmotically acquired water back to the freshwater environment (Barnes, et al., '88). Nematodes have a unique system made up of an excretory H cell and two canals lacking cilia, and an A, or secretory, cell. Both empty into a common duct at the secretory-excretory junction (Nelson, et al., '83).

Organisms lacking a blood vascular system or those with an altered blood vascular system generally possess a protonephridial system. A ciliary duct ends blindly in the body cavity in a set of specialized cells which form a weir at the end of the duct. The cilia beat causing a hydrostatic pressure gradient and fluid moves across a basement membrane to form an ultrafiltrate. The ultrafiltrate is modified into urine as it moves down the duct and is excreted (Ruppert and Smith, '88).

The metanephridial system is utilized by invertebrates with a blood vascular system. Podocytes, specialized

cells, surrounded by a basement membrane form the ultrafiltrate as the fluid moves from the blood vascular system across the basement membrane. The ultrafiltrate moves back across the membrane into the coelom. The metanephridial duct opens distally to the outside and proximally into the coelom with the modified filtrate moving through the duct to the outside (Ruppert and Smith, '88).

Malpighian tubules are found in insects, some arachnids and eutardigrades. These animals have no pressure gradient caused by a blood vascular system and utilize transporting epithelia in the tubules and the hindgut to regulate water and solutes. For example, in Milnesium tardigradum four Malpighian tubules project anteriorly from their point of entry into the pylorus, a transitional region between the midgut and hindgut (Dewel and Dewel, '80).

The organ that has been described here is unlike any other invertebrate osmoregulatory system. Evidence is provided by the presence of saccules that appear swollen when cryofixed and flattened when chemically fixed that it is functioning in this capacity. An important difference in the fixation methods is the time involved to complete fixation; cryofixation takes milliseconds whereas chemical fixation takes seconds. Since chemical fixation could destroy the enzymes responsible for the movement of water into the saccules before it "fixes" the position of the



membrane, it could allow enough time for the water in the saccules to be extracted by osmosis and for the saccules to collapse.

The lateral cells of the organ produce excess basement membrane which is detached and released from the cell and breaks away into the body cavity. This is extremely important because the basement membrane appears to have been exapted (Gould and Vrba, '82) in E. viridissimus and other echiniscids (Dewel and Dewel, '90) for a novel function. The basement membrane is a structure which has been conserved evolutionarily in metazoans (Fessler, et al., '84). It is composed of four major macromolecules. Type IV collagen serves as the supporting or anchoring mechanism for tissues in multicellular systems. Fibronectin is involved in cell adhesion and attachment of the basement membrane to the underlying cells. Proteoglycans, also involved in attachment of the basement membrane, have a variety of other functions depending on their form and are composed of a core protein with glycosaminoglycan side chains. Laminin also has receptor sites on the cell membrane and is arranged in a cross shape with cell binding domains and collagen binding domains (Kefalides, et al., '79).

Several functions are attributed to the basement membrane. These include filtration of large molecules in circulatory and excretory tissues, support of tissue layers and cell recognition during early development (Kefalides,

et al., '79). However, there is some evidence that the basement membrane is facilitating the transport of lipid. In a study on the insect Rhodnius by V. B. Wigglesworth ('88) the basement membrane is thickened only during the time the lipid is crossing the epithelium to the hemocoel. In addition, Ambrose and Riley ('88) describe a secretory system found in the pentastomids, a relic group of endoparasitic arthropods. Since special lipid secreting glands have a thickened basement membrane that follows deep infolds in the basal surface of the gland cell where lipid transport is occuring, there is evidence that the basement membrane is facilitating this transport. Although we have shown the material in the body cavity is not lipid per se, it may be interacting with basement membrane in a manner similar to lipid (Dewel and Dewel, personal communications). For example, proteoglycans contain both polar and nonpolar groups and can exert an emulsifying action on nonpolar compounds. Thus the structural similarities between the pentastomid glands and the tardigrade organ suggest a functional convergence.

However, in addition to aiding in the uptake of body cavity material in the infolds of the medial cell, the basement membrane is actually released into the body cavity to act as a dispersant. Cytochemical tests using cationic ferritin that labeled both the basement membrane of the lateral cell and the basement membrane strands in the body cavity indicate the presence of negatively charged



proteoglycans. Thus, the membrane appears to be carrying out two separate functions: dispersing the BCI and aiding in its transport into the medial cell. Further studies are needed to determine whether the same or different proteoglycans are responsible for the unique characteristics of the basement membrane of the organ.

This preliminary study of the morphology has revealed that this newly reported organ appears to have no known homologies within the animal kingdom. They are morphologically unlike other invertebrate osmoregulatory/excretory or secretory tissues and organs. There is morphological evidence suggesting that it is functioning in the important capacity of osmoregulation and both morphological and cytochemical evidence that it acts as an excretory organ for the transport of BCI to the endocuticle. Lateral cell have the ability to produce excess basement membrane which is then released into the body cavity. These strands come in contact with and fragment the body cavity material. This is quite different from the traditional functions credited to the basement membrane. An evolutionary novelty appears to have occured in the exaptation of the basement membrane allowing it to function in a previously unsuspected way. Further studies of these structures could provide important information to aid in the understanding of the successful radiation of organisms to terrestrial environments.

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## VITA

Brenda Gladene Roush was born in Wilmington, Delaware on August 26, 1964. Her family moved quite frequently and she attended several elementary schools until 1974 when they settled in Creston, North Carolina. Here she attended Riverview Elementary School and graduated from Northwest Ashe High School in June, 1982. The following August she entered Appalachian State University and in December 1986 she earned a Bachelor of Science degree in Biology.

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