IMMUNOHISTOCHEMICAL LOCALIZATION OF GONAD STIMULATING SUBSTANCE IN THE SEASTAR *Pycnopodia helianthoides*

by

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ABSTRACT

Spawning in seastars is initiated by gonad stimulating substance (GSS), a peptide found primarily in the radial nerve cords. GSS is thought to stimulate the ovarian follicle cells to produce 1-methyl adenine, which initiates oocyte maturation and spawning. Although much is known about the biochemical and physiological properties of GSS, little experimentation has been done to localize the site of production of GSS or the secretory pathway it takes to the ovary. It has been suggested that GSS is produced in the supporting cells of the radial nerve and moves to the ovary by diffusion through the coelomic fluid; however, most of the evidence for this has come from biochemical inference and histological tests, which may not be specific for GSS alone. The objective of this study was to develop a histochemical test specific for GSS so that it could be localized in the tissues of the seastar *Pycnopodia helianthoides*.

Polyclonal antibodies were developed that were specific for GSS purified by gel filtration and ion-exchange

chromatography. The antibodies were used on formalin fixed sections of radial nerve cords, axial organ, tube feet, ovarian wall and pyloric caeca. Binding sites of GSS antibodies were visualized using the indirect immunoperoxidase technique. Immunoreactivity, indicating the presence of GSS, was observed as discrete, brown granules that averaged 2 um in diameter.

In the radial nerve cords, GSS was found in the lower portion of the perihemal epithelium, which encloses the radial hemal sinus. GSS was not found in either the ectoneural or hyponeural nerves. Ultrastructural examination of the radial perihemal epithelium revealed an axonal plexus in the region where immunoreactivity was observed. The axons varied in size, but in general there were large (2 um diameter) and small (less than 1 um diameter) axons. The axons contained clusters of 100 nm diameter, electron-dense vesicles. The location of these axons, their size and distribution appeared to correspond to the size and distribution of the immunoreactive granules observed by light microscopy. It is possible that GSS is localized to the axon plexus of the radial perihemal epithelium.

The axial organ, which is an integral part of the hemal system, also showed immunoreactivity to GSS antibodies.

Immunoreactive granules similar to those observed in the radial perihemal epithelium were found in the axial perihemal epithelium. Ultrastructural examination of the axial organ revealed cells and axons that contained 100 nm diameter, electron-dense vesicles in the axial perihemal epithelium. The abundance and size of the vesicular clusters appeared similar to that of the immunoreactive granules observed by light microscopy; therefore, GSS in the axial organ may be localized to the vesicles in the axial perihemal epithelium.

Immunoreactive granules were found in the coelomic epithelium of the tube feet, mostly in the region that apposes the podial connective tissue. Ultrastructural correlates of the granules were not found in this study; however, previous works report 100 to 200 um diameter, electron-dense vesicles found in granulocytes. GSS may be localized to these cells in the coelomic epithelium of the tube feet.

Sections of ovarian wall and pyloric caecum were used as immunohistochemical controls because they do not contain GSS. As expected, no immunoreactivity was observed in either ovarian wall or pyloric caeca. The ultrastructure of the ovarian wall was examined to determine if there were differences between the ovarian perihemal epithelium, and

the perihemal epithelia of the radial nerve cord and axial organ. The ovarian perihemal epithelium was much reduced and did not contain the vesiculated axons or cells that may contain to GSS in the other tissues. Lack of vesicles in this region of the ovary may explain why no GSS is found in the ovarian wall.

Since GSS is not found in the ovary, except during spawning, it must be transported from its site of synthesis to the ovary. The proximity of GSS to the radial hemal sinus, the structural link between the radial and ovarian hemal sinus, and recent evidence for the tranlocative ability of the hemal system implicates the hemal system as being involved in GSS transport. I propose that GSS is secreted into the radial hemal sinus and axial hemal sinus, and is tranported to the ovary through the hemal sinus system.

That GSS is found in other tissues not directly connected with the ovaries (ie. the tube feet) suggests that GSS may have other physiological roles besides initiating spawning. One role could be the initiation of the characteristic spawning posture that seastars assume prior to spawning. Further study of GSS is needed to better define the physiological role of this hormone in asteroid neuroendocrine regulation.

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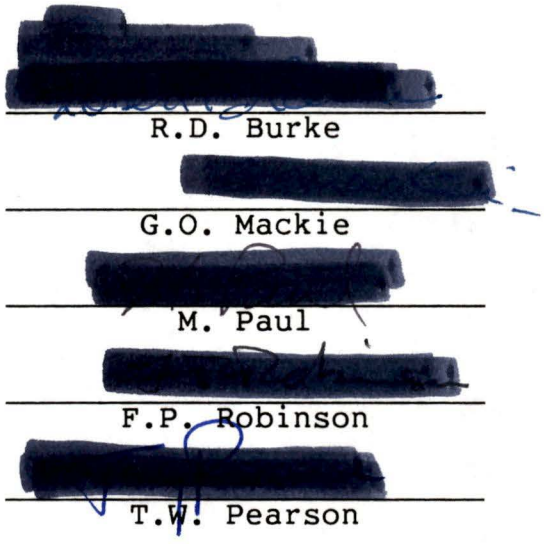

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I. INTRODUCTION

Most animal reproductive systems rely on hormones to control gonadal growth and gametogenesis (Golding, 1974). In asteroids, oocyte maturation and spawning appear to be controlled by a neuropeptide called gonad-stimulating substance (GSS), thought to be secreted by the radial nerves. GSS apparently stimulates ovarian follicle cells to synthesize and release 1-methyladenine (1-MA) which induces oocyte maturation and spawning (Kanatani, 1969, 1973; Hirai and Kanatani, 1971). These substances have been found in over twenty species of seastars (Kanatani, 1973), and a number of species of sea urchins and sand dollars have also been shown to have GSS and 1-MA activity (Kanatani, 1975; Cochran and Engleman, 1972). Recent evidence (Maruyama, 1984) has shown that the holothurian *Parastichopus japonicus* contains a GSS-like peptide in its radial nerve cords, and a 1-MA analogue in the ovaries.

The actions of GSS and 1-MA in asteroids are comparable to those of the gonadotropins and follicular hormones in vertebrates. The vertebrate pituitary hormones FSH and LH, like GSS, initiate the later stages of oogenesis, namely oocyte release and final maturation, by stimulating the

follicle cells surrounding the oocytes (reviewed by Berrill and Karp, 1976). The follicle cells respond by producing and secreting maturation-inducing substances which bring about germinal vesicle breakdown and final meiotic divisions, resulting in mature ova (Berrill and Karp, 1976). In vertebrates, the follicular hormone progesterone induces oocyte maturation (Berrill and Karp, 1976), while 1-MA serves an analogous function in asteroids (Schuetz and Biggers, 1967; Kanatani and Shirai, 1967).

Oogenesis and spawning have been extensively studied in asteroids but most of the emphasis has been on maturation events and the consequences of spawning after induction. Although a number of studies have been conducted on the spawning-inducing agent GSS, much of this has dealt with its biochemical and physiological effect at the ovary. The pathway of GSS from the nervous system to the reproductive system has only been briefly discussed in the literature, and most of the evidence for the proposed pathway of neuroendocrine spawning induction has been derived from biochemical inference and indirect results. There has been no direct demonstration of GSS *in situ*, and how it moves from the radial nerve (and possibly from other tissues) into the gonad. Elucidation of this mechanism is of fundamental importance in establishing the structural basis for neurologically regulated reproduction in asteroids.

A number of theories have been advanced as to how GSS effects the induction of spawning in asteroids. Chaet (1967) proposed that GSS in the radial nerve was released into the surrounding seawater and initiated spawning upon reaching a threshold level, acting somewhat like a pheromone. Unger (1962) proposed that GSS was secreted from the radial nerve into the hemal sinus where it was taken up by the water vascular system and released into the coelomic fluid around the ovaries. Kanatani (1973) proposed that GSS simply diffused into the coelomic fluid from the radial nerve and made its way through the ovarian wall to initate spawning. It is also possible that GSS is secreted from the nerves in the ovarian wall itself, since neurosecretory granules have been reported there (Schoenmakers *et al.*, 1981).

A. EARLY STUDIES ON GSS

The discovery by Chaet and McConnaughy (1959) that hot water extracts of radial nerve could be used to induce spawning in seastars led the way in studying the reproductive endocrinology of asteroids. Later work by Chaet (1964, 1967), Kanatani and Ohguri (1966), Kanatani (1975, 1980), and Schuetz and Biggers (1967, 1969) elucidated a hormonal mechanism controlling seastar reproduction.

This active substance in aqueous radial nerve extracts induced gamete shedding when injected into the coelom of ripe male and female seastars (Kanatani, 1973). Chaet (1967) called this radial nerve factor the gamete-shedding substance (GSS). When isolated fragments of ovaries or testes were maintained in seawater containing the extract, gametes began to discharge from the cut surfaces within 30 minutes of treatment (Kanatani, 1964; Kanatani and Ohguri, 1966; Kanatani and Shirai, 1969).

GSS-induced gamete release was ascribed to a factor in the nerve extract that caused the ovarian muscles to contract, thereby liberating eggs. This was based on length measurements of isolated ovaries during treatment with crude extracts, and on the observation that treated ovaries kept in calcium-free seawater did not discharge their eggs until removed to normal seawater (Mecklenberg and Chaet, 1964). Investigations into the effect of nerve extracts on contraction of ovarian tissue were made using isolates of the ovarian wall. Studies showed that the muscles did not respond to nerve extracts which had previously been gel filtered, yet the same extracts had a strong capacity to induce spawning in intact animals (Kanatani, 1967). Tests with known contraction-inducing agents, such as acetylcholine, potassium chloride and electrical stimulation, showed that while isolated preparations of

ovarian wall would undergo marked contractions when treated, the same agents failed to induce significant spawning in intact seastars (Kanatani, 1967; Kanatani and Shirai, 1969).

In seastars, oocytes within the ripe ovary remain in the late prophase stage of meiosis. In the ovarian preparations treated with GSS, it was found that oocytes would undergo germinal vesicle breakdown and the first maturation division, while the germinal vesicles of untreated oocytes remained intact. Therefore, GSS was thought to induce oocyte maturation (Chaet, 1966, 1967). However, Kanatani and Shirai (1967, 1970) observed that oocytes removed from the ovary and placed in GSS seawater failed to undergo maturation and their germinal vesicle remained intact. They also noted that isolated oocytes placed in the supernatant of GSS-incubated ovaries underwent complete germinal vesicle breakdown within 30 minutes, followed by polar body formation within 60 minutes. They concluded that induction of spawning and oocyte maturation could be attributed to the action of a second substance produced by the ovary under the influence of GSS (Kanatani., 1973).

By the early 1970's, it was apparent that neither gamete release nor oocyte maturation in seastars were not due to the direct action of GSS but that the factor isolated

from radial nerves stimulated the ovaries to produce a maturation-inducing substance (MIS)(Kanatani and Shirai, 1972). MIS is apparently produced in the follicular cells of the ovary in response to stimulation by GSS. This was shown by observations that oocytes with their follicular envelopes intact would undergo maturation in the presence of GSS while oocytes stripped of their follicle cells would not (Kanatani and Hirai, 1971; Hirai *et al.*, 1973). On this basis, Kanatani renamed the gamete-shedding substance, calling it the gonad-stimulating substance (GSS) instead (Kanatani, 1975).

B. CHEMICAL NATURE OF GSS

GSS is a heat-stable polypeptide that retains its activity after boiling for one hour at 100^{o}C. However, it is inactivated by treatment with proteolytic enzymes, such as trypsin, chymotrypsin, pepsin or pronase (Kanatani and Noumara, 1962; Chaet, 1964; Kanatani and Shirai, 1967; Schuetz, 1969). The addition of 0.2% ninhydrin to GSS also results in a loss of biological activity (Chaet, 1966).

Sephadex columns and ion-exchange chromatography on DEAE-Sephadex have proved the best methods for purifying GSS (Kanatani, 1973). Purified GSS has been shown to be biologically active at a concentration of 0.0096 ug/ml,

whereas the original crude extract was active at 6.6 ug/ml (Kanatani, 1971).

The molecular weight of *Asterias amurensis* GSS, as estimated from its amino acid composition, sedimentation equilibrium, and gel filtration on Sephadex G-50 columns is 2100 daltons. It consists of the following amino acids: aspartic acid (number of residues is 2), threonine (1), serine (6), glutamic acid (1), proline (1), glycine (4), alanine (2), valine (1), isoleucine (1), leucine (1), histidine (1), and ornithine (1). Since GSS does not contain cysteine or cystine, it seems to be a simple chain-like molecule. The isoelectric point of *A. amurensis* GSS is around pH 4.5 (Kanatani, 1973).

C. LOCALIZATION OF GSS

GSS is present in the radial nerves of male and female seastars and nerve extracts prepared from either induce discharge of gametes in both sexes (Kanatani and Ohguri, 1966). The content of GSS (expressed as activity per gram of dry weight of radial nerve tissue) in the radial nerves is equal in both sexes (Chaet, 1966; Kanatani and Ohguri, 1966), and is uniform along the proximal-distal axis of the radial nerve and within the nerve ring (Kanatani and Ohguri, 1966).

Initially GSS was considered to be confined to the radial nerve since extracts of other tissues failed to induce release of gametes (Chaet, 1966). However, Kanatani and Ohguri (1966) demonstrated that the tube feet and body wall of *A. amurensis* also contained GSS, although the content was several times lower than that of the radial nerves. Extracts of other tissues such as cardiac stomach, in which nervous tissue is plentiful, also showed some GSS activity, and the distribution of GSS seemed to correspond to the quantity of nervous tissue present in a given part of the body (Kanatani and Ohguri, 1966). However, extracts of pyloric caecum, which contains an extensive nerve plexus, failed to show GSS activity (Kanatani and Ohguri, 1966).

Evidence to suggest that GSS is a secretory substance relies on its detection in the coelomic fluid of naturally spawning seastars. The coelomic fluid of animals not discharging gametes does not show any activity (Kanatani and Ohguri, 1966; Kanatani and Shirai, 1970).

Various histological studies have been carried out to determine where GSS occurs in the radial nerve. Unger (1962) used chromalum-hematoxylin-phloxin stains and the paraldehyde-fuchsin method, and found numerous bipolar and multipolar ganglion cells containing fuchsinophilic granules in the radial nerve and circumoral nerve ring of

Marthasterias glacialis. The supporting cells underlying the radial nerve were also found to stain positively. Imlay and Chaet (1967) used similar histological techniques and found neurosecretory granules in three distinct areas of the radial nerve, including the epithelium of the hemal sinus. They suggested that these granules contained GSS. The subepidermal plexus of the radial nerve (which contains bipolar and multipolar cells) was reported by Atwood and Simon (1971) to contain fuchsinophilic granules ranging in diameter from 1 to 2 um. Similar granules were found in the basiepithelial plexus of the tube feet, body wall and cardiac stomach but not in the nerve plexus of the pyloric caecum, where no GSS activity was recorded. deAngelis *et al.*, (1972) subjected radial nerves to ultracentrifugation and electronmicroscopic examination. They were able to isolate 1 to 2 um granules, apparently from the supportive cell layer of the radial nerve, and show GSS activity in them.

D. TRANSPORT OF GSS

A number of hypotheses were advanced concerning the transport of GSS from the radial nerve to the gonads. Unger (1962) inferred the transport of fuchsinophilic granules from the supportive cells of the radial nerve along

supportive fibers in the nerve itself, presumably in the direction of the perihemal sinus. This led Kanatani to suggested that GSS is transported to the radial and transverse hemal canals, and into the water vascular system. From there it moves into the coelom where the gonads are suspended (Kanatani, 1973).

Chaet (1966) proposed another scheme for the route of GSS to the gonad. According to this, GSS is released from the oral surface of the radial nerve into the seawater in sufficient concentrations to elicit gamete release from its neighbour. A threshold level of GSS would be transported into the coelomic cavity of the original seastar to stimulate its gonads (Chaet, 1966). This suggests that GSS acts more as a pheromone than a hormone, but this mechanism has not been substantiated. Kanatani and Shirai (1968, 1969) showed that GSS in varying concentrations (25 to 200 ug dry nerve per ml) failed to enter the coelom and no spawning was induced. However, if the seastar's arm was first slit with scissors, spawning occurred within 2 hours (Kanatani, 1973).

The mechanism by which GSS is released from the nervous system is still obscure (Kanatani, 1973), however Kanatani (1973) proposes that once GSS is liberated from the nervous system it reaches the coelom and diffuses into the ovaries.

The follicle cells around the oocytes respond to GSS by producing MIS, identified as 1-methyladenine (1-MA). 1-MA stimulates dissolution the cementing substance between the cells causing disintegration of the follicular envelope (Kanatani, 1973). Contraction of the ovarian wall apparently cannot occur until 1-MA dissolves the cementing substance and makes the individual oocytes within the ovary freely movable (Kanatani, 1973). 1-MA is also believed to induce spawning posture, and brooding behaviour in those species that brood their young (Kanatani, 1973). In causing oocyte maturation, 1-MA first acts on the surface of the oocyte to bring about cytoplasmic maturation and the acquisition of fertilizability. This leads to production of a maturation-promoting factor (MPF), a cytoplasmic substance that causes breakdown of the germinal vesicle, completing oocyte maturation (Kanatani, 1975).

GSS probably binds to membrane-bound surface receptor sites on the follicle cell surfaces.(Kanatani, 1975). From here it may be moved into the follicular cytoplasm to induce *de novo* synthesis of 1-MA (Kanatani, 1975). There is no evidence to suggest, however, that GSS is a 1-MA precursor (Kanatani, 1975; Sano and Kanatani, 1980).

The system proposed by Kanatani (1973) was based on two experimental findings: GSS may reach the coelomic fluid

surrounding the gonads via connections between the radial hemal and water vascular canals; GSS is found in the coelomic fluid only in individuals undergoing spawning, and nonspawning or previously spawned individuals show no GSS activity (Kanatani and Ohguri, 1966). On this basis, Kanatani proposed that the coelomic fluid was the vehicle for GSS transport.

It is not clear that there is a common means for the radial hemal sinus and the radial water vascular canals to communicate with the coelomic fluid surrounding the gonads. Although Unger (1962) reported continuity between the radial hemal sinus, the radial water vascular system, and the coelom in *Marthasterias glacialis*, most workers (Gemmill, 1933; Hyman, 1955; Ubaghs, 1969) regard these three systems as isolated in asteroids. It seems unlikely that GSS diffuses into the gonadal coelom by this method.

The presence of GSS in the coelomic fluid of spawning individuals cannot be explained by Kanatani's hypothesis because there is a temporal anomaly between the documented action of GSS, and its role in the spawning mechanism. According to Kanatani, GSS stimulates 1-MA production which then elicits the observable action of spawning (the assumption of a spawning posture and the release of gametes). Yet the time period between application of GSS

and observable spawning activity *in vivo* and *in vitro* is between 30 and 60 minutes. Thus it would be expected that GSS activity should be detected in the coelomic fluid of a ripe individual at least 30 minutes prior to spawning; however, this has not been demonstrated. An alternate explanation for finding GSS activity in the coelomic fluid of spawning individuals is that excess GSS may leak out of the gonad after spawning has already begun. Further, it is not clear whether the reported spawning-inducing capacity of coelomic fluid from spawning individuals is the result of GSS or 1-MA. Clearly, the mechanism of GSS-induced spawning required further investigation.

E. PURPOSE

This thesis research was undertaken to answer two questions: Where is GSS produced in the radial nerve cords, and how does it get to the ovary to initiate spawning? To answer these questions the specific objectives of this research were;

1. To purify enough GSS so that polyclonal antibodies could be raised in rabbits.
2. To use the rabbit antisera to localize GSS in the radial nerve cords and other tissues reported to show GSS activity, and to see if GSS is present in the ovary itself.

3. To use the rabbit antisera to establish whether or not the hemal system could be involved in GSS transport.

The working hypothesis for this study was that GSS is secreted into the radial hemal sinus from the radial nerve. From there it is transported through the hemal system via the periesophageal hemal ring to the aboral hemal ring, where it is distributed to the ovaries by the genital hemal sinuses.

II. PURIFICATION OF GSS

A. MATERIALS AND METHODS

The procedures adopted for purifying GSS were modified from the methods of Kanatani *et al.*, (1971). The principal differences were the bioassay method, more extensive gel filtration and ion-exchange chromatography, and analysis of the final product using HPLC.

1. Preparation of Lyophilized Radial Nerve

Between May and July, 1982, 256 specimens of *Pycnopodia helianthoides* were collected from local waters at Victoria and Bamfield, B.C., using SCUBA, and kept in marine aquaria at ambient sea temperature before dissection. All specimens collected had central disc diameters greater than 10 cm. Radial nerve tissue was dissected from both male and female seastars.

Radial nerves were isolated from each ray by opening the aboral surface with a midline incision. The ambulacrum was cut along the ambulacral groove to expose the nerve on the oral surface. Nerves were removed with fine forceps and placed in 10 ml of distilled water (dw). Isolated nerves were sliced into small pieces and homogenized with an electric tube and pestle tissue grinder. Homogenates were

poured into petri dishes and frozen at -20°C, then lyophilized at 10 uT and -50°C for 48 hr. The lyophilized radial nerve (35 g) was stored in a desiccator at -20°C for 6 months.

2. Chemical and Physical Processes

All chemicals and organic solvents were of commercially available reagent grade except triethylamine, formic acid and acetonitrile, which were HPLC grade. 1-Methyladenine (1-MA) was obtained from Sigma Chemical Co. Sephadex G-50, G-25 and G-10, and DEAE Sephadex A-25 were obtained from Pharmacia Fine Chemicals, Inc. A Waters MCH-10 protein column was used for peptide analysis. All experiments were carried out at 8°C unless otherwise stated.

Protein concentrations were determined by the method of Lowry *et al.*, (1951). Chloride concentrations were measured using Sigma reagents for the determination of chloride (Sigma Chemical Co., Kit No. 830-T).

Chromatography columns were made of ground glass with a uniform inside diameter of 2.6cm. Flow adaptors were used to suspend the gels in the columns. A single speed, peristaltic pump was used to move eluants through the columns. Flow rate was adjusted by a screw clamp placed on the column input line. Protein elution was monitored with a UV absorbtiometer. Fractions were collected with an

automatic fraction collector, which could be calibrated to collect desired fraction volumes.

3. Bioassay

Ovaries from the purple starfish, *Pisaster ochraceus*, were used to assay for GSS activity. Female starfish were collected intertidally from Telegraph Bay, Victoria, B.C., during March to August, 1983. Ovaries from ripe females were isolated by opening the aboral body wall at the midline of each ray proximal to the central disc. The ovaries were transported to the laboratory in containers of seawater at 4°C. Most bioassays were conducted on the same day of collection, though ovaries remained viable for 24 hours if stored at 4°C.

Whole ovaries were rinsed with filtered seawater (FSW) then cut into 1 cm fragments. Each of the fragments was washed three times with FSW to remove loose oocytes, then placed in individual compartments of a multiwell plate. Each compartment was filled with 0.9 ml FSW and 0.1 ml of the sample to be assayed. The plates were incubated for 1 hr at 12°C. After incubation, the fragments were observed for oocyte expulsion, and the amount of GVBD was measured in each compartment. The average percent GVBD from 3 replicates of a random sample of oocytes was assumed to represent the GSS activity in a test sample.

Two assay methods were employed: The first was to detect the presence of GSS activity during the purification procedure. Samples (0.1 ml) from each eluted fraction were tested in accordance with the above procedure. As controls, Samples (0.1 ml) of FSW, 10^{-6}M 1-MA (Sigma Chemical Co.), and the appropriate blank buffer were applied to separate ovarian fragments and incubated along with the test samples. In cases where pyridine acetate buffers were used, the test samples were first dried under a heat lamp then redissolved in the same volume of FSW. After 1 hr, the fragments were examined for the presence of GSS activity.

The second method of bioassay involved serial dilutions of a known quantity of protein from crude extracts and purified GSS to determine the minimum dose required to induce oocyte expulsion and GVBD in the ovarian fragments. Dilutions ranged from 1 mg of protein/ml to 0.001 ug of protein/ml. FSW and 1-MA were used as controls. There were 3 replicates of each dilution and each control. Incubation and examination were carried out as before.

B. RESULTS AND DISCUSSION

1. Bioassay

The activity of test samples, as expressed as percentage of GVBD after one hour, was measured by averaging three separate counts of 100 oocyte samples. Only oocytes released from the ovarian fragments were examined. Fragments that did not release oocytes after one hour were opened and their oocytes examined. These always showed less than 20% GVBD, and many were disintegrated or mishapened. Since most of the controls also showed less than 20% GVBD and similar oocyte disruption, the test fragments that did not release oocytes after one hour were equitable with the controls, and recorded as GSS-absent.

The extent to which GVBD would occur when GSS was present in the sample has been found to be dose dependent (Kanatani *et al.*, 1971), with certain limitations. If after one hour less than 70% GVBD was observed, the oocytes of that sample would undergo no further GVBD. Samples showing activity above 70% GVBD would normally show 100% GVBD after two hours. Therefore, greater than 70% GVBD after one hour was chosen as a high level of GSS activity, and samples showing less than 70% GVBD after one hour were not used in subsequent purification steps.

The minimum dose of crude extract required to induce greater than 70% GVBD after 1 hour was 5.0 ug protein/ml. The minimum dose of purified GSS required to induce greater than 70% GVBD after 1 hour was 0.002 ug protein/ml. Thus the activity of pure GSS was 2500 times that of crude radial nerve extract.

2. Preparation of Crude Extracts

The lyophilized radial nerves were ground to powder with a mortar and pestle. The powder was homogenized in 500 ml of cold (4^{o}C) acetone using a Sorvall Omnimixer. The suspension was filtered on coarse filter paper in a Buchner funnel, and the residue washed with 1 l of cold acetone. The filter paper, with the residue, was then transferred to a vacuum chamber and the remaining acetone evaporated *in vacuo*.

The dried residue was homogenized in 200 ml of 0.01M NaCl until suspended. The suspension was transferred to a hot water bath (90^{o}C) for 30 minutes, then cooled in an ice bath. The cooled suspension was centrifuged at 20,000 x g for 50 minutes at 2^{o}C. The supernatant was collected and designated Crude Extract I. The precipitate was resuspended in 200 ml of 0.01M NaCl and centrifuged as before; its supernatant was designated Crude Extract II. The precipitate was resuspended for a final time in 100 ml of 0.01M NaCl and and centrifuged to obtain Crude Extract III.

After the acetone extraction of lyophilized radial nerve an orange pigment was extracted in the filtrate, and a yellow residue was collected on the filter paper. After acetone evaporation, samples of both the filtrate and residue were tested for GSS activity. Only the yellow residue showed GSS activity, a result in accord with previous studies (Kanatani *et al.*, 1971). The yellow colour of the GSS-active residue was due to a soluble non-proteinaceous pigment. This pigment was eluted in the same volume as GSS during the initial gel filtrations on Sephadex G-50 and G-25.

3. Gel Filtration on Sephadex G-50

Crude extracts were concentrated to one half their original volume and applied to a long Sephadex G-50 column (2.6cm x 83cm). The gel was equilibrated with 0.05M NaCl in 2% 1-butanol, which was also used as the eluant for upward flow elution. The flowrate for applying the samples was 30 ml/hr, and the flowrate for elution was 60 ml/hr. Fraction size was 10 ml. After filtration, 0.1 ml samples of each fraction were removed for protein determination and assayed for GSS activity.

Active fractions from each crude extract that showed greater than 70% GVBD were pooled and lyophilized.

The protein elution profile for Crude Extract I on Sephadex G-50 (Fig. 1) was typical for all crude extracts. A large, multicomponent protein peak appeared after void volume and ended at 260 ml of eluant. A smaller, broader peak appeared between 400 and 550 ml of eluant. GSS activity was found within this peak between 400 and 500 ml of eluant. The GSS-active fractions (No.s 40 to 50) were pooled and lyophilized.

Lyophilizates of Crude Extract I were dissolved in 50 ml of 0.05M NaCl in 2% 1-butanol and applied to the same column for recycling chromatography. Flowrates and fraction sizes were the same as before. Fractions were collected after two cycles and analysed for protein and GSS activity as before. Lyophilizates from Crude Extracts II and III were also subjected to recycling chromatography in this manner.

Protein analysis after two cycles showed a large peak containing high GSS activity, though the peak was poorly resolved(Fig. 2A.). Elution profiles of Crude Extracts II and III showed similar results. The active fractions from all crude extracts were pooled and lyophilized for a final run on the Sephadex G-50 column.

Active fractions from the three crude extracts were dissolved in 50 ml of eluant and subjected to a final run on

the G-50 column. All parameters were the same as before except fraction size, which was 5 ml. Better resolution of the peak containing GSS activity was observed in Figure 2B after the third gel filtration on Sephadex G-50. GSS activity was consistently eluted within 400 to 500 ml of eluant throughout all G-50 runs, indicating that GSS had not been broken down by gel filtration on G-50. Active fractions 80 to 99 were pooled and lyophilized.

4. Gel Filtration on Sephadex G-25

The active lyophilizate was dissolved in 30 ml of 0.1M pyridine acetate buffer, (PAB), which was prepared by adjusting a solution of 0.1M pyridine and 2% 1-butanol to pH 5.0 with 1N acetic acid. The sample was applied to a 2.6cm x 62cm Sephadex G-25 column equilibrated with the same buffer. Sample application flowrate was 30 ml/hr, and elution flowrate was 60 ml/hr. Flow was applied upward. Fraction size was 10 ml. After elution, 0.1 ml samples were collected from each fraction and protein content was determined, using PAB as a blank standard. Samples (0.1 ml) collected for bioassay were first dried under a heatlamp to remove the buffer, since pyridine inhibits spawning (Kanatani *et al.*, 1971). The dried samples were redissolved in 0.1 ml of FSW and assayed in the usual manner. Samples (0.1 ml) of blank buffer were treated the same way and used as controls, as well as FSW.

Sephadex G-25 and PAB at pH 5.0 gave better resolution of the GSS-active portion of the lyophilizate from the last G-50 filtration. Figure 3A shows the protein elution profile of the lyophilizate after filtration. Two peaks were resolved; a small peak eluted between 150 and 200 ml of eluant, and a second, larger peak eluted between 250 and 460 ml of eluant. GSS activity was found within the second peak at 310 to 390 ml of eluant. The active fractions were pooled and lyophilized.

The active lyophilizate was dissolved in 10 ml of PAB (pH 5.0). This was applied to a 2.6cm X 60cm Sephadex G-25 column equilibrated with the same buffer. Application flowrate was 15 ml/hr. Elution flowrate was decreased to 30 ml/hr to increase resolution. The fraction size was 10 ml. Fractions collected after filtration were assayed for protein and GSS activity as before. A single peak was resolved (Fig. 3B.). GSS activity was eluted between 300 and 380 ml of eluant. The active fractions were pooled and prepared for ion-exchange chromatography.

5. Ion-Exchange with DEAE Sephadex A-25

A 2.6 cm x 56 cm DEAE Sephadex A-25 column was equilibrated with PAB (pH 5.0) containing 0.05M NaCl. The pooled active fractions (80 ml) from the previous filtration were diluted with PAB to a final chloride concentration of

0.05M, and applied to the column at a flowrate of 30 ml/hr. The column was washed with 300 ml of starting buffer at a flowrate of 60 ml/hr. After this a linear salt gradient was applied using 0.05M NaCl in PAB in the first reservoir, and 0.3M NaCl in PAB in the second reservoir. The total volume of the gradient was 1 l. Finally the column was washed with 500 ml of 1.0M NaCl in PAB. Fraction size was 10 ml. Protein determination and bioassays were carried out as before. In addition, every tenth fraction was analyzed for chloride concentration.

GSS was eluted isocratically within a multicomponent peak [The isocratic pH of GSS is around 4.5 (Kanatani *et al.*, 1971), and proteins elute from DEAE Sephadex gels within 0.5 units of their isocratic points (Pharmacia Fine Chemicals Inc., 1982)]. Significantly, the pigment that accompanied GSS in gel filtration was not eluted under these conditions until the chloride concentration was 0.25M; thus it was possible to remove the pigment from the GSS active sample(Fig. 4).

The pooled active fractions (110 ml) were lyophilized and dissolved in an equal volume of PAB adjusted to pH 6.5. The final chloride concentration was 0.05M. This was applied to a 2.6 cm x 54 cm column of DEAE Sephadex A-25 equilibrated with the same buffer. Application flowrate was 15 ml/hr. Fraction size was 10 ml. The column was washed

with 500 ml of starting buffer at a flowrate of 30 ml/hr, then a linear salt gradient was applied using 500 ml of 0.05M NaCl in PAB in the first reservoir and 0.3M NaCl in PAB in the second reservoir. After this, the column was washed with 1 l of 1.0M NaCl in PAB. Fractions were assayed as before.

Figure 5 shows the result of ion-exchange of the GSS-active portion obtained from the previous run. No peaks were observed during the wash, and did not appear until the start of the gradient. Peaks were not observed during the wash and none appeared until the start of the gradient. At this pH, GSS was retained by the gel and was eluted within three peaks between 780 and 930 ml of eluant, at a chloride concentration from 0.15 to 0.17M.

A final ion-exchange run was carried out using a 2.6 cm x 50 cm DEAE Sephadex A-25 column equilibrated with 0.1M NaCl in PAB (pH 6.0). The pooled active fractions (160 ml) were lyophilized and redissolved to give a final salt concentration of 0.1 M, and applied to the column at a flowrate of 15 ml/hr. The column was washed with 500 ml of starting buffer at a flowrate of 30 ml/hr, then a linear salt gradient was applied with 250 ml of 0.1M NaCl in PAB in the first reservoir and 250 ml of 0.2M NaCl in PAB in the second. Finally the column was washed with 500 ml of 1.0M NaCl in PAB.

Protein was not eluted from the column until the start of the gradient (Fig. 6A.). GSS activity was found within a large peak eluted between 625 and 675 ml of eluant, at a chloride concentration of ranging from 0.12 to 0.14M. GSS was thus eluted at a lower ionic strength the previous run, probably due to the 0.5 unit drop in pH.

6. Gel Filtration on Sephadex G-25

The pooled active fractions (50 ml) were applied to a 2.6 cm x 53 cm Sephadex G-25 column equilibrated with PAB, at a flowrate of 15 ml/hr. Fraction size was 7.5 ml and the elution flowrate was 17 ml/hr. A single peak was resolved (Fig. 6B.) in which GSS-activity eluted between 255 and 290 ml of eluant. The active fractions were pooled (45 ml) and applied to a second 2.6 cm x 49 cm Sephadex G-25 column equilibrated with PAB. Application flowrate was 15 ml/hr and elution flowrate was 30 ml/hr. Fraction size was 10 ml. GSS activity was eluted in a single peak between 240 and 300 ml of eluant (Fig. 7A.), and almost the entire peak showed GSS activity greater than 70% GVBD. Active fractions were pooled (60 ml) and lyophilized.

7. Desalting on Sephadex G-10

The active lyophilizate was dissolved in 2 ml of PAB and applied to a 2.6 cm x 22 cm Sephadex G-10 column

equilibrated with PAB. Application flowrate was 15 ml/hr and elution flowrate was 30 ml/hr. Fraction size was 3.5 ml. GSS activity was eluted within a single peak between 35 and 75 ml of eluant, and the entire peak showed GSS activity greater than 70% GVBD (Fig. 7B.). Lyophilization yielded 2 mg of a fine white powder assumed to be pure GSS. This was placed in a labelled vial and stored in a desiccator at -20^{o}C.

8. Reverse Phase High Performance Liquid Chromatography (HPLC)

A Waters MCH-10 HPLC column (4 mm x 300 mm) was connected to a Varian 5000 liquid chromatograph and washed with an acetonitrile gradient in dw. The initial concentration of acetonitrile was 60%, decreasing to 10% after 30 min, the increasing to 60% after 1 hr. A second wash with an acetonitrile gradient in triethylammonium formate (TEAF) was applied with an initial acetonitrile concentration of 60%, decreasing to 10% after 20 min, then increasing to 60% after 1 hr, and finally decreasing to 10% after 90 min. The column was then ready for use.

TEAF was used as the mobile phase, and was prepared by adjusting a 1.0M solution of triethylamine to pH 6.5 with 0.25M formic acid. The TEAF was filtered on a Sep-Pak C-18 cartridge to remove impurities.

A blank run was performed using a solution of 200 ul dw and 600 ul TEAF to determine mobile phase artifacts. The chromatograph was programmed to deliver a flowrate of 1 ml/min at 66 atm and 24 C. A UV detector linked to a chart recorder was set at a wavelength of 280 nm, with a slit width of 4 nm. The sensitivity of the chart recorder was set to 0.05 AUFS at 1 mV. 10 min after the start of the run, an acetonitrile gradient in TEAF was applied from 10% to 60% over 1 hr. The column was then washed as previously described.

For the peptide run, 20 ug of the active lyophilizate from the previous gel filtration were dissolved in 200 ul of dw and mixed with 600 ul TEAF. This was applied to the column under the same conditions as before. Fraction size was 1 ml. After the run, 0.1 ml samples from each fraction were dried under a heat lamp, redissolved in 0.1 ml FSW, and assayed for GSS activity.

Figure 8 shows the elution profile of the purified sample. The initial deflections are injection artifacts; the small negative deflection at 10 min indicates the start of the acetonitrile gradient. A single peak was observed after 30 min, a sample of which induced 98% GVBD after 1 hour. No other peaks were observed throughout the rest of the elution or during the final wash of after the run.

These results indicated that the substance obtained from gel filtration and ion-exchange chromatography of radial nerve crude extracts was pure GSS.

FIGURE 1

Gel filtration of *P. helianthoides* radial nerve extract (Crude Extract I) on a long Sephadex G-50 column. Absorbance is calculated as the total amount of protein in each fraction. Activity above 70% GVBD was found in fractions 40 to 50.

1

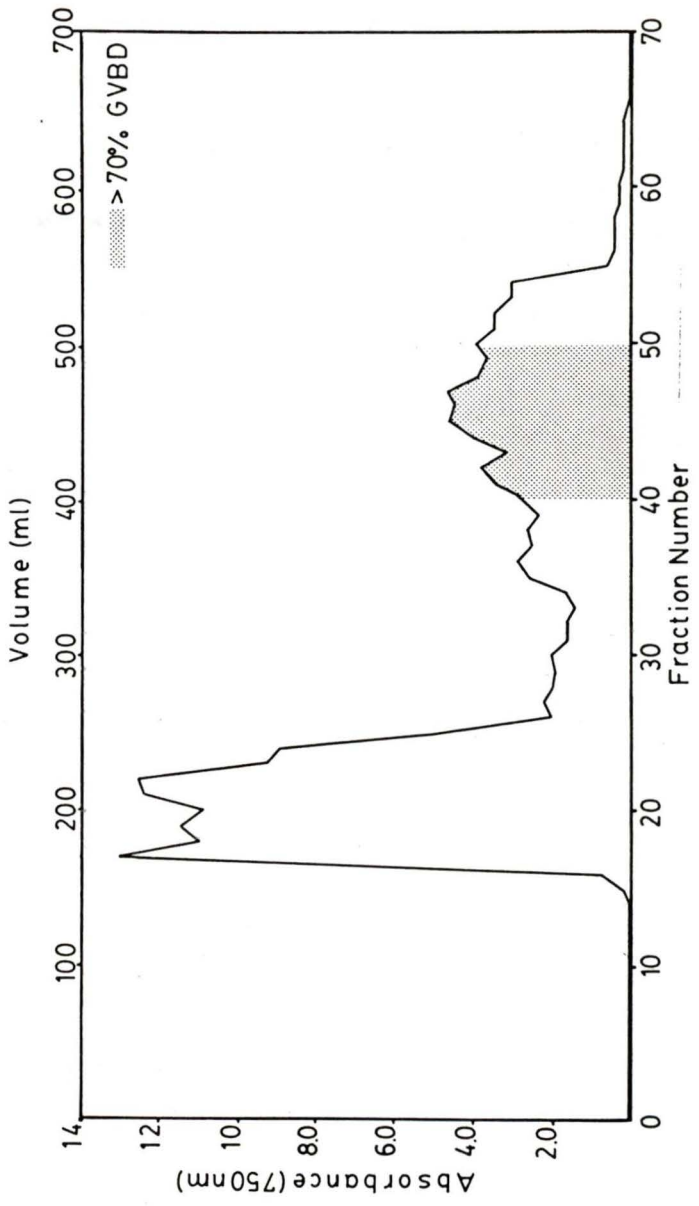
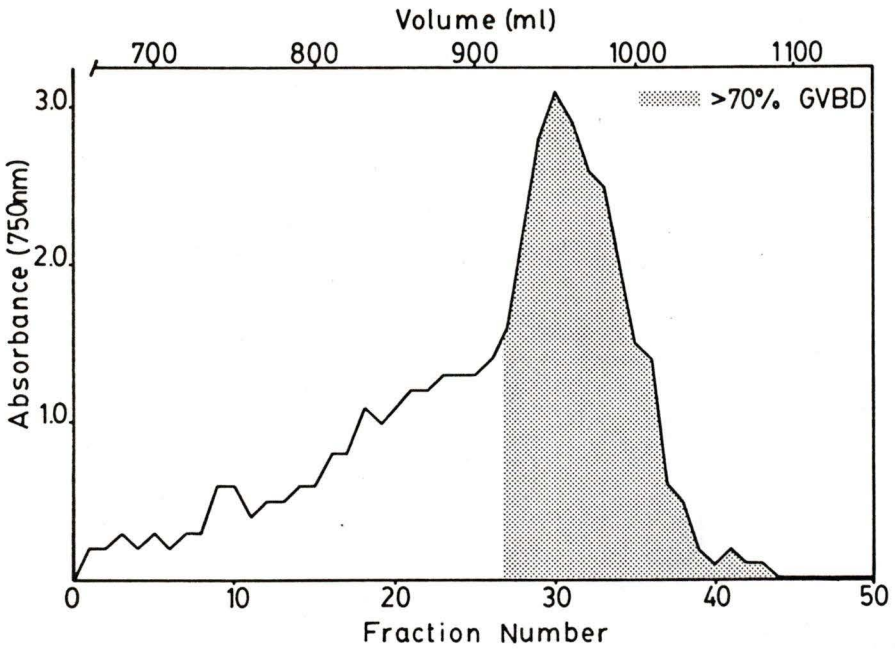

Volume (ml)
100
200
300
400
500
600
700
Absorbance (750nm)
14
12
10
8.0
6.0
4.0
2.0
0
10
20
30
40
50
60
70
Fraction Number
>70% GVBD

FIGURE 2

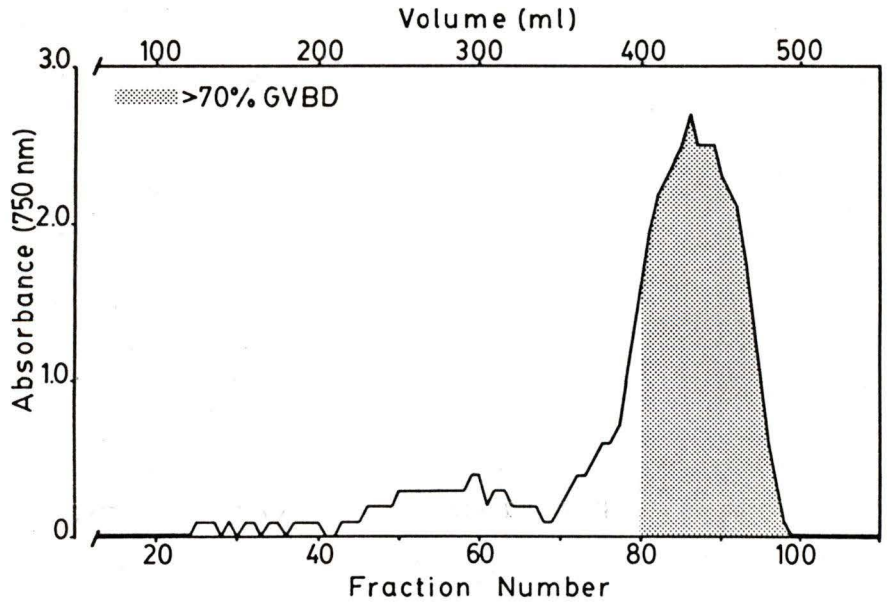
A. Gel filtration of active fractions from Crude Extract I after two cycles on a long Sephadex G-50 column. Fractions 26 to 44 showed activity above 70% GVBD.

B. Gel filtration of pooled GSS-active fractions from all crude extracts obtained from the previous gel filtration on a long Sephadex G-50 column.

2


Volume (ml)
700
800
900
1000
1100
>70% GVBD
Absorbance (750nm)
3.0
2.0
1.0
0
10
20
30
40
50
Fraction Number

A


Volume (ml)
100
200
300
400
500
>70% GVBD
Absorbance (750 nm)
3.0
2.0
1.0
0
20
40
60
80
100
Fraction Number

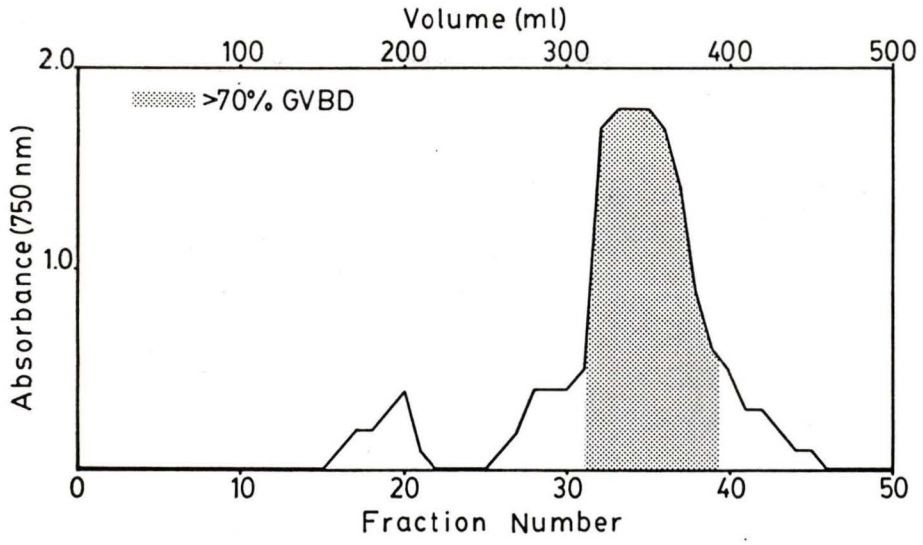
B

FIGURE 3

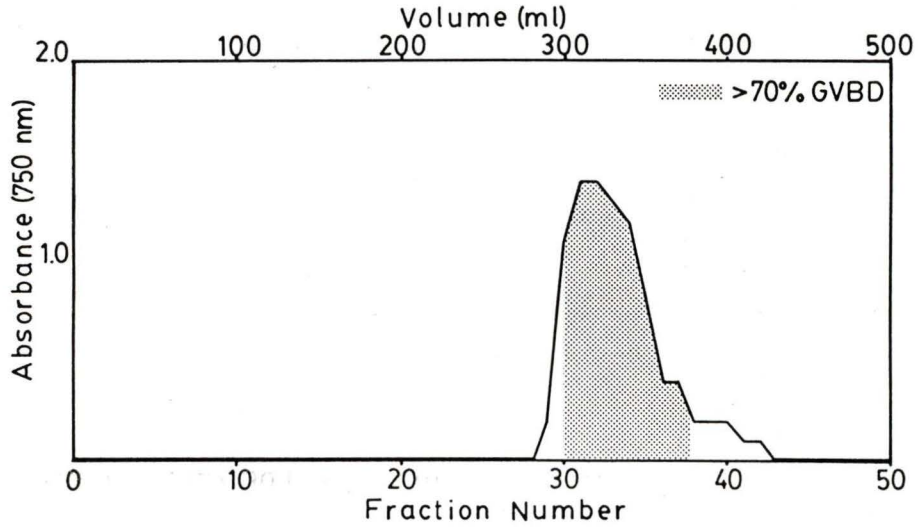
A. Gel filtration of pooled active fractions (Nos. 80 to 99 in Fig. 3A. on a Sephadex G-25 column. Greater than 70% GVBD was found in fractions 31 to 39.

B. Further gel filtration of pooled active fractions from the previous gel filtration on a Sephadex G-25 column. Fractions 30 to 38 showed activity above 70% GVBD.

3


Volume (ml)
100
200
300
400
500
2.0
1.0
>70% GVBD
Absorbance (750 nm)
0
10
20
30
40
50
Fraction Number

A


Volume (ml)
100
200
300
400
500
2.0
1.0
>70% GVBD
Absorbance (750 nm)
0
10
20
30
40
50
Fraction Number

B

FIGURE 4

First ion-exchange chromatography of GSS sample obtained from the previous gel filtration, on a DEAE Sephadex A-25 column. A linear NaCl gradient was used at pH 5.0.

4

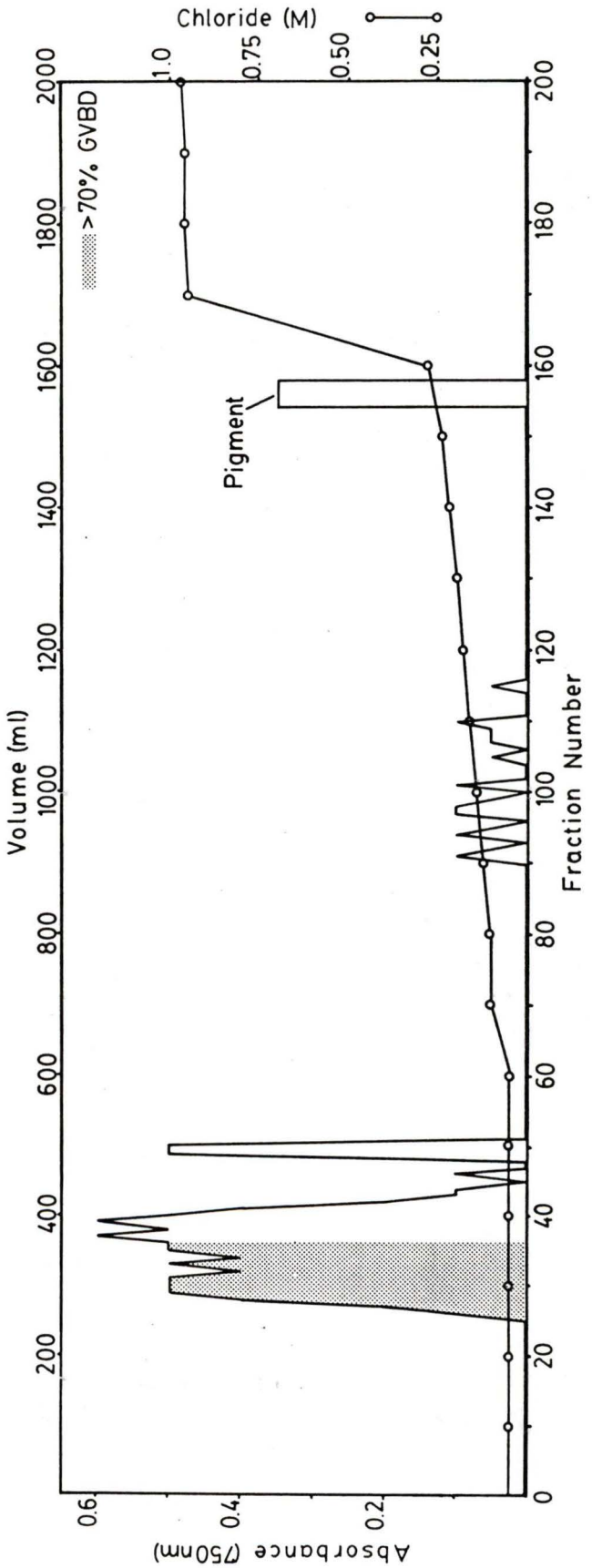

Chloride (M)
1.0
0.75
0.50
0.25
>70% GVBD
Pigment
Volume (ml)
Fraction Number
Absorbance (750nm)
0.6
0.4
0.2
0
200
400
600
800
1000
1200
1400
1600
1800
2000
20
40
60
80
100
120
140
160
180
200

FIGURE 5

Second ion-exchange chromatography of fractions 25 to 36 (Fig. 5) on a DEAE Sephadex A-25 column. A linear NaCl gradient was used at pH 6.5.

5

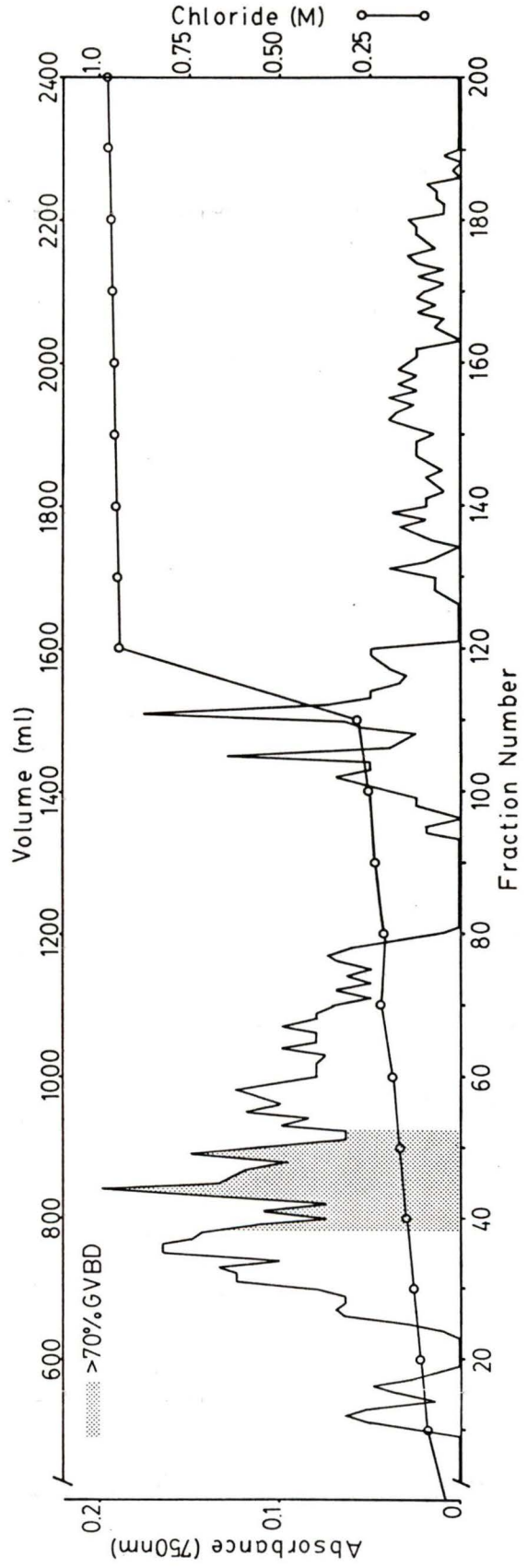
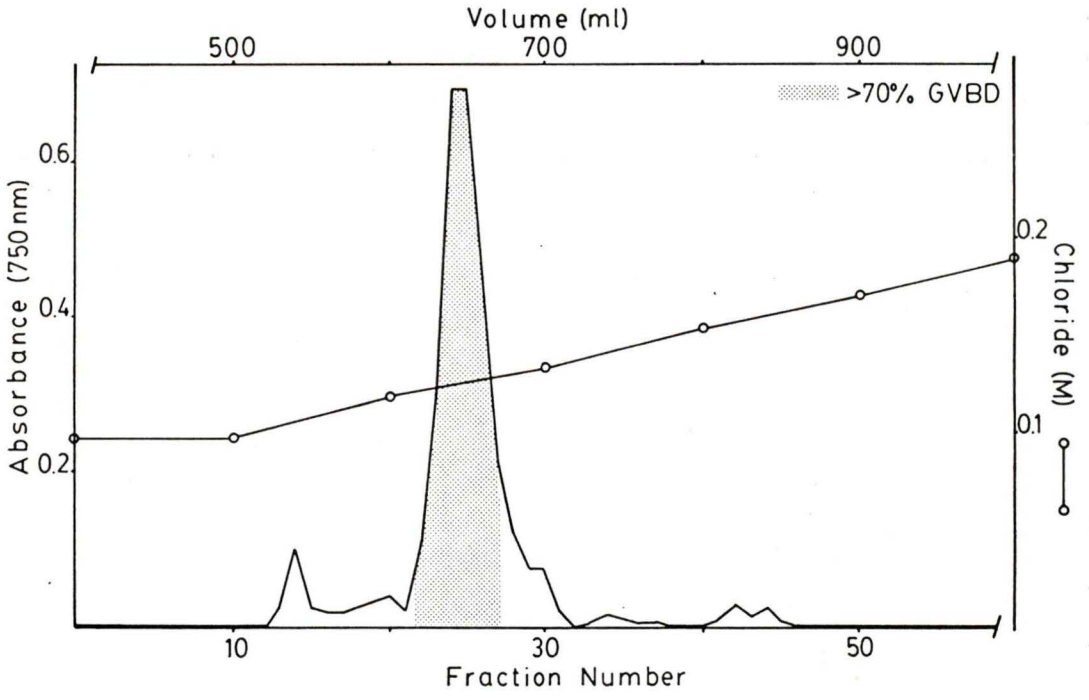

Chloride (M)
1.0
0.75
0.50
0.25
Volume (ml)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Fraction Number
200
180
160
140
120
100
80
60
40
20
>70% GVBD
Absorbance (750nm)
0.2
0.1
0

FIGURE 6

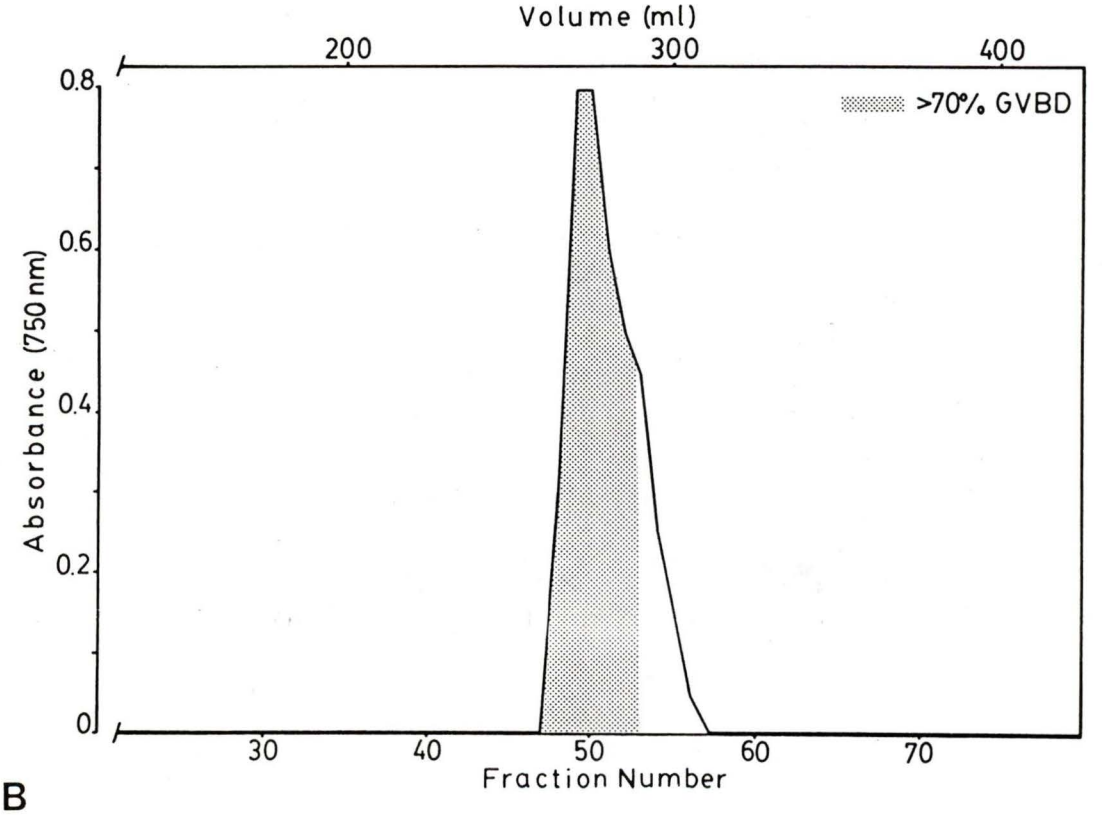
A. Final ion-exchange chromatography of GSS sample (Fractions 38 to 52 in Fig. 6) on a DEAE Sephadex A-25 column. A linear NaCl gradient was used at pH 6.0.

B. Gel filtration of GSS sample (Fractions 22 to 27 in Fig. 7A. on a Sephadex G-25 column. Fractions 48 to 53 showed activity greater than 70% GVBD.

6


Volume (ml)
500
700
900
>70% GVBD
Absorbance (750 nm)
0.6
0.4
0.2
0.2
0.1
Chloride (M)
10
30
50
Fraction Number

A


Volume (ml)
200
300
400
>70% GVBD
Absorbance (750 nm)
0.8
0.6
0.4
0.2
0
30
40
50
60
70
Fraction Number

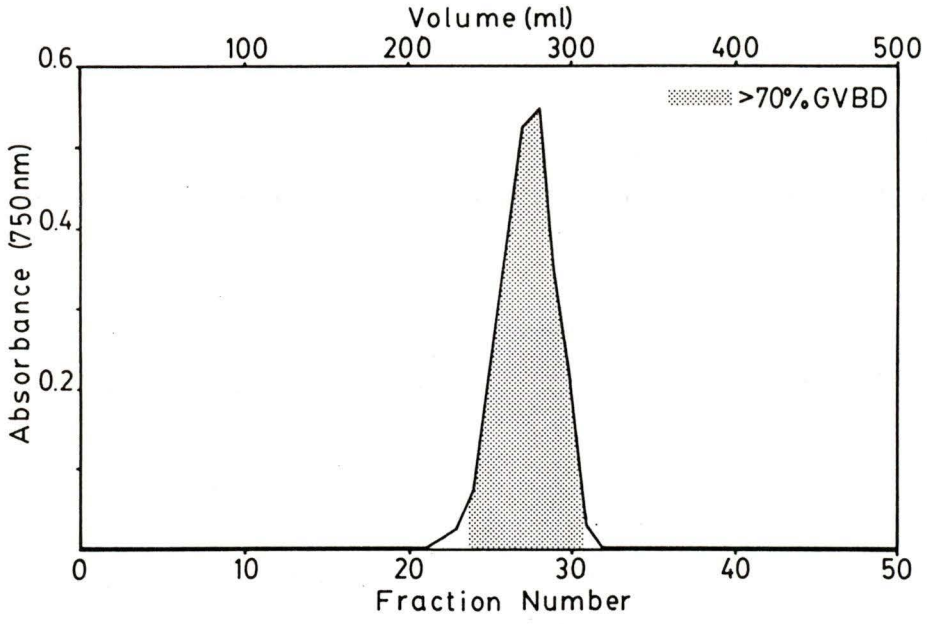
B

FIGURE 7

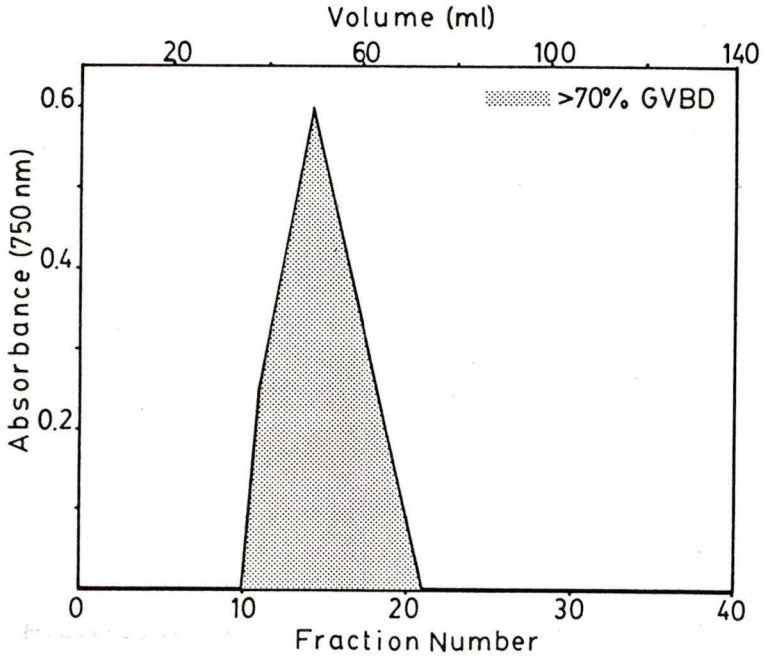
A. Gel filtration of GSS sample obtained from the previous gel filtration on a Sephadex G-25 column. Activity above 70% GVBD was found in fractions 24 to 30.

B. Desalting gel filtration of GSS on a Sephadex G-10 column. Greater than 70% GVBD was found in fractions 10 to 21.

7


Volume (ml)
100
200
300
400
500
0.6
0.4
0.2
Absorbance (750nm)
>70% GVBD
0
10
20
30
40
50
Fraction Number

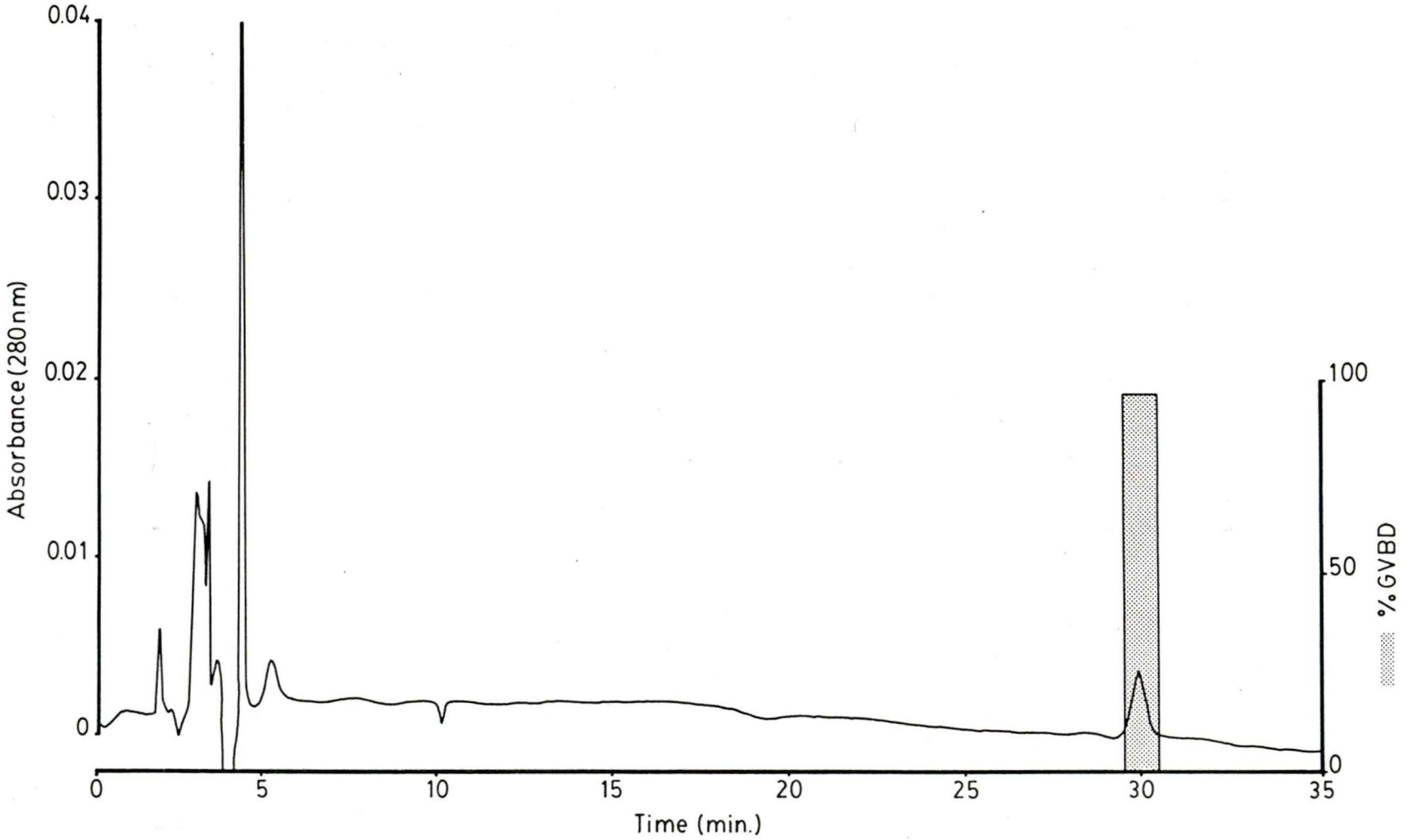
A


Volume (ml)
20
60
100
140
0.6
0.4
0.2
Absorbance (750 nm)
>70% GVBD
0
10
20
30
40
Fraction Number

B

FIGURE 8

Reverse phase high performance liquid chromatography of a small sample of GSS obtained from gel filtration and ion-exchange chromatography. An increasing acetonitrile gradient was used. A single peak was found to have GSS activity.


0.04
0.03
0.02
0.01
0
Absorbance (280nm)
100
50
0
% GVBD
0
5
10
15
20
25
30
35
Time (min.)

III. PREPARATION OF GSS ANTISERA

A. MATERIALS AND METHODS

1. General

Two female New Zealand white rabbits (5 and 7 kg body weight) were obtained from Cedar Glen Farm, Victoria B.C., and housed in the animal room at the University of Victoria. The animals were acclimated for 3 months prior to treatments.

Bovine thyroglobulin (BTg), 1-ethyl-3-(3',dimethylamino propyl) carbodiimide hydrochloride (EDC), Freund's complete and incomplete adjuvants, Hydroxylamine, and sodium azide were obtained from Sigma Chemical Co. Coarse agar was obtained from BDH. Venisystems Butterfly-21 and Butterfly-19 Infusion Kits were obtained from McGill and Orme Surgical Supply, Victoria, B.C.

2. Conjugation of GSS to Bovine Thyroglobulin (BTg)

The first conjugation reaction was carried out according to the method of Skowsky and Fisher (1972). The initial molar ratio of GSS:BTg:EDC was 118:1:200. GSS (1 mg) and 5 mg of BTg were dissolved in 150 ul of dw. EDC (50 ul at a concentration of 4.62 mg/ml) were added and the

reactants incubated at 22°C for 18 hr. 200 ul of 1.0M Hydroxlamine were added and the conjugate solution (400 ul final volume) was diluted with 4.6 ml of normal saline to give a final peptide concentration of 200 ug/ml. This was divided into 10, 0.5 ml aliquots, labeled Conjugate A, and stored at -20°C.

The second conjugation reaction was performed according to the method of Davis and Preston (1981). The initial molar ratio of GSS:BTg:EDC was 1:0.01:10. Three phosphate buffer solutions were used: Low Phosphate Buffer (LPB) was prepared from a 12.5mM solution of monobasic sodium phosphate ($NaH_2PO_4.H_2O$) adjusted to pH 5.0 with 1N HCl; High Phosphate Buffer (HPB) consisted of a 0.2M solution of $NaH_2PO_4.H_2O$ adjusted to pH 8.0 with 10N NaOH; Phosphate Buffered Saline (PBS) was prepared from mono- and dibasic sodium phosphate (pH 7.0) with 8.5% (w/v) NaCl.

The reaction was as follows: GSS (500 ug) was dissolved in 100 ul LPB. 50 ul of EDC in dw were added to give a final concentration of 10mM EDC. The solution was incubated at 22°C for 2 min, then 100 ul of BTg in HPB was added to give a final concentration of 0.01mM BTg. This mixture was incubated at 22°C for 18 hr, then diluted to 5 ml with PBS to give a final peptide concentration of 100 ug/ml. This was stored at -20°C in 0.5 ml aliquots labeled Conjugate B.

3. Collection of Sera and Inoculation

Blood was collected by bleeding each rabbit from a central ear vein using either a Butterfly-21 or Butterfly-19 Infusion kit. Topical treatment of the ear with xylene induced local dilation of the vein. This was cannulated and the blood collected in sterile 10 ml test tubes. After collection, the ear was thoroughly washed with warm water to remove the xylene, and treated with Vaseline to prevent drying of the skin.

Serum was prepared according to the method of Campbell *et al.*, (1970). Freshly drawn blood was allowed to clot at room temperature for 6 hr, then stored overnight at 4°C to allow the clot to contract. The serum was decanted and centrifuged at 1000 x g for 30 min at 4°C. The supernatant was removed by pipette. To remove lipids, the serum was equilibrated to 0°C and centrifuged at 20,000 x g for 50 min at 0°C. The lipids formed a solid disc on top of the supernatant and the underlying lipid-free serum was removed with a pipette. Sodium azide (0.1% w/v) was added to the serum as a preservative. Sera were divided into 0.5 ml aliquots, labeled, and stored at -20°C.

Inoculations of antigen were carried out using the multiple intradermal injection method of Vaitukaitus (1981). Inocula were prepared by mixing conjugate solutions 1:1 with

Freund's complete or incomplete adjuvants. The mixture was emulsified in two linked 5 ml syringes. The contents were passed between both syringes by alternate strokes of the plungers until the fluid thickened.

The fur from the back of each rabbit was shaved, and the skin wiped with 50% ethanol. The inoculum (20 to 50 ul) was injected intradermally, producing a small lump. Injections were repeated over the entire back of the animal until the entire dosage had been administered. The animal's back was washed with warm water after each treatment.

4. Inoculation and Bleeding Schedule

Sera were coded for identification using a four-part sequence of the form *AXBY* where *A* specified the rabbit identity, *X* the number of inoculations, *B* the conjugate antigen code, and *Y* the number of bleeds after the last inoculation.

Inoculations and bleeds were carried out over an 11 week period, according to the schedule outlined in Table 1. 10 ml preimmune sera were collected from each rabbit 3 months prior to the inoculations with antigen. Booster inoculations of antigen were usually administered 2 days after the last bleed to avoid shocking the animal. In cases where the animal showed an adverse skin reaction to inoculations, boosters were suspended until the skin healed.

Likewise, bleeds were delayed if the veins were found unsuitable for cannulation.

5. Immunoassay

The double immunodiffusion test (Ouchterlony, 1968) was used to detect the presence of GSS antibodies in sera, and determine their titre and specificity. Microplate agar gels (Crowle, 1973) were used as the diffusion media. 1.5% agar was prepared by dissolving 1.5 g of coarse agar in 100 ml of Borate buffered saline (BBS) (8.5% NaCl; pH 8.4) at 100^{o}C. The liquid agar was filtered twice through a Whatman #1 filter cartridge linked to a preheated syringe. The filtered agar was stored in 5 ml batches at 4^{o}C until use. Microplate gels were poured as outlined in Figure 9a.

Test samples were applied to the gels by means of the plexiglass template shown in Figure 9b. The design of the template allowed sera from both rabbits to be tested on the same gel so that results could be compared independent of differences between microplates. To detect GSS antibodies, sera were diluted 1:1 with BBS and diffused against pure GSS (20 ug/ml in BBS). Preimmune sera and blank BBS were used as controls. All volumes were 10 ul of sample/well. Gels were incubated at 4^{o}C for 36 hr, then examined for precipitin lines. Positive sera were divided into two batches and stored in 0.5 ml aliquots at either 4 or -20^{o}C.

To determine titre in positive sera, GSS (20 ug/ml in BBS) was diffused against various dilutions of sera in BBS. To determine specificity, positive antisera were diffused against pure GSS, and extracts of radial nerve, tube feet, pyloric caeca, and ovarian wall. Cross reactivity of antisera to the carrier molecule was determined by diffusion against BTg (20 ug/ml in BBS).

B. RESULTS AND DISCUSSION

1. Detection of GSS Antibodies

No antibodies were detected in sera 28 days after the initial inoculation of 200 ug of Conjugate A in Freund's complete adjuvant (Table 1). The animals were given booster inoculations with 100 ug of Conjugate A in Freund's incomplete adjuvant after 30 days. Sera taken 7 and 14 days (7 and 19 days for rabbit *A*) after the first booster were also negative for GSS antibodies. However, 7 days after a second booster of 100 ug of Conjugate B in Freund's incomplete adjuvant, *A* sera showed detectable GSS antibodies, and continued to show a positive reaction during the rest of the treatment. Single precipitin lines (Fig. 10A) indicated interaction of serum antibodies with a single antigenic species (Crowle, 1973). Rabbit *S* did not show any detectable GSS antibodies throughout the treatment,

even though it received a third booster of Conjugate B two months after the initial inoculation.

Both animals showed cross reactivity to the carrier protein BTg, beginning 28 days after the initial inoculation. Rabbit *S* continued to produce BTg antibodies throughout the treatment; no BTg antibodies were detected in rabbit *A* after 60 days of treatment.

TABLE 1. INOCULATION, BLEEDING AND SCREENING SCHEDULE: *AB*, GSS antibody; *Ag*, dosage (GSS w/v); *BL*, bleed; *FC*, Freund's Complete Adjuvant; *FI*, Freund's Incomplete Adjuvant; *IN*, inoculation.

DAY	*RABBIT*	*TREATMENT*	*AB*	*CODE*
1	A	IN; 200 ug Ag; FC; 2 ml total		----
1	S	IN; 200 ug Ag; FC; 2 ml total		----
14	A	BL; 10 ml obtained	-	A1A1
14	S	BL; 10 ml obtained	-	S1A1
28	A	BL; 10 ml obtained	-	A1A2
28	S	BL; 10 ml obtained	-	S1A2
30	A	IN 100 ug Ag; FI; 1 ml total		----
30	S	IN 100 ug Ag; FI; 1 ml total		----
37	A	BL; 5 ml obtained	-	A2A1
37	S	BL; 7 ml obtained	-	S2A1
44	S	BL; 5 ml obtained	-	S2A2
46	S	IN; 100 ug Ag; FI; 1 ml total		----
49	A	BL; 7 ml obtained	-	A2A2
51	A	IN; 100 ug Ag; FI; 1 ml total		----
53	S	BL; 10 ml obtained	-	S3B1
58	A	BL; 10 ml obtained	+	A3B1
60	S	BL; 6 ml obtained	-	S3B2
62	S	IN; 100 ug Ag; FI; 1 ml total		----
65	A	BL; 5 ml obtained	+	A3B2
69	S	BL; 10 ml obtained	-	S4B1
72	A	BL; 10 ml obtained	+	A3B3
76	S	BL; 5 ml obtained	-	S4B2

2. Titre of GSS Antisera

Three sera from rabbit *A* had detectable GSS antibodies after the second booster inoculation. These were: A3B1; A3B2; and A3B3. Titre in the three sera were determined by the maximum amount they could be diluted with BBS and still show detectable precipitin lines after double diffusion against pure GSS (Fig. 10B). Antiserum A3B2 was diluted 1:25 with BBS and still produced precipitin lines. Antisera A3B1 and A3B3 had maximum dilutions of 1:10 and 1:15, respectively (Table 2). It was concluded that antiserum A3B2 had the highest GSS antibody titre.

TABLE 2. DETERMINATION OF GSS ANTIBODY TITRE: Antibody titre was measured by the maximum amount an antiserum could be diluted with borate buffered saline and still produce precipitin lines in immunodiffusion.

ANTISERUM CODE	*DILUTION* 1:5	1:10	1:15	1:20	1:25	1:30
A3B1	+	+	-	-	-	-
A3B2	+	+	+	+	+	-
A3B3	+	+	+	-	-	-

3. Specificity of GSS Antisera

Antisera specificity was tested by diffusion against tissue extracts of radial nerve and tube feet, tissues known to contain GSS, and extracts of pyloric caeca and ovarian wall, which do not contain GSS. Crude tissue extracts were prepared according to the method for extracting GSS from radial nerve (Chapter 2). The concentration of protein in all extracts was 200 ug/ml. The results are shown in Table 3: Antisera reacted with radial nerve and tube foot extracts, but not with pyloric caeca or ovarian wall extracts. The reactive tests produced single precipitin lines, indicative of interaction with a single antigenic species (Crowle, 1973). The precipitin line for radial nerve extract was denser than the precipitin line for tube foot extract (Fig. 10C), indicating a difference between the amounts of GSS present in these two tissues. This result was consistent with previous observations that although GSS is present in tube feet, it's concentration is much lower than that in the radial nerve (Kanatani, 1973).

TABLE 3. SPECIFICITY OF GSS ANTISERA: Antisera were diffused against tissue extracts of radial nerve, *RN*, tube feet, *TF*, pyloric caeca, *PC*, and ovarian wall, *OW*.

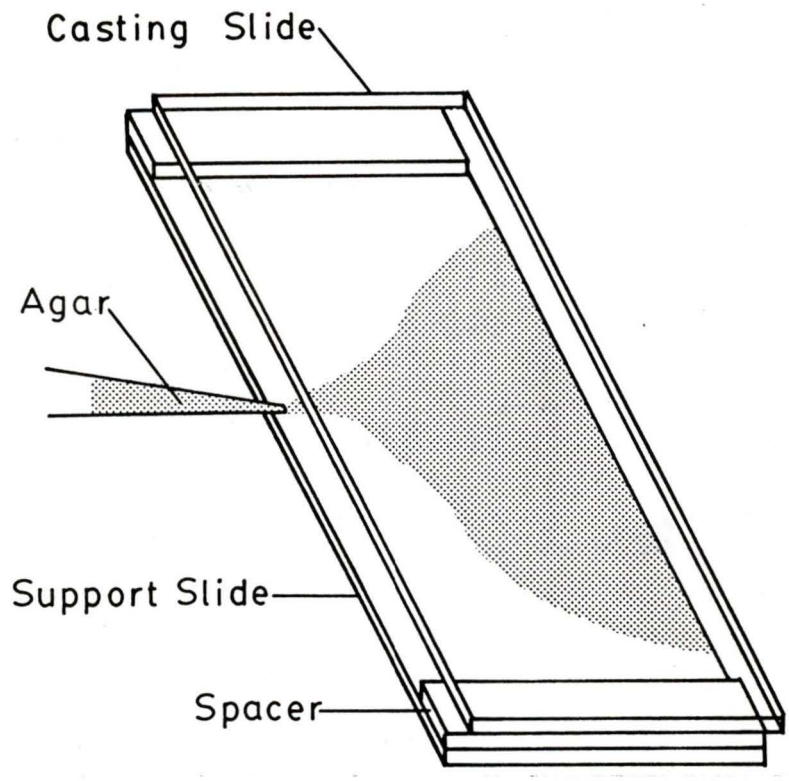
ANTISERUM CODE	*TISSUE EXTRACT*			
	RN	*TF*	*PC*	*OW*
A3B1	+	+	-	-
A3B2	+	+	-	-
A3B3	+	+	-	-

FIGURE 9

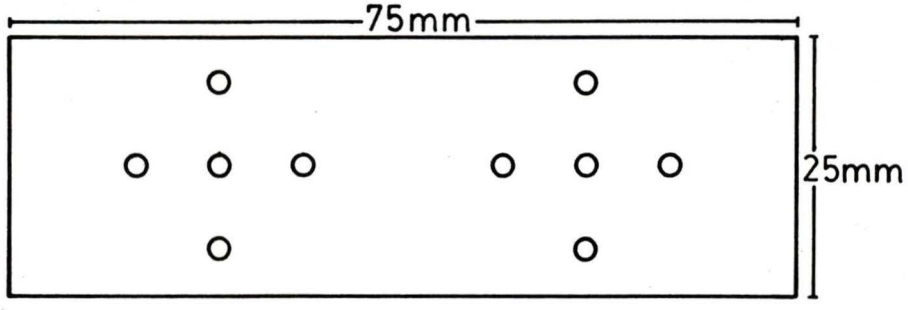
A. PREPARATION OF MICROPLATE GELS: Each gel was prepared using two glass microscope slides. The supporting slide was coated with a thin layer of 1.5% agar to bind the gel; the casting slide was sprayed with a thin layer of silicon to permit easy removal after casting. A 1 mm thick spacer was placed at each end of the supporting slide and the casting slide clamped on top, slightly offset from the edge of the supporting slide to form a pouring ledge. Hot 1.5% agar was pipetted between the two slides, which were then aligned. The agar was allowed to gel at room temperature for 30 min, then the casting slide was removed.

B. PLEXIGLASS TEMPLATE FOR APPLYING SAMPLES: The top view of the template shows the pattern for immunodiffusion of the samples. Holes bored in the template were 2 mm in diameter and 3 mm in depth. The distance between each center well and its peripherals was 10 mm. The side view shows the arrangement of the microplate gel and the template. The template was layed over the gel and taped to the supporting slide. Holes (1 mm diameter) were punched in the gel beneath each sample well of the template. The total volume of each sample well was 10 ul.

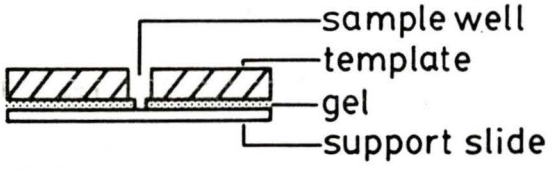
9



A



Top View



Side View

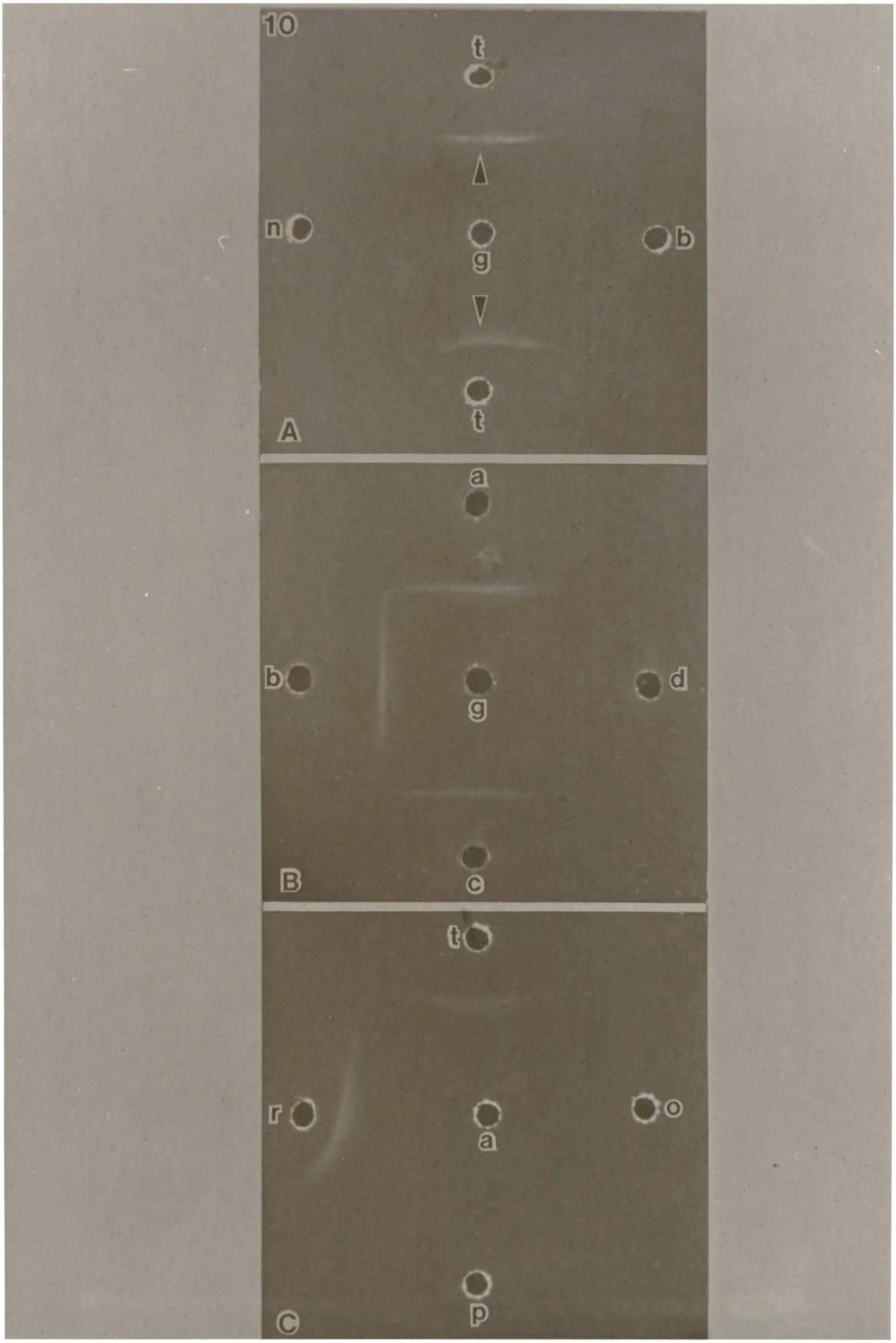
B

FIGURE 10

A. Immunodiffusion of serum A3B1, *t*, pure GSS, *g*, preimmune serum, *n*. and BBS, *b*. Single precipitin lines (arrows) at the interface of GSS and serum diffusion indicate the presence of GSS antibodies. Single lines represent a single precipitating system, indicating interaction of antibodies with a single antigen species (Crowle, 1973).

B. Immunodiffusion of GSS, *g*, against antiserum A3B2 diluted with BBS. Dilutions are 1:15, *a*, 1:20, *b*, 1:25, *c*, and 1:30, *d*. No lines are observed at dilution 1:30. The maximum amount an antiserum could be diluted and still produce precipitin lines was taken as a measurement of its titre.

C. Immunodiffusion of antiserum A3B2, *a* against extracts of radial nerve, *r*, tube feet, *t*, ovarian wall, , and pyloric caeca, *p*. Preciptin lines indicating the presence of GSS antigen and antibodies only occur at the diffusion interfaces of radial nerve extract and tube foot extract, and antiserum. The precipitin line for tube feet was less dense than that for radial nerve extract, indicating less GSS present in tube feet.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IV. LOCALIZATION OF GSS

A. MATERIALS AND METHODS

1. General

Specimens of *P. helianthoides* were collected between October and December, 1983, from local waters at Victoria, B.C. They were held in marine aquaria at 10^{o}C, and dissected within 1 week of collection. Tissue samples of axial organ, ovaries, pyloric caeca, radial nerve cords and tube feet from the females, and radial nerve cords from the males were prepared for immunohistochemical analysis, and light and electron microscopy. All chemicals and solvents used were of commercially available reagent grade. Bovine serum albumin (BSA), 3, 3'-diaminobenzedine (DAB), Goat antirabbit IgG conjugated to horseradish peroxidase (HRP), and Protein A were obtained from Sigma Chemical Co.

2. Procedures for Immunohistochemistry

Tissues were fixed using a modification on Gomori's Method (Humason, 1971). Tissues were cut into 5 mm^3 pieces and fixed in 4% paraformaldehyde in FSW for 10 min at 4^{o}C, rinsed with dw, then postfixed in cold

(-20°C) acetone overnight. Tissues were transferred through two 1 hr changes of petroleum ether at 4°C, allowed to equilibrate to room temperature, then infiltrated with liquid paraffin *in vacuo* for 30 minutes, followed by embedment in fresh paraffin. Sections (6 to 10 um) were cut and mounted on slides subbed with a mixture of 0.1% gelatin in 0.1% Chromium potassium sulfate. The slides were dried with a blow dryer and either processed immediately or stored at 4°C until use.

Prior to staining, the sections were cleared in xylene (2 x 3 min) and rehydrated through a graded acetone series, rinsed with dw (2 x 3 min) and then washed with PBS (3 x 3 min). In some cases, the final PBS wash contained 0.1% Triton X-100 to enhance staining.

Sections for staining were preincubated with 0.1% BSA in PBS for 5 min then incubated with rabbit antisera diluted 1:50 to 1:100 with PBS. Incubation was carried out at 4°C for 18 hr, or at 22°C for 2 hr. After washing with PBS (3 x 3 min), the sections were incubated with Goat antirabbit IgG conjugated to HRP diluted 1:50 with PBS. Incubation was carried out for 1 to 2 hr at 22°C. Sections were then washed with PBS (3 x 3 min).

Staining was developed according to the method of Van Noorden and Polak (1983). Sections were incubated with 0.4% DAB and 0.012% H_2O_2 (added just before use) in PBS for 7 to 10 min, followed by 3, 3 min rinses in PBS. The slides were mounted, examined and photographed with Koehler illumination on a light microscope.

Controls for nonspecific binding included pretreatment of preimmune sera and Goat antirabbit IgG with 0.1% Protein A, to block F_C binding, and pretreatment of tissue sections with 0.1% BSA. Control sections for staining were incubated with preimmune sera, without rabbit antisera, with rabbit antisera alone, or with Goat antirabbit IgG alone. Endogenous peroxidase activity was blocked by pretreating the tissue sections with 0.15% H_2O_2 in PBS. All stained sections were viewed and photographed with identical illumination and exposures.

3. Procedures for Light and Electron Microscopy

Tissues were cut into 1 mm^3 pieces and fixed in 2.5% glutaraldehyde (pH 7.4) in Millonig's Phosphate Buffer (MPB) for 2 hr followed by 3 x 5 min rinses in MPB, then postfixed in 1% Osmium tetroxide in MPB for 1 hr. After rinsing in MPB (3 x 5 min +1 x 30 min), the tissues were dehydrated through a graded ethanol series,

two rinses in propylene oxide (30 min each), and infiltrated overnight in a 1:1 mixture of propylene oxide and catalysed Poly/Bed. Embedment in Poly/Bed was carried out the next day. The Poly/Bed was polymerized at 65^{o}C for 24 hr.

For light microscopy, 1.0 um sections were cut with a diamond knife on a MT5000 ultramicrotome, and mounted on glass slides. The sections were stained with Richardson's stain, and viewed with a light microscope. Sections were photographed using green and cyan filters to enhance contrast.

For electron microscopy, 60 to 50 nm sections were cut and mounted on parlodion-coated, 75 mesh copper grids. Sections were stained with an aqueous solution of uranyl acetate for 45 min, followed by lead citrate for 7 min. Stained sections were viewed and photographed with a Philips EM-300 transmission electronmicroscope at 60 kV.

B. GSS IN THE RADIAL NERVE CORD

1. Anatomy and Histology of the Radial Nerve Cords

The radial nerve cords are located on the oral surface along the midline of each arm. The cords are continuous with the epidermis and appear as thickened ridges in the oral ambulacral groove. The cords terminate proximally in a circumoral nerve ring that surrounds the mouth in the central disc and unites all the radial cords. This comprises the central nervous system in asteroids (Smith, 1965).

The radial nerve cord itself has three components: The ectoneural nerve is the most prominent feature, and is usually referred to as the radial nerve. To avoid confusion between this and the term radial nerve cord, the term ectoneural nerve will be used to describe this component. The second component is the hyponeural nerve, also referred to as Lange's nerve, which is separated from the ectoneural nerve by a connective tissue layer. The third component is the radial hemal sinus, which runs longitudinally through the perihemal space (Fig. 11A).

The ectoneural nerve is composed of three layers (Figs. 11B & C): The outermost layer is the epithelial

layer, which is between 20 and 30 um thick. Beneath this lies the superficial nerve plexus, averaging 12 um thick. The largest component of the ectoneural nerve is the deep nerve plexus, which varies in thickness from 75 to 100 um.

In the epithelial layer, apical microvilli arising from the epithelial cells infiltrate the glycocalyx and end in oral terminals (Fig. 12A). There are three types of cells in the epithelial layer. The largest are the mucous gland cells, which average 25 um in length (Fig. 12B). These cells are filled with large (2 to 3 um) mucus granules, and have a small nucleus that lies basally in cell.

The second cell type is the supporting cell (Fig. 12C). The cell body is located orally in the epithelium. The cell body contains an oval shaped nucleus, and numerous small granules. These cells give rise to the supporting fibers, which run from the epithelium through to the basal surface of the deep plexus. The supporting cell granules average 1 um in diameter, and are found in the apical and basal portion of the cell body, and also at intervals along the supporting fibers in the deep plexus (Fig. 13D).

The third cell type is the sensory cell (Fig. 12D). These cells are characterized by round nuclei with one or two folds. The sensory cells bodies lie basally in the epithelium, and send an apical process to the oral surface and a basal process into the superficial plexus (Smith, 1971).

The superficial plexus underlies the epithelium, though there is no distinct boundary between them (Fig. 13A). The axons of the superficial plexus are between 150 to 300 nm in diameter, and have no particular direction in the plexus, running transversally as well as longitudinally in the nerve cord. They usually contain a few clear and dense vesicles, which average 50 nm in diameter.

The axons of the deep plexus are somewhat larger, averaging 300 nm in diameter. These axons predominantly run longitudinally in the nerve cord (Fig. 13B). They contain numerous mitochondria, and clear and dense vesicles: The dense vesicle are between 60 and 100 nm in size; the clear vesicles are slightly larger, averaging 120 nm in size. Some deep plexus axons contain either dense or clear vesicles (Fig. 13C), or mixtures of both types.

The hyponeural nerve is located aborally in the nerve cord (Fig 14A), separated from the ectoneural nerve by a connective tissue layer. It is usually 20 to 25 um thick. The apices of the hyponeural cell bodies extend into the perihemal canal, and overlie an axon plexus and a muscle fiber layer adjacent to the connective tissue. The axons of the hyponeural nerve typically run transversally in the cord (Fig. 14B). They are larger than the axons of the ectoneural nerve, between 500 and 700 um in diameter. The axons contain small, dense vesicles that are 150 um in size. Granules that are 700 nm in diameter can be found in the axons. Vesiculated cell bodies are also found in the axon plexus (Fig.14D).

The hyponeural cell bodies are 6 to 8 um in size and contain light and dark granules, which are 400 um in diameter. Clear vesicles are also present. The rough endoplasmic reticulum of these cells is well pronounced (Figs. 14C & D).

2. Immunohistochemistry and Ultrastructure

Formalin fixed sections were subjected to immunohistochemistry as previously described. The results are shown in Figure 15. Immunoreactivity was characterized by the development of brown granules. No

immunoreactivity is observed in any of the control sections, including those treated with preimmune serum (Fig. 15A). Sections treated with antiserum A3B2 show immunoreactivity principally in the perihemal epithelium of the hemal sinus, with smaller amounts occuring in the perihemal epithelium lining the ectoneural nerve (Fig. 15B). No immunoreactivity was observed in the ectoneural nerve, the connective tissue layer, or the hyponeural nerve.

Immunoreactive granules in ten tissue sections were measured. The granules range between 1 to 4 um in diameter, though it is difficult to tell if what appears to be a large granule is not a close cluster of smaller granules. Ten granules were counted from each section, and the average granule size is 2 um.

The granules do not appear to be distributed uniformly in the perihemal epithelium, but usually occur in small groups of 2 to 3, with intervening spaces between groups (Fig.15C). The granules are found in the basal portion of the perihemal epithelium, closely apposed to the hemal sinus (Fig. 15D). No granules are found in the hemal sinus itself.

Since the tissues were unstained (except for the immunostain) histological features of the sections were

not preserved, so it is difficult to determine what cytological components are reacting with GSS antisera in the test. The fixation method chosen does not extract GSS, while more conventional fixation techniques (such as Bouin's fixative) remove GSS activity from the tissue. However, the fixation method used in this study does not give the best preservation of tissue.

Ultrastructurally the perihemal epithelium is a simple, low columnar epithelium (Fig. 16A). The cells are spatulate in outline and joined apicolaterally by intermediate junctions. They average 3 um in length. The epithelial cells contain numerous granules similar to those found elsewhere in the radial nerve cord. The nucleus is usually basal in the cell body. Beneath the cell layer is a 1 to 2 um layer composed of axons and muscle fibers. This layer overlies a prominent basal lamina averaging 200 nm in thickness. The basal lamina apposes the hemal sinus.

The size of the axons varies but there appear to be two types: Large axons range from 1 to 3 um in diameter; small axons are less than 1 um in diameter, and usually average 200 nm in size (Figs. 16B & C). The large axons, and some of the small axons, contain round, electron-dense vesicles that average 100 nm in diameter.

No such axons or vesicles are found elsewhere in the perihemal epithelium.

The large vesiculated axons commonly occur in groups of 2 or 3 (Figs. 17A & B) and there are areas where no axons can be seen. The axons are also in close proximity to the basal lamina. I could not determine whether or not the epithelial cells give rise to the axons.

Scanning electronmicrographs of the perihemal epithelium reveal that the outside (in the perihemal space) is flagellated (Fig. 17C). In cross section (Fig.17D) what appears to be an axonal tract is observed. Cell bodies within this tract, and separate from the epithelial cells, appear to give rise to the axons. The inner (hemal sinus side) of the epithelium shows a dense, fibrous matrix that was not revealed by transmission electronmicroscopy.

C. GSS IN OTHER TISSUES

1. The Axial Organ

The axial organ arises orally from the periesophageal hemal ring and runs aborally with the stone canal of the water vascular system. Aborally it

gives rise to the gastric hemal tufts, which permeate the wall of the cardiac stomach and pyloric caeca. It joins the aboral hemal ring, which gives rise to the genital hemal strands, before terminating in a dorsal sac (Fig. 22).

Histologically, the axial organ presents a complex lacunar system bound by an extensively folded perihemal epithelium (Fig. 18A). Because of the extensive convolutions, the axial organ is difficult to interpret at the light microscope level. Essentially, hemal lacunae are bound by a perihemal epithelium exposed to the perihemal canal within the axial sinus. Within the folds of the epithelium, the epithelial cells appear to secrete a mucoid substance that occludes the perihemal space in the folds.

Immunostaining of fixed sections of axial organ tissue reveals immunoreactive granules, when treated with GSS antiserum (Figs. 18C &D). Tissue treated with preimmune serum does not show immunoreactivity. The immunoreactive granules are confined to the epithelium of the axial organ, although occasional granules do occur in the axial hemal sinus (Fig. 18D). Measurements of granule size taken from 10 separate sections shows that the average size is 1 um, smaller than that

commonly found in the radial perihemal epithelium. The granules are also less abundant in the axial epithelium. They appear in clusters as found in the radial perihemal epithelium.

Ultrastructural examination of cross sections of the axial organ shows that it consists of a simple cuboidal epithelium, underlain principally by muscle fibers and a rugous basal lamina, which encloses the hemal sinus (Fig. 19A). In the perihemal space, large, membrane bound bodies are observed. The cuboidal epithelial cells average 5 um in size and contain numerous dense and clear inclusions. The are joined basolaterally by intermediate junctions; the nuclei lie basally (Fig. 19B).

Periodically, vesicular clusters are observed, and higher power electronmicrographs reveal cells (Fig. 19C) or axons (Fig. 19D) contained electron-dense, 100 nm vesicles, similar to those found in the radial perihemal epithelium. The vesiculated cells are usually 4 um long, and their axons clusters 1.5 um in size. The axons range from 200 nm to 1 um in diameter. The vesiculated cells and axons appear to correspond in size and distribution to the immunoreactive granules observed by light microscopy.

2. The Tube Feet

The tube feet arise from the oral surface in rows along the arm. The tube foot wall is composed of three layers: An outer epidermis, which is folded and made up of a tall columnar epithelium and nerve plexus; an medial connective tissue layer; and an inner coelomic epithelium primarily composed of reticulocytes oriented in circular and longitudinal directions (Fig. 20A).

Treatment of sections of tube foot wall shows no immunoreactivity in control sections (Fig. 20B) but the presence of immunoreactive granules in sections treated with GSS antiserum (Figs. 20AC&D). The immunoreactive granules are found in the inner coelomic epithelium, appearing in spaces between the reticulocytes. No immunoreactive granules are found in the outer epidermis or the connective tissue layer. The granules appear most numerous near the boundary of the connective tissue layer and the coelomic epithelium, and decrease in number toward the water vascular canal. They average 2 um in size. Ultrastructural examination of the tube foot wall failed to demonstrate any ulstrastructural correlates to the immunoreactive granules. No cells or axons containing vesicles of any kind were found in the coelomic epithelium.

3. The Ovaries and Pyloric Caeca

As well as immunological controls, sections of ovarian wall and pyloric caeca were subjected to immunohistochemistry as tissue controls. These tissues do not contain GSS (Kanatani, 1973). No immunoreactivity is found in either the ovarian wall or sections of the pyloric caeca.

The ovarian wall was examined ultrastructurally (Fig. 21A).. The paired ovaries are suspended in the major coelom of each ray. Each ovary consists of a central racus from which branch lobulate acini. The ovarian wall consists of an outer coelomic (visceral) epithelium, composed of a single layer of cuboidal cells 5 um in size. They appose a basal lamina that overlies a connective tissue layer of collagen, usually 2 um thick. This is followed by a second basal lamina, which apposes a cell and muscle fiber layer. A third basal lamina separates this layer from the genital hemal sinus, which is bound on the other side by a fourth basal lamina. The fourth basal lamina apposes the germinal epithelium of the ovary, which gives rise to the oocytes.

Vesiculated axons are rare in the ovary and confined to the coelomic (visceral) epithelium, the

outermost layer (Figs. 21B&C). The axons are 1 to 1.5 um in diameter, and contain electron-dense vesicles that range from 100 to 200 um in size.

FIGURE 11

A. Light micrograph of a cross section of the radial nerve cord stained with Richardson's stain. Connective tissue, *c*, ectoneural nerve, *e*, hemal sinus, *h*, hyponeural nerve, *l*, and the perihemal canal, *p*. Bar = 100 um.

B. Light micrograph of a cross section of the oral portion of the ectoneural nerve stained with Richardson's stain. Deep plexus, *d*, epithelial layer, *e*, glycocalyx, *g*, and superficial plexus, *s*. Bar = 50 um.

C. Light micrograph of a cross section of the aboral portion of the ectoneural nerve stained with Richardson's stain. Connective tissue layer, *c*, and the hyponeural nerve, *l*. Bar = 50 um.

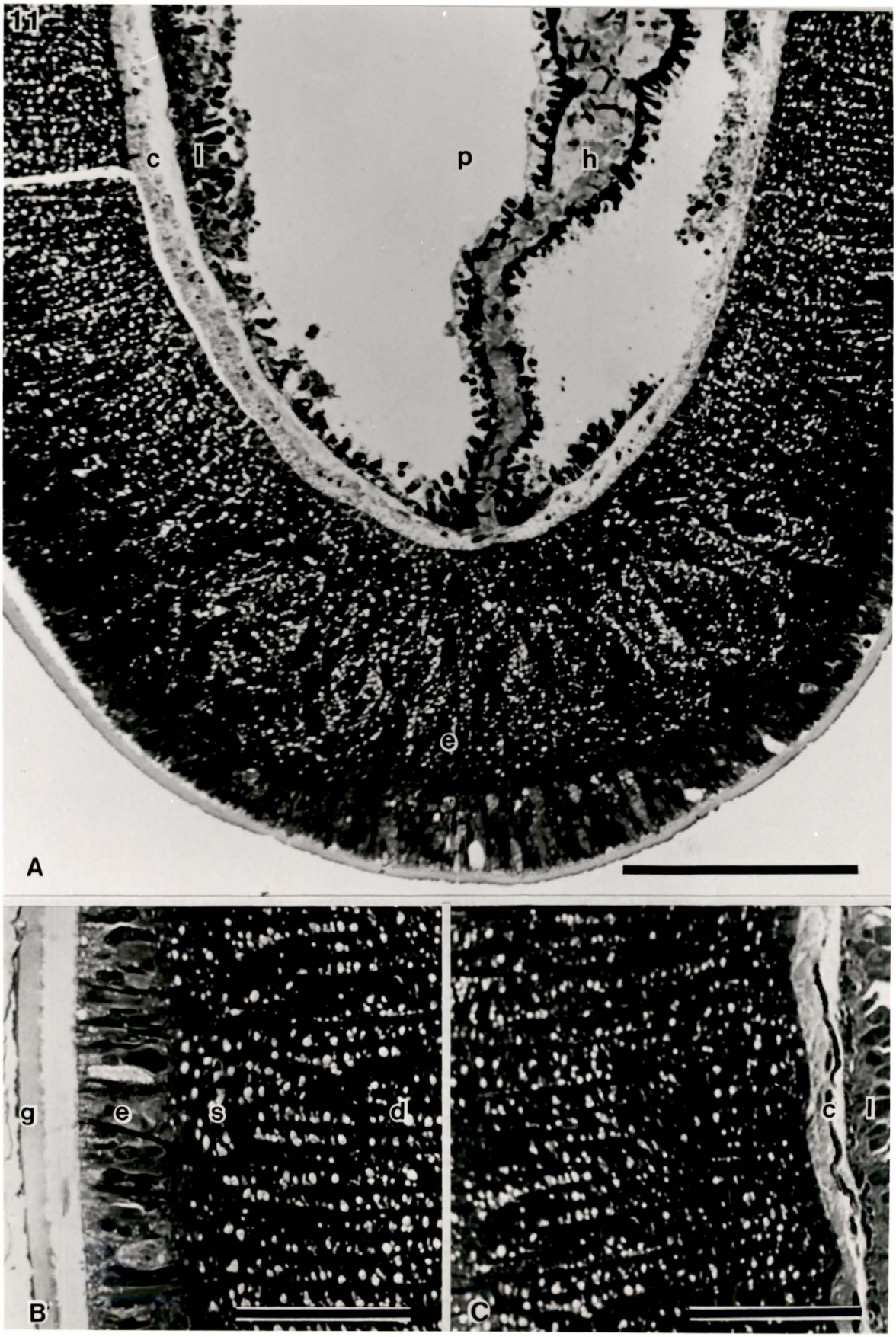
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FIGURE 12

A. Transmission electronmicrograph of a cross section of the ectoneural glycocalyx. A single cilium, *c*, and the apical portion of an epithelial cell, *e*, are shown. Microvillus, *m*. Bar = 2 um.

B. Transmission electronmicrograph of a mucus gland cell in cross section. Glycocalyx, *g*, mucus granule, *m*, and mucus gland cell nucleus, *n*. Bar = 5 um.

C. Transmission electronmicrograph of a supporting cell in cross section. Supporting fiber, *f*, granule, *g*, and nucleus, *n*. Bar = 1 um.

D. Transmission electronmicrograph of a sensory cell body in cross section. Golgi apparatus, *g*, and nucleus, *n*. Bar = 1 um.

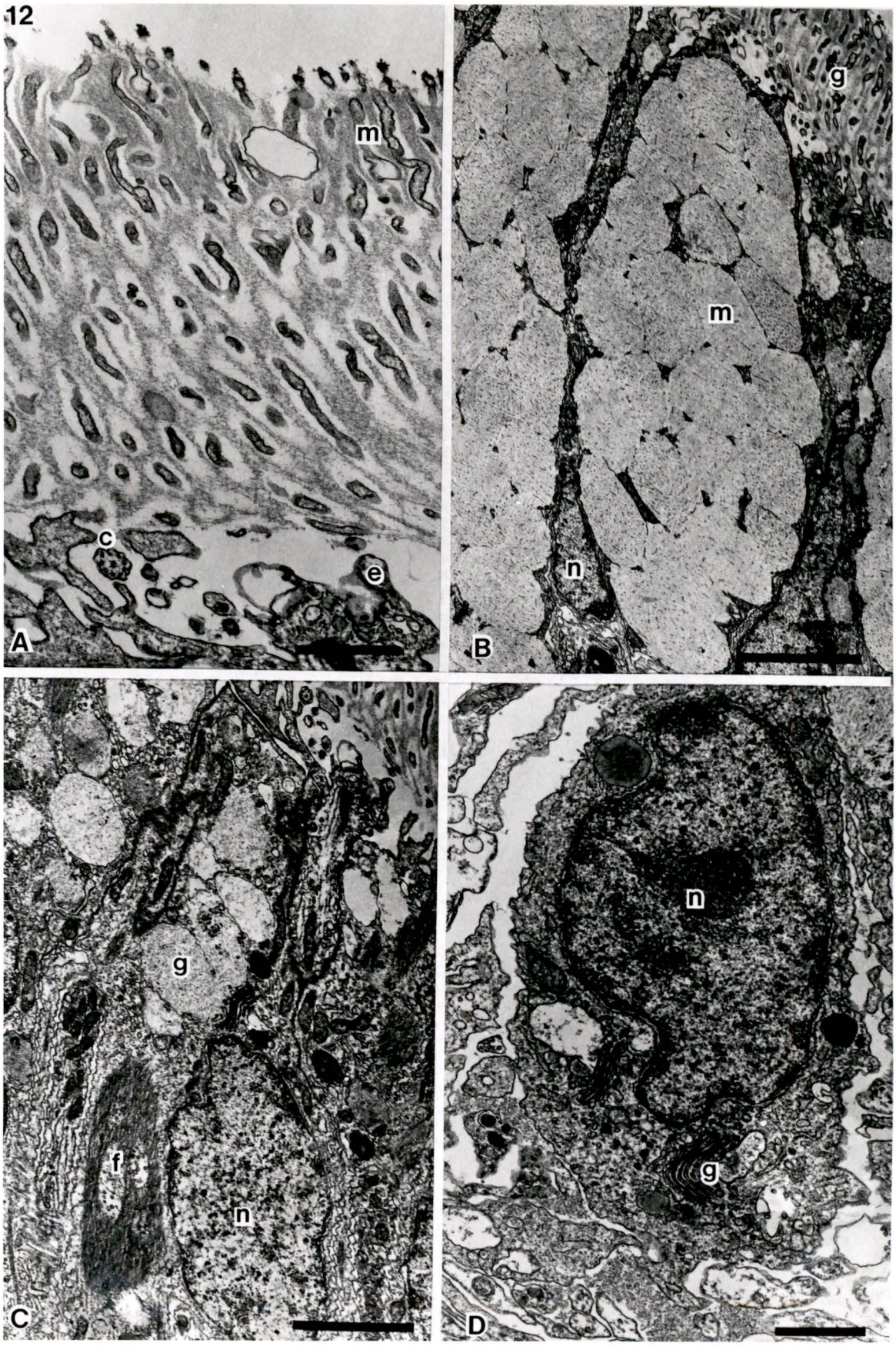
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FIGURE 13

A. Electron micrograph of the junction of the epithelial layer with the superficial plexus in cross section. Cell body of a supporting cell, *c*, supporting fiber arising from the supporting cell, *f*, granule in the supporting cell, *g*, and the axons of the superficial plexus, *s*. Bar = 2 um.

B. Electronmicrograph of the axons in a cross section of the deep plexus. Mitochondrion, *m*. Bar = 1 um.

C. Higher power electronmicrograph of the deep plexus axons showing clear and dense vesicles. Mitochondrion, *m*. Bar = 1 um.

D. Electronmicrograph of a supporting fiber, *f*, in the deep plexus. Fiber granule, *g*. Bar = 1 um.

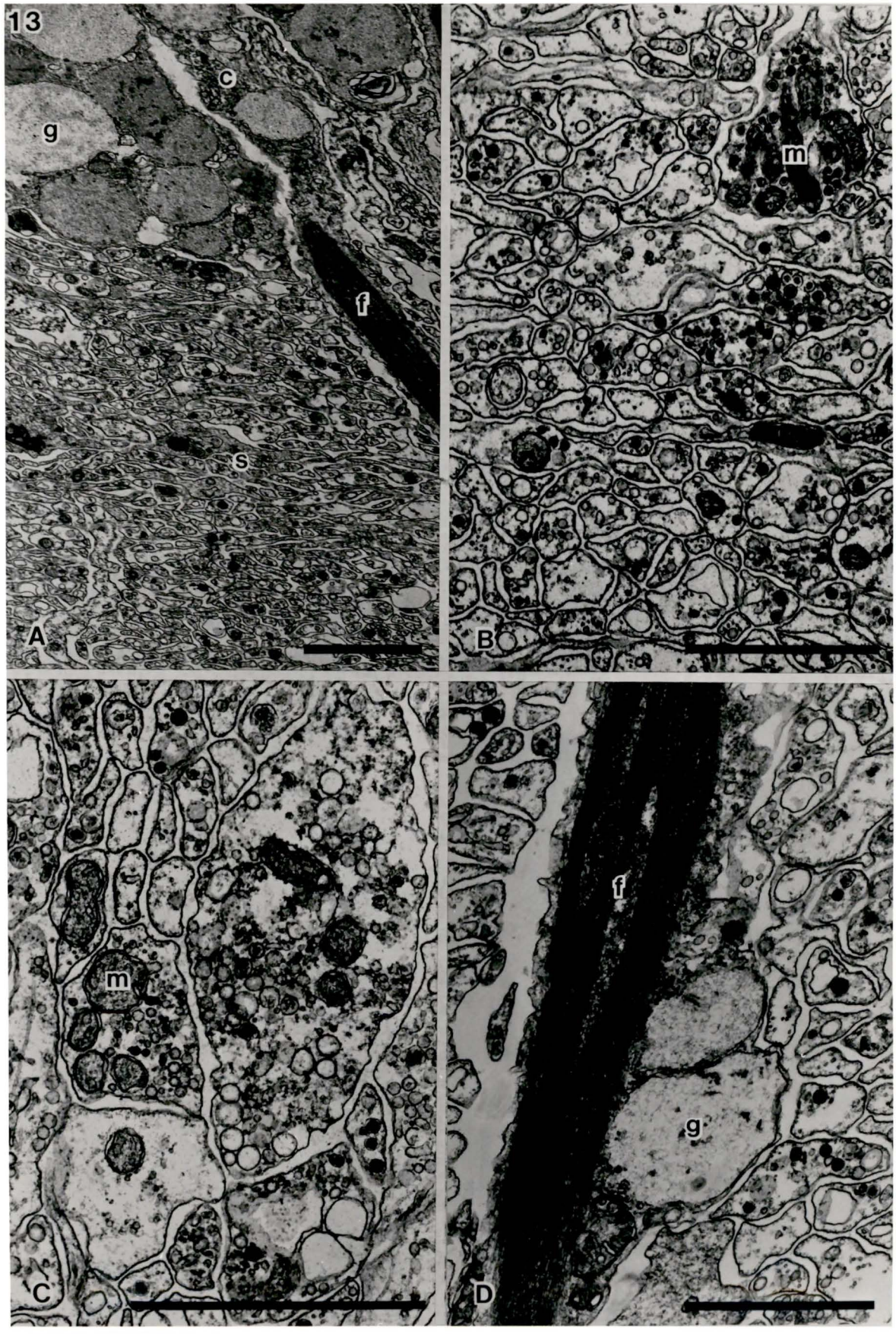
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FIGURE 14

A. Light micrograph of the radial nerve cord showing the hyponeural nerve, *l*, in cross section stained with Richardson's stain. Connective tissue layer, *c*, ectoneural nerve, *e*, and the perihemal space, *p*. Bar = 100 um.

B. Higher power light micrograph of the hyponeural nerve in cross section. Axonal plexus, *a*, cell body, *c*, and muscle fibers, *m*. Bar = 50 um.

C. Transmission electronmicrograph of the hyponeural nerve in cross section. Transverse axon, *a*, granule, *g*, cell nucleus, n vesiculated cell, *v*. Bar = 5 um.

D. Transmission electronmicrograph of a hyponeural cell body in cross section. Clear granule, *c*, dark granule, *g*, cell nucleus, *n*, small clear vesicle, *v*, and rough endoplasmic reticulum, *r*. Bar = 1 um.

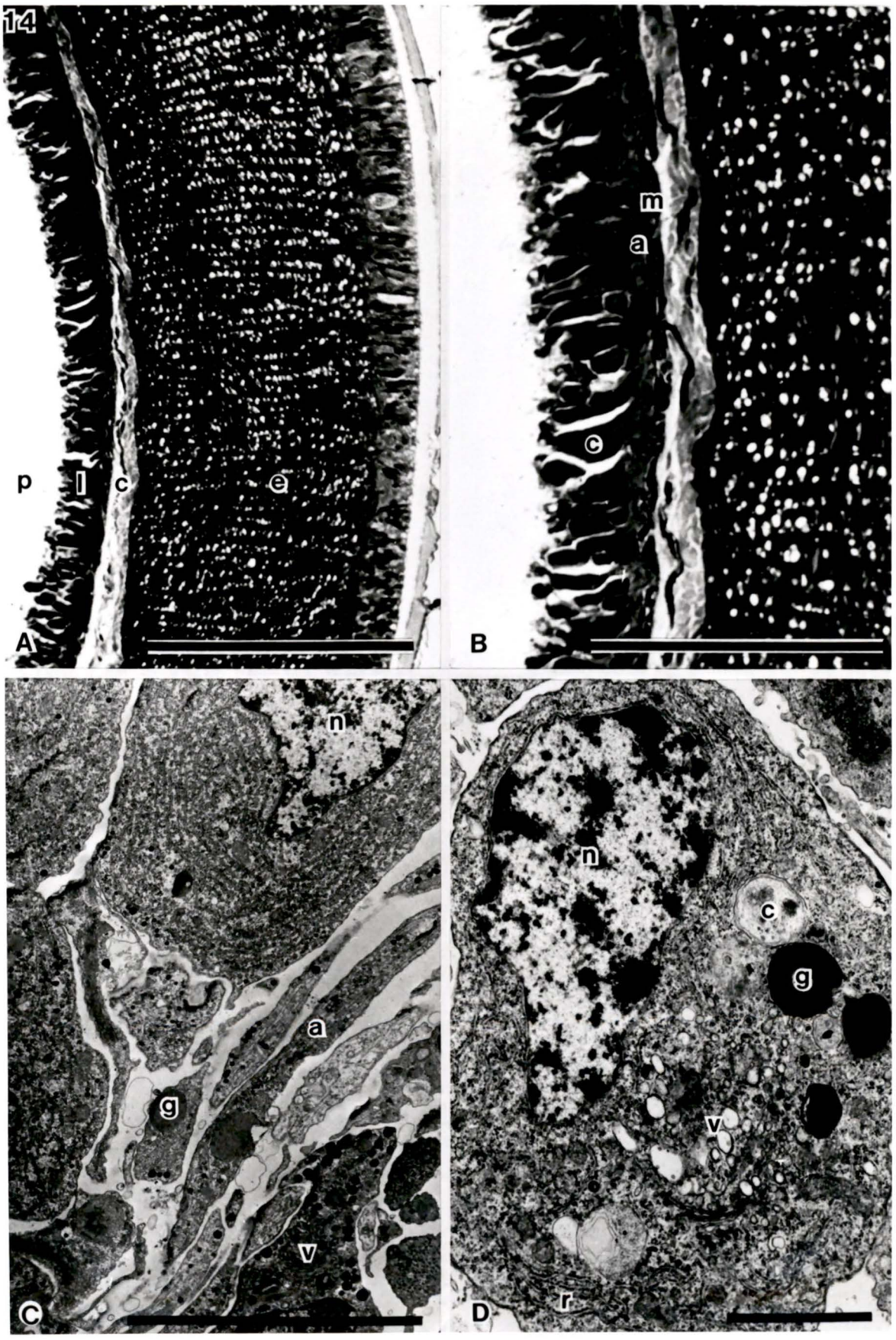
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FIGURE 15

A. Light micrograph of the radial nerve cord in cross section. The tissue was unstained but treated with preimmune serum, and does not show immunoreactivity. Ectoneural nerve, *e*, hemal sinus, *h*, hyponeural nerve, *l*, and the perihemal epithelium, *p*. Bar = 100 um.

B. Light micrograph of the radial nerve cord in cross section, treated with antiserum A3B2. Immunnoreactivity occurs as granules, *g*, and are found only in the perihemal epithelium. Ectoneural nerve, *e*, hemal sinus, *h*, and the hyponeural nerve, *l*. Bar = 100 um.

C. Higher power light micrograph of the perihemal epithelium, *p*, and the hyponeural nerve, *l*, in cross section. Immunnoreactive granules, *g*, appear in the perihemal epithelium and are absent in the adjacent hyponeural nerve. Hemal sinus, *h*. Bar = 25 um.

D. High power light micrograph of the perihemal epithelium in cross section. Immunoreactive granules, *g*, appear in the basal portion of the epithelium. Hemal sinus, *h*. Bar = 5 um.

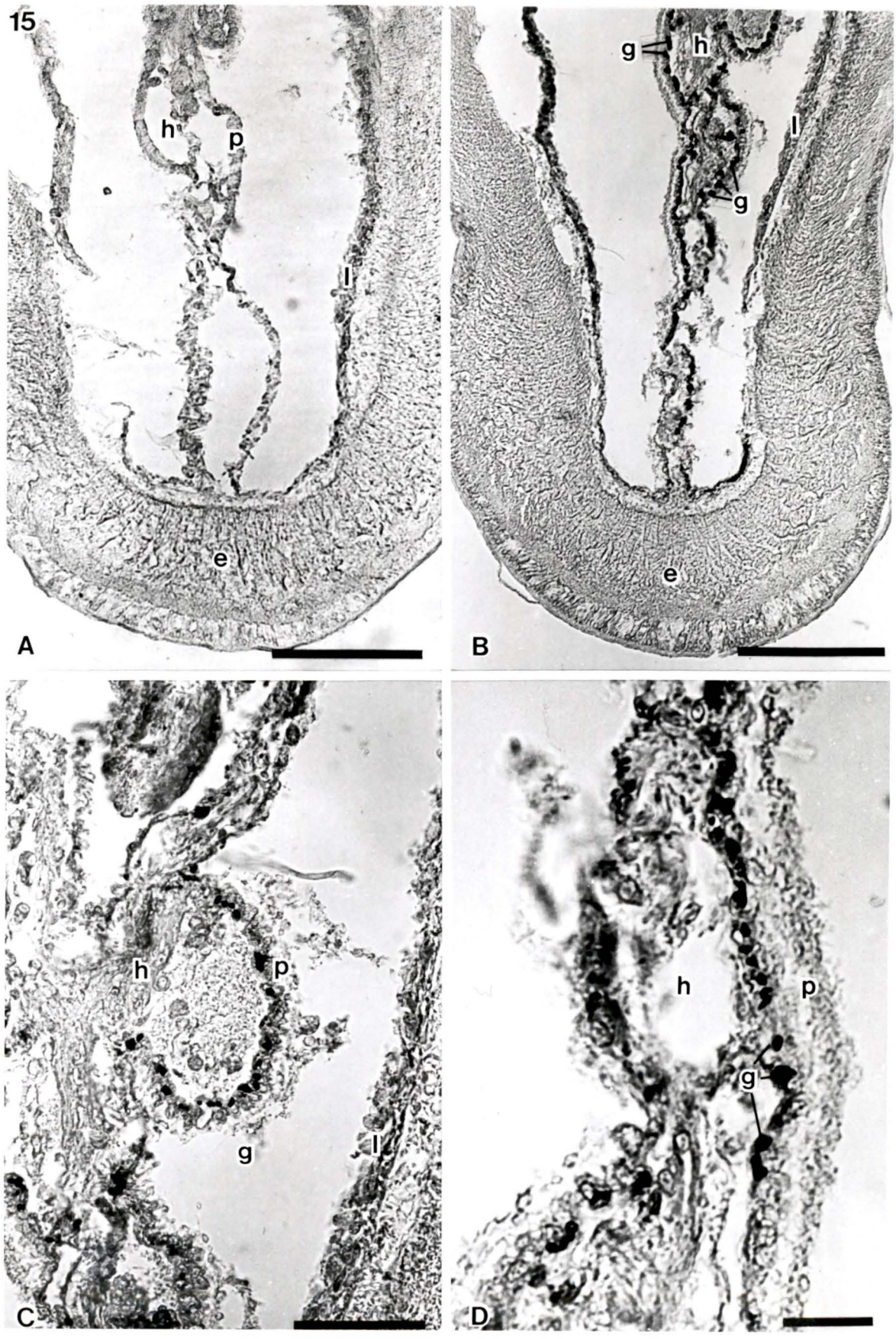
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FIGURE 16

A. Transmission electronmicrograph of a cross section of the perihemal epithelium. Axons, *a*, basal lamina, *b*, epithelial cell body with numerous granules, *c*, intermediate junctions between cells, *j*, hemal sinus, *h*, muscle fiber, *m*, epithelial cell nucleus, *n*, and the perihemal canal, *p*. Bar = 1 um.

B. Electronmicrograph of the lower portion of the perihemal epithelium in longitudinal section. Vesiculated axon, *a*, basal lamina, *b*, granules, *g*, hemal sinus, *h*, and a muscle fibre, *m*. Bar = 5 um.

C. High power electronmicrograph of small axons containing electron-dense vesicles, *v*, in the lower portion of the perihemal epithelium. Muscle fiber, *m*. Bar = 1 um.

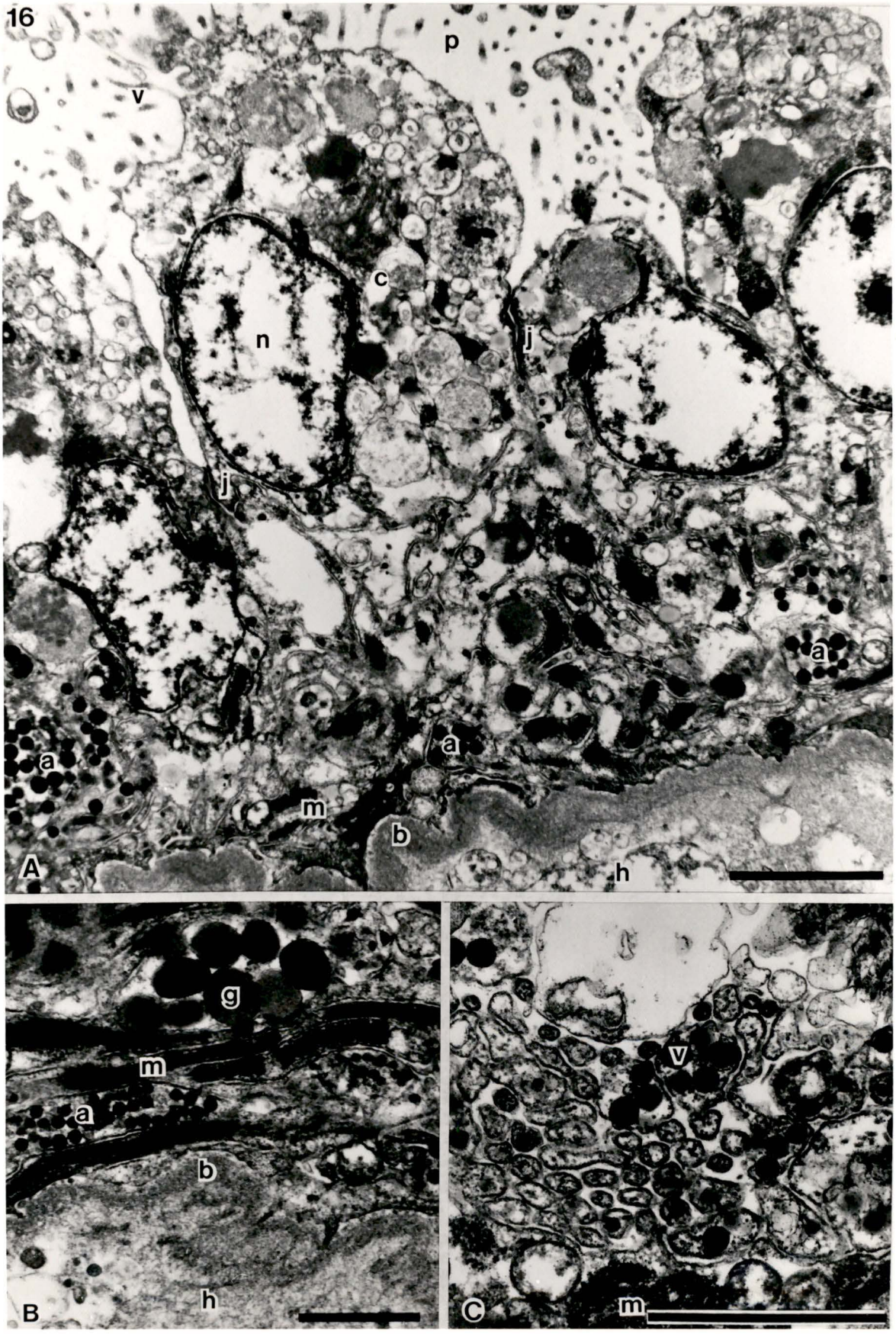
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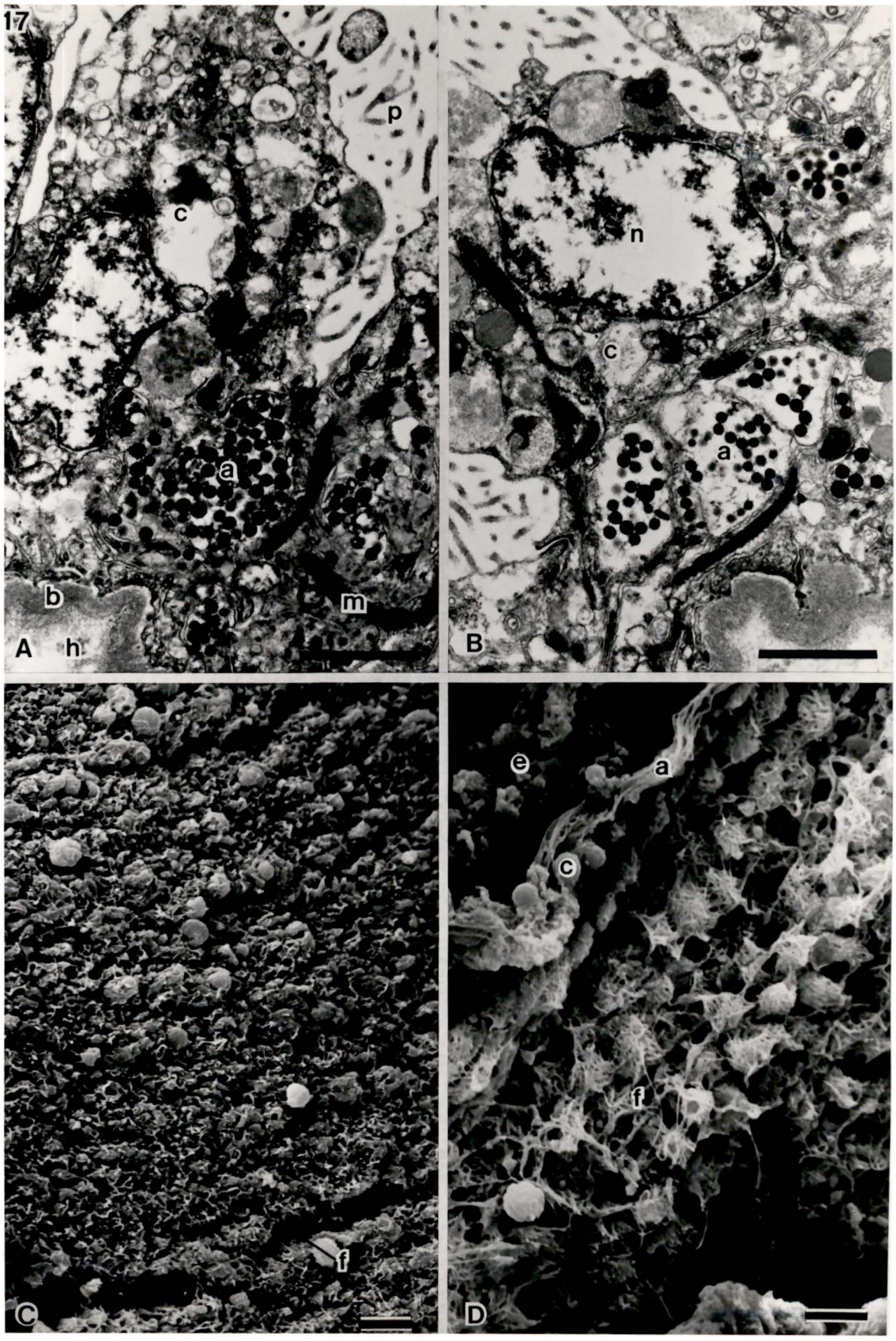
FIGURE 17

A. Electronmicrograph of a cross section through the perihemal epithelium showing large vesiculated axons, *a*. Basal lamina, *b*, epithelial cell body, *c*, hemal sinus, *h*, muscle fiber, *m*, and the perihemal canal, *p*. Bar = 2 um.

B. Electronmicrograph of a cross section through the perihemal epithelium showing a cluster of large vesiculated axons, *a*, in the basal portion. Epithelial cell body, *c*, epithelial cell nucleus, *n*. Bar = 2 um.

C. Scanning electronmicrograph of the outside of the perihemal epithelium showing flagella arising apically from the cells, *f*. Bar = 10 um.

D. Scanning electronmicrograph of the inside of the perihemal epithelium showing the epithelial cell bodies, *e*, and cell bodies, *c* that appear to give rise to an axon tract, *a*. Fibrous layer beneath the cell bodies, *f*. Bar = 10 um.


17
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b
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C
f
D

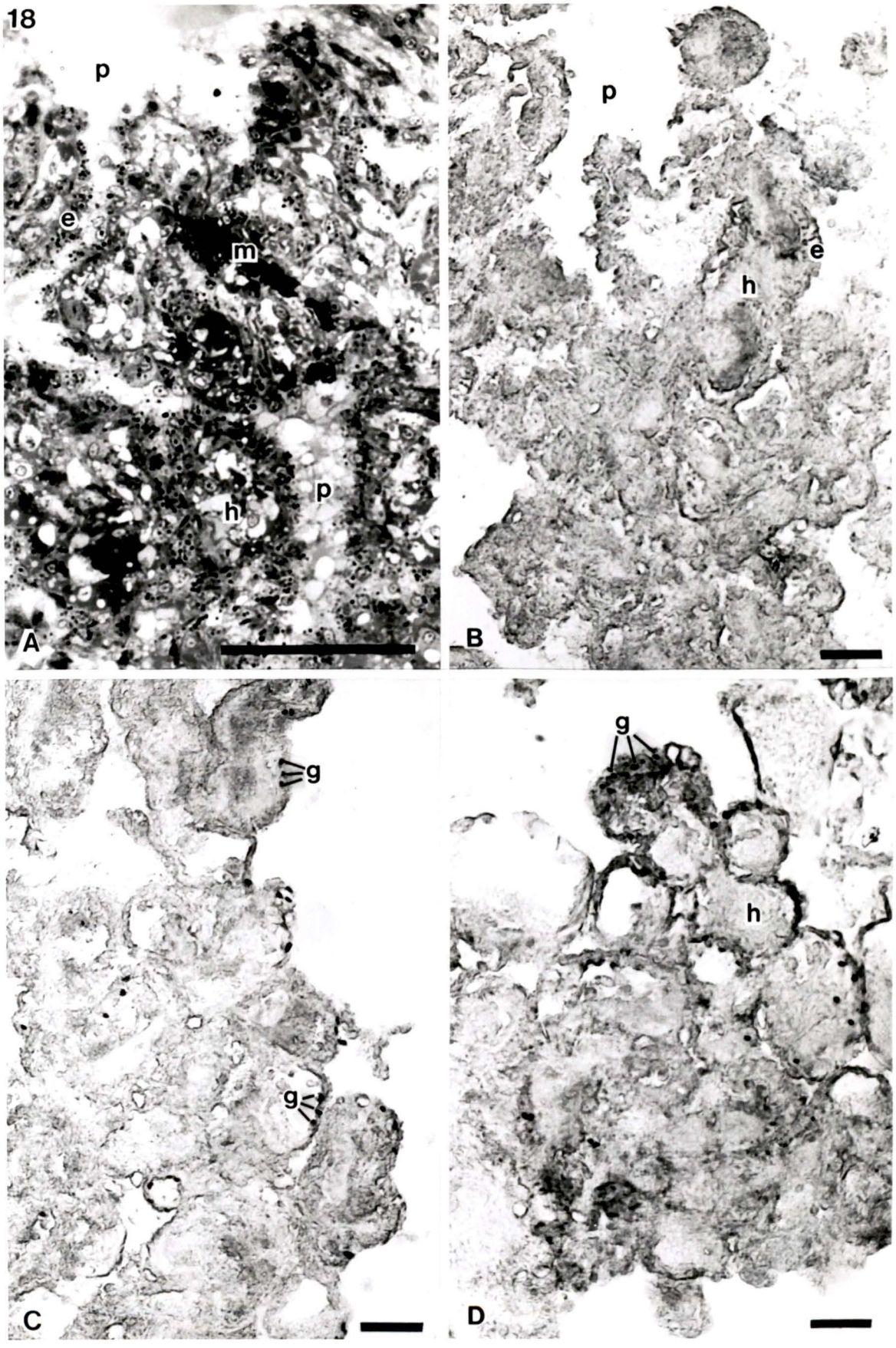
FIGURE 18

A. Light micrograph of a cross section of the axial organ stained with Richardson's stain. Epithelial layer, *e*, hemal sinus, *h*, muscle fiber, *m*, and the perihemal canal, *p*. Bar = 50 um.

B. Light micrograph of the axial organ in cross section. The tissue was unstained but was treated with preimmune serum, and shows a lack of immunoreactivity. Axial epithelium, *e*, axial hemal sinus, *h*, and the perihemal space, *p*. Bar = 10 um.

C. Light micrograph of the axial organ in cross section. The tissue was treated with antiserum A3B2 and shows immunoreactivity in the form of granules, *g*, in the epithelial layer. Bar = 10 um.

D. Light micrograph of another region of the axial organ treated with antiserum A3B2, showing immunoreactive granules, *g*, in the epithelium. Hemal sinus, *h*. Bar = 10 um.


18
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B
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C
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h
D

FIGURE 19

A. Low power electronmicrograph of the axial organ epithelium in cross section. Basal lamina, *b*, epithelial cell body, *c*, axial hemal sinus, *h*, muscle fiber, *m*, and a vesicular cluster, *v*. Bar = 5 um.

B. Higher power electronmicrograph of the axial epithelium in cross section. Basal lamina, *b*, epithelial cell granule, *g*, axial hemal sinus, *h*, intermediate junction, *j*, epithelial cell nucleus, *n*, and the perihemal canal, *p*. Bar = 2 um.

C. Electronmicrograph of a portion of the axial epithelium containing a vesiculated cell, *c*. Basal lamina, *b*, axial hemal sinus, *h*, nucleus, *n*, and electron-dense vesicles, *v*. Bar = 2 um.

D. Electronmicrograph of axons, *a*, in the axial epithelium. Basal lamina, *b*, epithelial cell granule, *g*, hemal sinus, *h*, and a muscle fiber, *m*. Bar = 1 um.

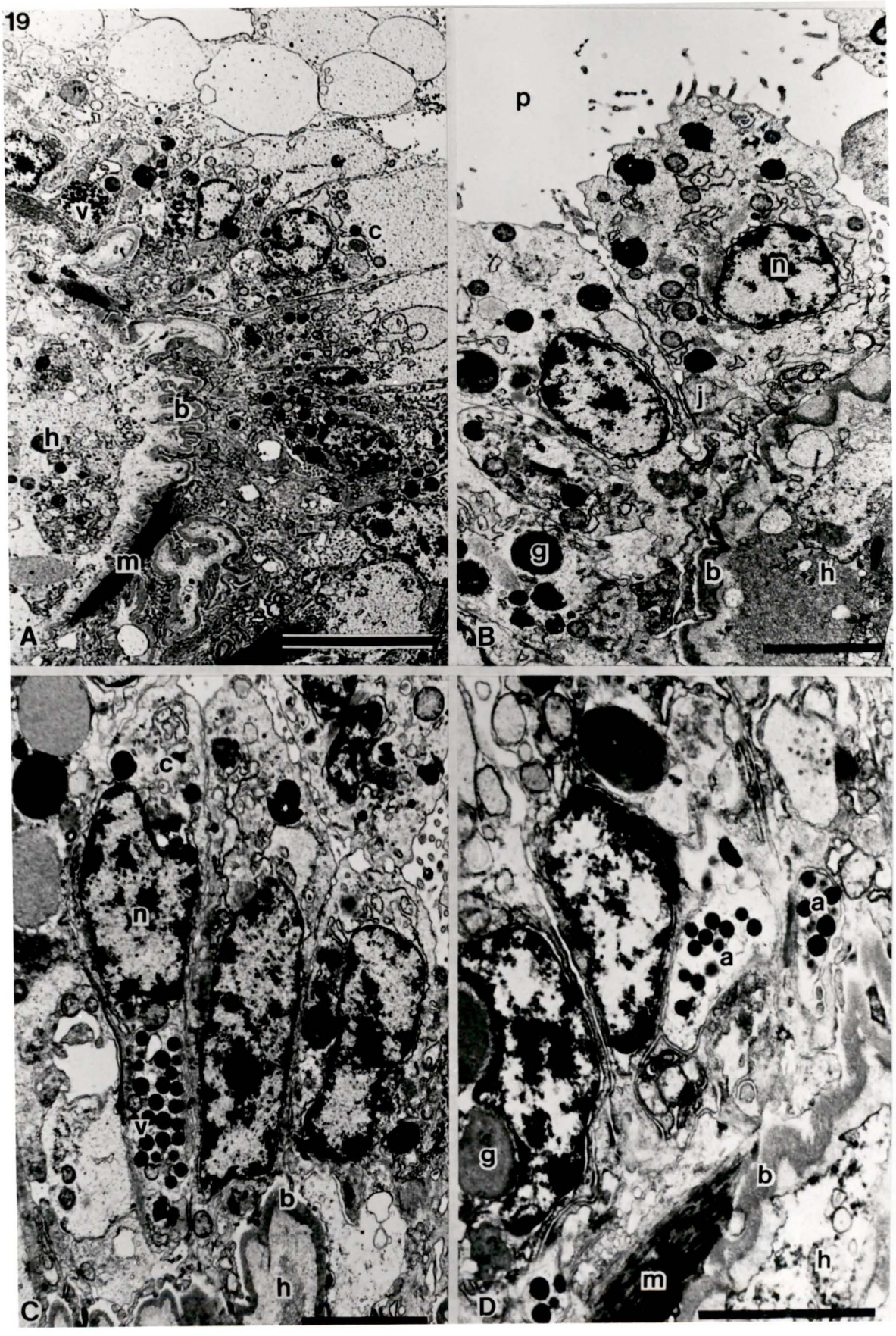
19
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FIGURE 20

A. Light micrograph of the tube foot wall in longitudinal section stained with Richardson's stain, showing the outer epithelium, *o*, connective tissue layer, *c*, and the inner coelomic epithelium, *i*. Bar = 100 um.

B. Light micrograph of the tube foot wall in longitudinal section treated with preimmune serum. No immunoreactivity is observed. Connective tissue layer, *c*, inner coelomic epithelium, *i*, and outer epithelium, *o*. Bar = 20 um.

C. Light micrograph of a longitudinal section of the tube foot wall treated with antiserum A3B2. Immunoreactivity is observed as dark granules, *g*, in the inner coelomic epithelium, *i*. Connective tissue layer, *c*, outer epithelium, *o*. Bar = 20 um.

D. Light micrograph of another longitudinal section of the tube foot wall treated with antiserum A3B2. Immunoreactivity occurs as dark granules, *g*, near the connective tissue layer, *c*. Inner coelomic epithelium, *i*, and outer epithelium, *o*. Bar = 20 um.

20

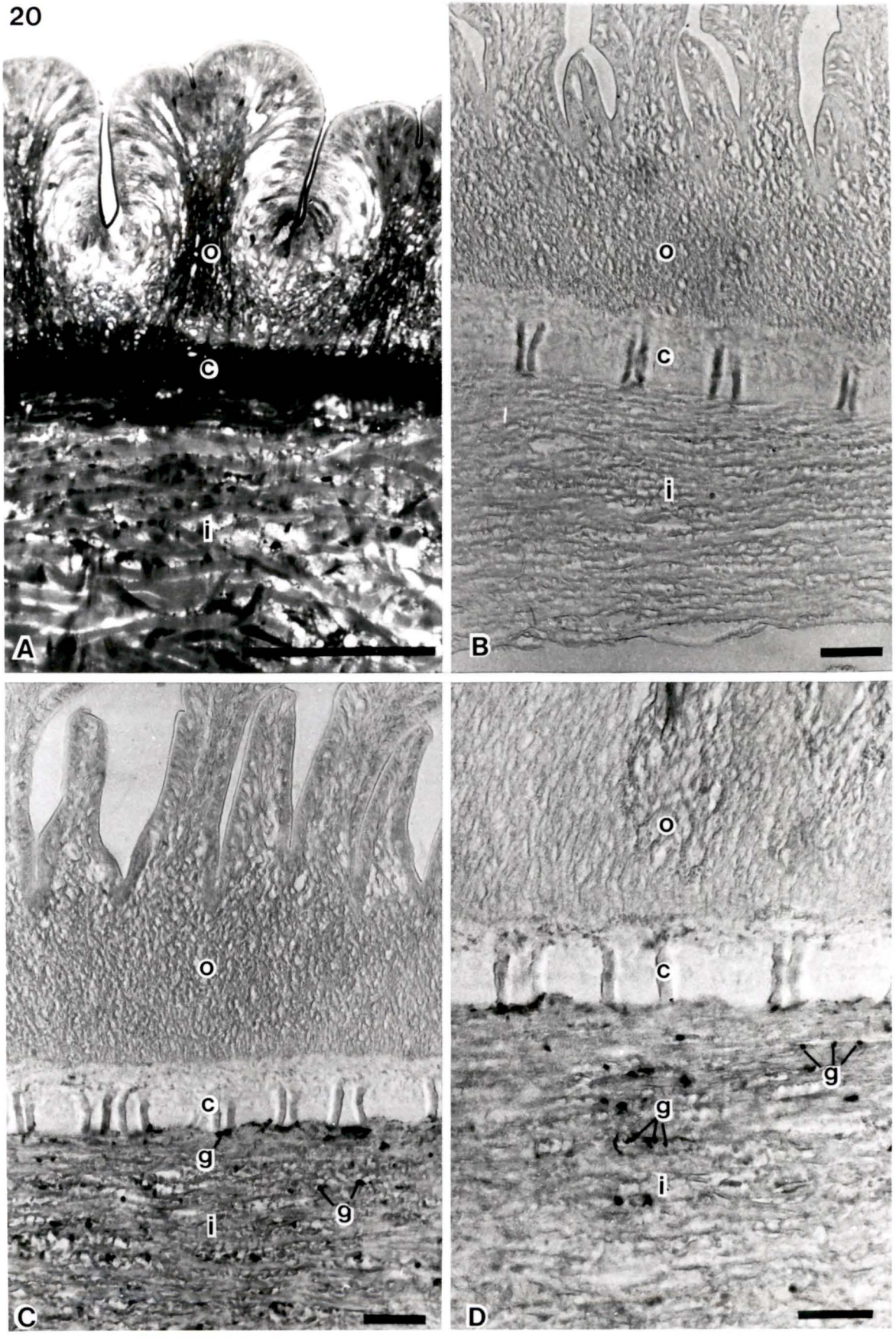
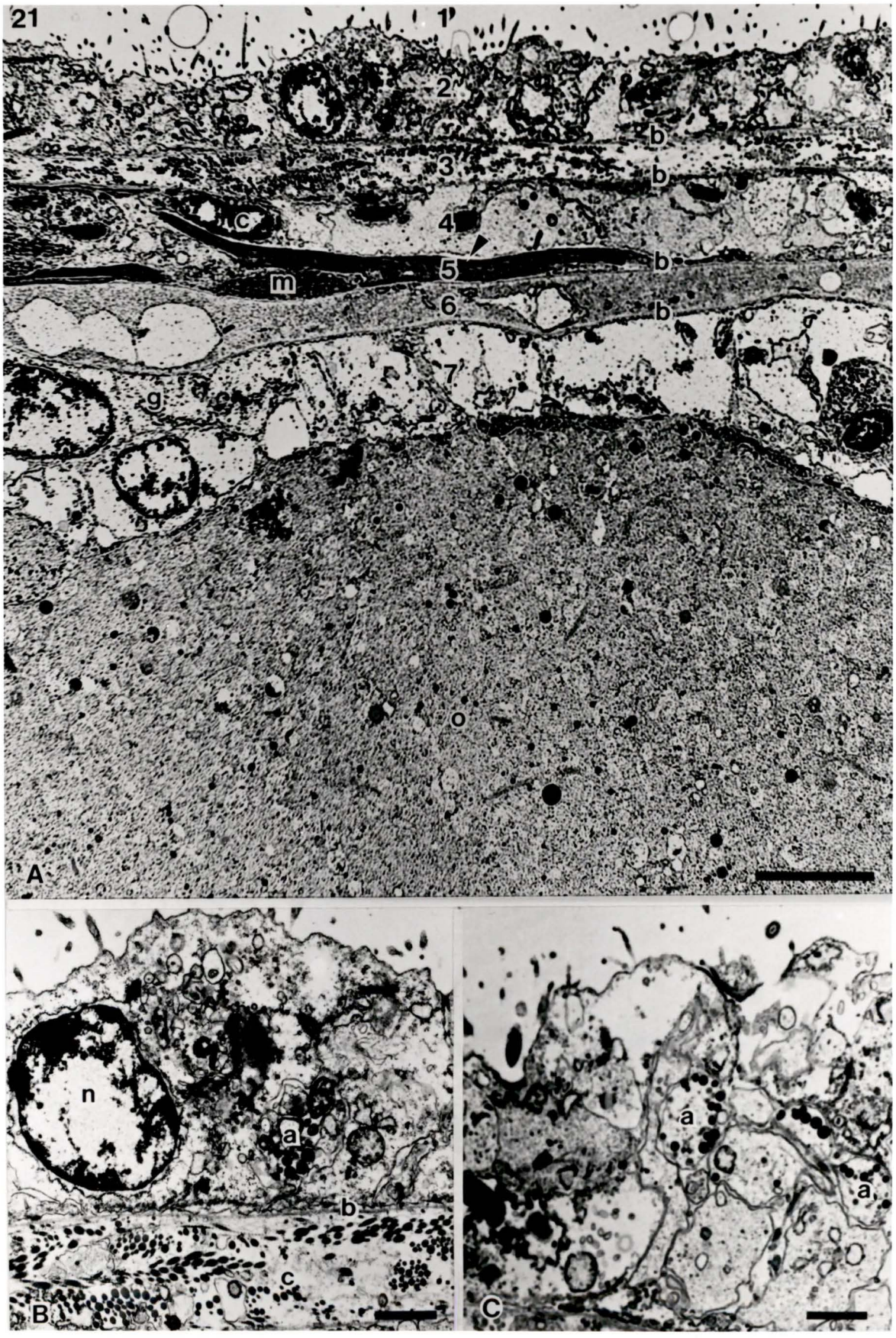


FIGURE 21

A. Electronmicrograph of the ovarian wall in cross section, showing the coelom, *1*, coelomic epithelium, *2*, connective tissue layer, *3*, cell and muscle fiber layer, *4* and *5*, genital hemal sinus, *6*, and the germinal epithelium, *7*. Basal laminae, *b*, epithelial cell, *c*, germinal epithelial cell, *g*, muscle fiber, *m*, and a portion of an oocyte, *o*.

B. Electronmicrograph of a vesiculated axon in the coelomic epithelium. Axon, *a*, basal lamina, *b*, and epithelial cell nucleus, *n*. Bar = 1

C. Another region of the coelomic epithelium showing small axons with a few dense vesicles. Axons, *a*. Bar = 1 um.


21
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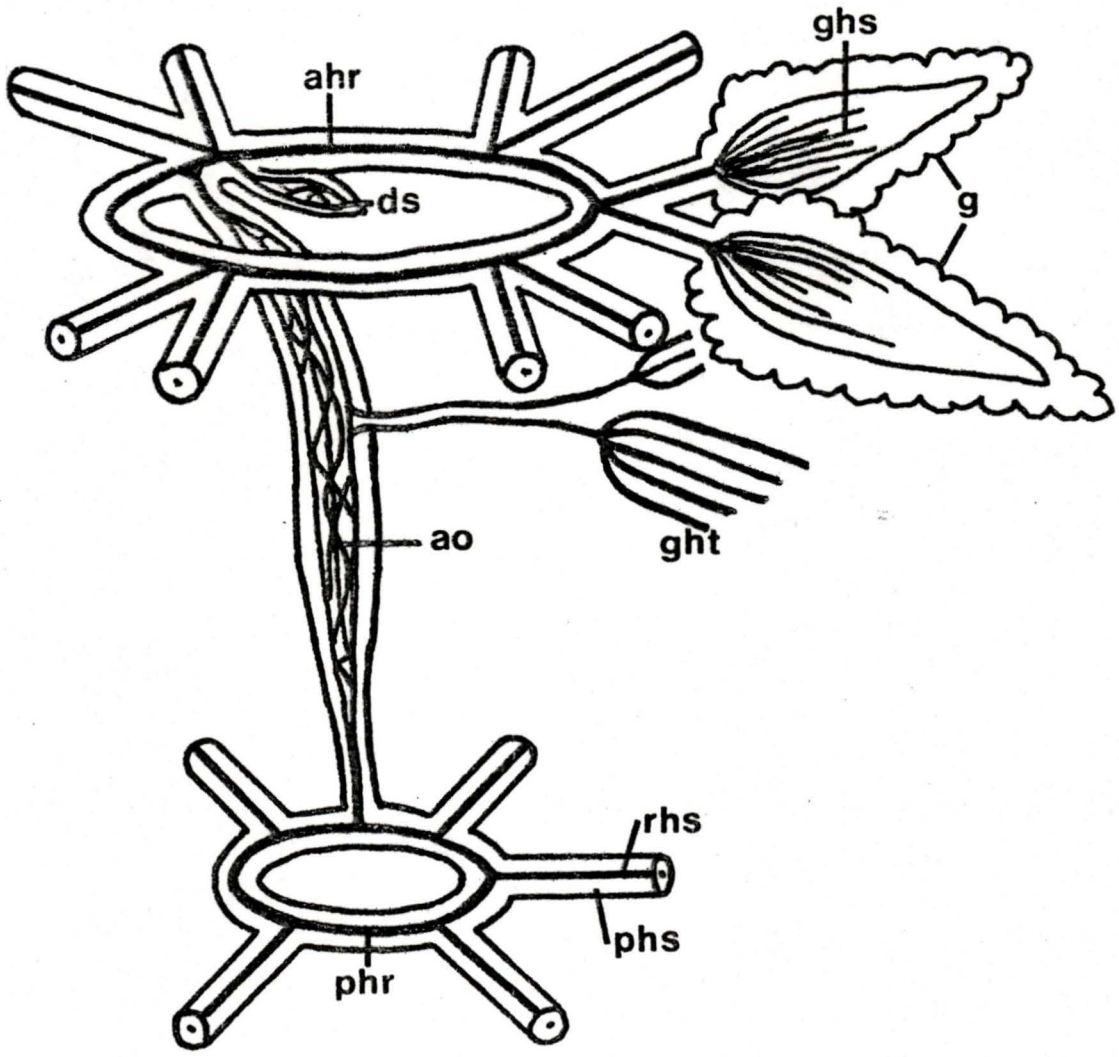
C

FIGURE 22

Schematic drawing showing the main components of the asteroid hemal system. Aboral hemal ring, *ahr*, axial organ, *ao*, dorsal sac, *ds*, gonads, *g*, genital hemal strands, *ghs*, gastric hemal tufts, *ght*, periesophageal hemal ring, *phr*, perihemal space (canal), *phs*, and the radial hemal sinus, *rhs*.


ghs
ahr
ds
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phs
phr

D. DISCUSSION

1. Localization of GSS

Previous studies on the radial nerve cords relied on histochemical techniques to correlate the presence of neurosecretory substances with the localization of GSS. Unger (1962) investigated the radial nerve cords of *Marthasterias glacialis* for neurosecretory substances and found that numerous bipolar and multipolar ganglion cells in the ectoneural nerve contained granules that stained with Gomori's chrome hematoxylin-phloxin and Gabe's paraldehyde-fuchsin techniques. He also found granules in the supporting cell layer and along the supporting fibers that stained with paraldehyde-fuchsin. He proposed that GSS was in the fuchsinophilic granules of the supporting cells and migrated along their fibers through the plexus.

Since these techniques could not distinguish GSS from other neurosecretory substances, Unger's proposition was based on the assumption that GSS was the only neurosecretory substance in the nerve; however, the granules of the supporting cells were not stained by Gomori's method, yet the granules of the ganglion cells were, indicating a difference between the contents of the two cell types, and the possibility of more than one neurosecretory substance in the ectoneural nerve. Given this consideration, it is unlikely that GSS could be localized by the methods used.

Imlay and Chaet (1965) used numerous histochemical tests to localize a neurosecretory substance in the radial nerve cords of *Asterias forbesi*. They found 1 to 2 um neurosecretory granules in three distinct areas of the nerve cord; The supporting cell (epidermal) layer of the ectoneural nerve, the apical portions of cells in the coelomic epithelium of both the ectoneural and hyponeural nerves, and the apices of cells in the coelomic (perihemal) epithelium of the hemal sinus. Knowing GSS to be a peptide, they used a variety of specific staining tests to elucidate the chemical nature of the neurosecretory substance in the granules. The ninhydrin-Schiff reaction indicated that the substance was proteinaceous. Tests involving the performic acid-alcian blue reaction indicated the presence of cystine, and tests for other amino acids suggested an absence of arginine, catecholamines, indoles, tryptophan and tyrosine. They found that ester sulfates and nucleoproteins were present in the substance by using acidified methylene blue and alcian blue. They correlated the presence of this proteinaceous neurosecretory substance with the presence of GSS in the radial nerve cord.

The chemical nature of GSS, as determined by Kanatani *et al.*, (1971) indicates that the neurosecretory substance identified by Imlay and Chaet might not be GSS but another neurosecretory substance. The amino acid composition of GSS

did not contain cystine, nor was there report of the the presence of ester sulfates or nucleoproteins (Kanatani & Ohguri, 1971), as indicated by Imlay and Chaet's alcian blue test. The alcian blue test is also an indicator of the presence of mucopolysaccharides (Humason, 1972). Isolation and bioassay of the putative GSS granules was not reported, so it was not established that the neurosecretory substance was GSS. Imlay and Chaet's findings do suggest, however, that more than one neurosecretory substance is found in the radial nerve cord.

A different approach was carried out by deAngelis and coworkers (1972), who isolated granules containing GSS activity from homogenates of radial nerve cord in *Asterina pectinifera*. Homogenates of radial nerve cord were centrifuged differentially and the GSS active fractions subjected to sucrose density gradient ultracentrifugation. Electronmicroscopic examination of the active fraction showed it contained granules that were similar to those found in the supporting cells of the ectoneural nerve. They next extirpated a portion of the supporting cell tissue of the ectoneural nerve in *Marthasterias glacialis*, and reported its GSS activity higher than the remainder of the nerve cord. Based on these findings, they suggested that GSS was produced by the supporting cells of the ectoneural nerve, and that these cells act physiologically as neurosecretory cells.

Although a GSS active fraction consisting of various size granules was isolated, it was not clear whether the bioassay reflected activity of the substances in the granules, or solubilized GSS that may have been present in the supernatant of the active fraction. The criteria by which the granules of the isolated fraction and those found in the supporting cells were compared was not reported, and it was difficult to form a correlation based on size because of the osmotic effect the sucrose gradient had on the isolated granules, and the effect of the fixation on the cellular granules (deAngelis *et al.*, 1972). Granules of various sizes are found throughout the radial nerve cord and are not exclusive to the supporting cells. Also, since whole nerve cords were homogenized, it was not clear that the GSS active granules came from the supporting cells, or some other region of the cord.

The extirpated supporting cells were reported to have greater GSS activity relative to the remainder of the nerve, however the published data indicated the opposite. It was probable that this was an editorial error. However, removal of a certain group of cells from the ectoneural nerve would be difficult because there are no distinct boundaries between the regions of the ectoneural nerve, nor do supporting cells exclusively comprise that portion of the nerve removed.

Application of immunohistochemical techniques specific to GSS enables direct localization of GSS, *in situ*, in tissues of *P. helianthoides*. Antibodies specific for GSS bind to native GSS molecules in the tissues. These binding sites can be subsequently visualized using the indirect immunoperoxidase technique. Briefly, this involves binding a second antibody to the GSS antibodies in the tissue. This second antibody is conjugated to horseradish peroxidase, an enzyme that catalyzes the reaction of hydrogen peroxide with an electron donor; in this case, 3, 3' Diaminobenzidene (DAB). The reduction product of DAB forms a coloured compound that precipitates from solution at the site of the antibody reaction, thus localizing the antigen (Van Noorden and Polak, 1983). The advantage of this method over other histochemical techniques for localizing neurosecretory substances is that this test is specific for GSS and does not rely on inference.

The immunoreactive granules observed in GSS-positive tissue indicate the presence of GSS, and so will be refered to as GSS granules. The results show that GSS is localized primarily in the perihemal epithelium of the radial hemal sinus, with smaller amounts occuring in the coelomic epithelium of the radial nerve cord. The staining is discrete, suggesting that GSS may be packaged or in some way bound in distinct areas, and is not spread diffusely

throught the tissue. Since GSS is soluble in aqueous solutions, unbound GSS would be removed during the staining technique. Therefore, GSS is probably contained within membrane-bound granules or vesicles. No GSS granules (or any other immunoreactivity) are found in the other components of the radial nerve cord including the ectoneural nerve, which was previously believed to contain GSS secreting cells (Unger, 1962; Imlay and Chaet, 1965; deAngelis *et al.*, 1972; Kanatani, 1973).

GSS granules are confined to the basal third of the perihemal epithelium, between the epithelial cell bodies and the basal lamina. No GSS granules are found in the cells, or in the hemal sinus.

Assuming that GSS is membrane bound, the perihemal epithelium was investigated ultrastructurally to determine if there were any components of this region that could contain GSS. The basal third of the perihemal epithelium lies directly beneath the epithelial cells and consists of a mixture of vesiculated axons and muscle fibers, which lie adjacent to the basal lamina. All of the axons observed contain clusters of electron-dense vesicles that have an average diameter of 100 nm. The axons themselves vary in size from 0.5 um to 3 um, the average diameter being 2 um. The average diameter of the axons correlates with the

average diameter of the GSS granules observed by light microscopy.

The vesiculated axons are not distributed uniformly throughout the perihemal epithelium, but commonly occur in groups of two or three with intervening spaces between groups. In some cases, numerous axons are found together in discrete areas, suggesting a plexus. The GSS granules observed by light microscopy also show this distribution; groups of two or three granules are usually observed, but there are areas where their density is great enough to form a solid band of stain. The correlation between the location, size and distribution of the GSS granules with the vesiculated axons suggests that GSS may be localized in the vesicles of the axons.

Vesiculated axons in perihemal epithelia were described by Doyle (1967) in the holothurian, *Cucumaria*, but no such studies have been made on asteroid systems and, prior to this study, it was not known that vesiculated axons existed in the perihemal epithelium of seastars. The perihemal axons may originate from a continuation of the hyponeural nerve, since they share a common epithelium (Cobb, 1970) and axons similar to the perihemal axons are found in the hyponeural nerve. However, no GSS granules are found in the hyponeural nerve. Also, the axons of the hyponeural nerve

primarily run transversally from the nerve cord to innervate the tube feet (Cobb, 1970). On the other hand, the perihemal axons appear to primarily run longitudinally in the nerve cord. It is also possible that the perihemal axons arise from the basal portion of the epithelial cells. In this case, the perihemal epithelium would be more properly described as a nerve, and constitute a third nervous component of the radial nerve cord. Further ultrastructural study of the perihemal axons is needed to elucidate their origin.

GSS granules are also found in the epithelium of the axial organ, and the coelomic lining of the tube feet, though smaller in size and of less abundance. Ultrastructural examination of the epithelium of the axial organ reveals cells and axons that contain electron-dense, 100 nm diameter vesicles similar those of the radial perihemal epithelium. Their distribution and abundance appears similar to that of the GSS granules observed by light microscopy, and suggests that GSS in the axial organ is localized to these cells and axons. The GSS granules in the tube feet are distributed primarily in the basal region of the coelomic epithelium, in spaces between the reticulocytes. Wood and Cavey (1981) report that granulocytes, so called because they contain 100 to 200 nm diameter, electron-dense granules, are common to these

spaces. The function of the granulocytes is as yet unknown, but their distribution as described by Wood and Cavey appears to correspond to that of the GSS granules found in the coelomic epithelium. Unfortunately, ultrastructural examination of the tube foot coelomic epithelium failed to demonstrate granulocytes in this study. Further examination is required to determine ultrastructural correlates for GSS localization in the tube feet.

GSS granules were not found in the wall of the ovary, confirming earlier reports that bioassay of this tissue shows an absence of GSS (Kanatani, 1973). The ovarian wall is derived from two coelomic epithelia: the outer wall is formed by the visceral coelomic epithelium lining the coelom in which the ovaries are suspended (Schoenmakers *et al.*, 1981). The inner wall is a derivative of the perihemal epithelium and the hemal sinus. During ovarian development, outgrowths of the aboral perihemal ring enter the outer visceral coelomic sac of the ovary, forming the inner sac. Germ cells migrate through the perihemal space into the developing ovary and come to lie innermost, forming the germinal epithelium (Hyman, 1955). Thus, the genital coelom in the ovarian wall is a remnant of the perihemal space and is bound on either side by derivatives of the perihemal epithelium.

This area was examined ultrastructurally for cells and axons similar to those of the perihemal epithelium of the axial organ and radial nerve cord. No vesiculated axons can be found in this region of the ovary, nor are there any cells containing vesicles similar to those found in the radial perihemal epithelium. The perihemal epithelium of the ovary appears much reduced; muscle fibers predominate, though a few cells can found periodically throughout this layer. Absence of the ultrastructural correlates to GSS in the ovary could explain why no GSS is detected here.

Axons do exist in the ovary in the outer epithelium, as shown in this study and previously by Schoenmakers *et al* (1981). These axons contain similar vesicles to those observed in the perihemal epithelium. These axons are believed to function as innervators to the ovarian muscles, and bring about contraction of the ovarian wall during spawning (Schoenmakers *et al.*, 1981). This area was not immunoreactive with GSS antibodies, so it is unlikely that these axons could contain GSS.

Sections of pyloric caeca were also absent of immunoreactivity, and bioassay of these tissues show that GSS is absent here (Kanatani, 1973). Pyloric caeca were used as controls throughout this study because of their absence of GSS; most other asteroid tissues (apart from the

ovarian wall) do show the presence of GSS to some degree (Kanatani, 1973). Pyloric caeca extracts do not react with GSS antisera in immunodiffusion, but this is not conclusive that the antisera contain antibodies that react exclusively with GSS. It is possible that the antibodies could react with other asteroid peptides that are similar to, or share a common epitope with GSS. Immunoreactivity with an asteroid tissue devoid of GSS would indicate that the serum contained antibodies specific for other asteroid substances. The negative reaction with pyloric caeca in both the immunodiffusion and immunohistochemical tests gives a strong indication that the antisera developed in this study were specific for GSS.

2. Transport of GSS

GSS is not found in the ovarian wall and therefore must be moved from its site of production in the radial perihemal epithelium to the ovary to initiate spawning. GSS is found primarily in the radial perihemal epithelium, in close proximity

to the radial hemal sinus. This immediately suggests that the hemal system is involved in translocating GSS, and I propose that GSS is secreted into the radial hemal sinus and is transported to the ovary through the hemal sinus system.

There are structural and functional aspects that support this hypothesis.

a) Development and Structure of the Hemal Sinus System

The development and adult structure of the hemal system has been previously described (Gemmill, 1914; Hyman, 1955; Ubaghs, 1969). The tissues that make up the system are derived from the left larval somatocoel (Hyman, 1955). Prior to metamorphosis, the left somatocoel accompanies the growth of the hydrocoel, which give rise to the water vascular canals. Five diverticula bud from the wall of the ventral horn of the somatocoel and divide into two branches in the interray between the developing radial water vascular canals. These diverticula eventually fuse oral to the radial water vascular canals to form the radial perihemal canals. This fusion creates a central septum in the perihemal canal, that will contain the hemal tissues (Hyman, 1955). In the central disc, outgrowths of the diverticula fuse to form the outer portion of the periesophageal ring (Ubaghs, 1969).

During metamorphosis, the axocoel gives rise to the axial sinus that will house the axial organ and stone canal, and grows orally to form inner periesophageal ring, which fuses with the outer portion formed by the perihemal diverticula (Hyman, 1955). This explains the connection

between the periesophageal ring and the axial sinus in the adult.

The dorsal horn of the left somatocoel accompanies the developing axocoel to form the axial organ and its hemal branches. Aborally it forms the aboral perihemal ring, which gives off two genital branches into each ray (Hyman, 1955). These branches enlarge distally into sacs and become the inner part of the ovary, as previously described.

The axial organ consists of a hemal plexus bound by a perihemal epithelium. Aborally it terminates in a dorsal sac, and ends orally in the periesophageal ring (Hyman, 1955). The hemal plexus of the axial organ can be considered the central part of the hemal system (Hyman, 1955). Hemal tissues arise from the developing axial organ and infiltrate the perihemal septa throughout the perihemal canals. In the radial perihemal canals, transverse hemal branches are sent out to the primary tube feet (Hyman, 1955). In addition, the axial organ sends out hemal connectives, the gastric hemal tufts, to the cardiac stomach (Hyman, 1955) and pyloric caeca (Ubaghs, 1969). The gastric hemal tufts are the only part of the hemal system not enclosed in a coelomic channel (Hyman, 1955). Absence of perihemal derivatives in the pyloric caeca explains why no GSS is found in these organs.

Apart from its association with the axocoel, the hemal system is entirely derived from the left somatocoel, and so arises as a separate system from other coelomic derivatives such as the water vascular system. It is apparent that continuity between the hemal system and major organs is established early on in development, and is retained after metamorphosis in the adult form. The hemal system provides a pathway for the transport of GSS to the ovaries.

b) Function of the Hemal Sinus System

Until recently, little was known about the function of the hemal sinus system in asteroids. Unlike echinoids and holothurians, which have a prominent hemal system, the hemal system of asteroids is not very conspicuous (Hyman, 1955).

Gemmill (1914) observed rhythmic pulsations in the dorsal sac of the axial organ in *Asterias rubens* and proposed that the axial organ could function to move fluids throughout the hemal canals, thus constituting a circulatory system. Later histological examinations revealed that the hemal system does not possess the normal characteristics of a circulatory system and it appears to be unnecessary for that role since movement of respiratory gases and nutrients are carried out by the coelomic fluids (Ferguson, 1984). The hemal system in asteroids was considered vestigial.

Evidence that the hemal system can function in translocation was first reported by Ferguson (1970) in a study on nutritive physiology in asteroids. Specimens of *Echinaster echinophorus* were injected with C^{14}-labelled amino acids and subjected to autoradiographic study. Though most of the label appeared to be translocated by the coelomic fluids, a significant amount was taken up by the radial hemal strands and the connective tissue-hemal plexus of the tube feet (Ferguson, 1970). It was postulated that active uptake by the perihemal canals was responsible for the movement of the labelled material into the hemal tissues.

Broertjes *et al.*, (1979) found evidence that materials from the digestive organs was actively taken up by the hemal system in *Asterias rubens*, and later (1980) proposed that the hemal system was involved as a transport route for vitellogenesis in the ovaries. Walker (1980) studied the anatomy of the ovary and found that genital hemal sinus underwent seasonal changes associated with gametogenesis and vitellogenesis. He hypothesised that the gonads depended on the hemal system for the collection and storage of nutrients (Walker, 1980).

The translocatory ability of the hemal system has been demonstrated by Ferguson (1984). Specimens of *Echinaster*

graminicolus were fed C^{14}-labeled nutrients and subjected to autoradiographic analysis over a period of 2 days. Ferguson found that the tracer was transported from the cardiac stomach into the hemal sinus, and was subsequently distributed throughout the hemal system. The tracer moved from the hemal sinus of the digestive glands into the axial organ. From there it was transported to the genital hemal sinus, and the oral hemal ring where it was distributed to the radial hemal strands and the tube foot hemal plexus (Ferguson, 1984). He concluded that the hemal system plays a significant role in the distribution of nutritive material within the body.

Ferguson's findings show that the hemal system is capable of transporting substances throughout the asteroid body, and also show that the hemal system is continuous between organs and body regions. It is possible that GSS secreted into the hemal sinus could be moved throughout the hemal system, and thus reach the ovaries. This would be the most efficacious route since the genital hemal sinus directly apposes the germinal epithelium, which gives rise to the oocytes. GSS would come into direct contact with the oocytes without having to traverse the ovarian wall.

It is possible that GSS is not secreted into the hemal sinus but instead is secreted into the perihemal canal. GSS

could still reach the ovary by this route and enter through the genital coelom. However, Ferguson found no tracer in the perihemal canals, indicating that the hemal tissues were responsible for taking up substances from the perihemal fluids (Ferguson, 1984). Further, GSS is localized in the basal third of the perihemal epithelium, probably in the axons that appose the basal lamina. GSS would be released closer to the hemal sinus than the outer perihemal space. Isolation and bioassay of hemal material would determine if GSS were present in the hemal sinus. However, if GSS were secreted into the perihemal canal, Ferguson's findings indicate that it would eventually be taken up by the hemal sinus.

With regard to the mechanism of GSS transport through the hemal system, Ferguson proposes that continuous exchange of materials between the perihemal fluid and the hemal tissues could produce a net transport. The hemal sinus is filled with congealed material in thin-walled sinuses, and appears unsuitable for axial flow (Ferguson, 1984). The walls of the perihemal canals are made up of flagellated cells oriented to create local circulation (Walker, 1979). Materials in the hemal sinus could move back into the perihemal fluid for transport; thus the hemal tissues act as a storage reservoir, and the actual transport is accomplished by perihemal circulation (Ferguson, 1984).

It still must be established if it is possible for substances in the radial hemal sinus to reach the genital hemal sinus. Ferguson's findings indicate that substances move centrifugally from the axial organ to the periphery. This is probably a result of the site of origin of the tracer. Labelled substances taken up by the tube feet have been shown to move into the radial hemal sinus and are translocated proximally, toward the central disc (Ferguson, 1970). Unfortunately, Ferguson did not examine the axial organ or ovaries in this earlier study, but it seems likely that substances in the hemal system move in both directions. It may be possible to cannulate the distal portion of the radial hemal sinus and inject a suitable marker. Tracing the progress of the marker through the hemal system by serial sections may determine if it is possible for substance to reach the ovaries from the radial hemal sinus.

3. The Physiological Role of GSS

So far, it is only known that GSS functions in initiating spawning by acting on the ovarian follicle cells, but it may have other physiological roles as well. The fact that GSS can be found in other tissues not directly associated with the ovaries, such as tube feet, body wall, and cardiac stomach (Kanatani, 1973), may mean that it

affects other organ systems. One role could be the induction of the spawning posture prior to the release of gametes, and may explain why GSS is found in tube feet. Spawning posture is characterized by a coordinated movement of the arms that gradually elevates the central disc above the substrate. This posture is assumed for one or more hours. GSS released in the tube feet may effect a prolonged motor response that results in this behaviour. Alternatively, GSS may act on the hyponeural nerve, which controls coordinated movement of the tube feet (Smith, 1965). GSS may stimulate the hyponeural nerve which in turn coordinates the tube feet in the assumption of spawning posture.

4. Future Studies on GSS

Although GSS can be localized by immunohistochemical techniques, the cells that produce it could not be resolved by light microscopy. The application of immunohistochemistry to ultrastructural studies, using a suitable electron-dense marker such as ferritin, would determine what elements of the perihemal epithelium and other tissues produce GSS. This application would also be useful in localising the GSS binding sites on the ovarian follicle cells, and may elucidate how GSS affects these cells to synthesize and release 1-MA. Another approach

would be to label the GSS molecule itself. This technique would make it possible to determine if GSS binds to cells of other tissues, and may give clues to other physiological roles for GSS.

The amino acid composition of GSS is already known (Kanatani and Ohguri, 1971), but the amino acid sequence is still to be determined. Characterisation of the GSS molecule would allow synthetic GSS to be prepared, and free the investigator from the cumbersome task of preparing GSS from biological sources. It would also allow for a comparison between GSS and reproductive hormones in other animal systems.

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IMMUNOHISTOCHEMICAL LOCALIZATION OF GONAD STIMULATING SUBSTANCE IN THE SEASTAR *Pycnopodia helianthoides*

Author:

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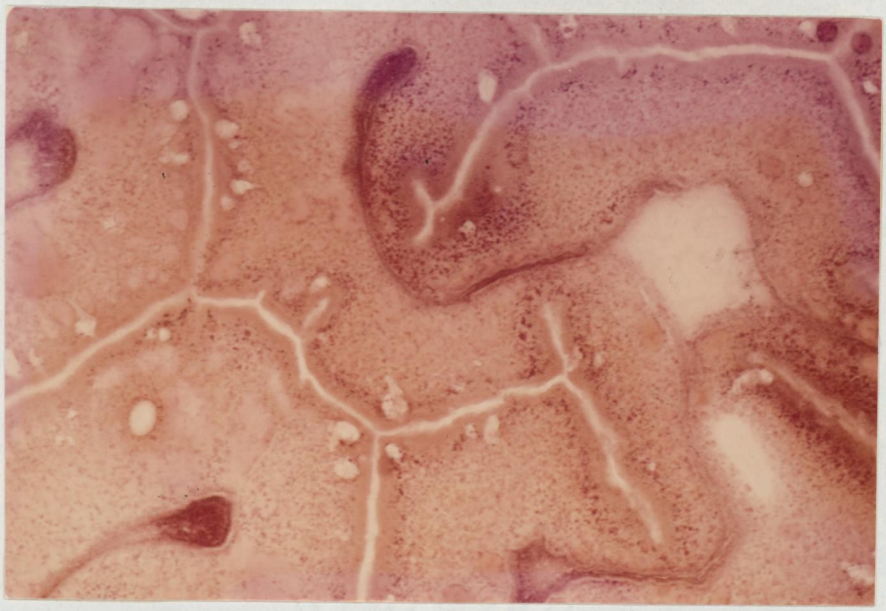
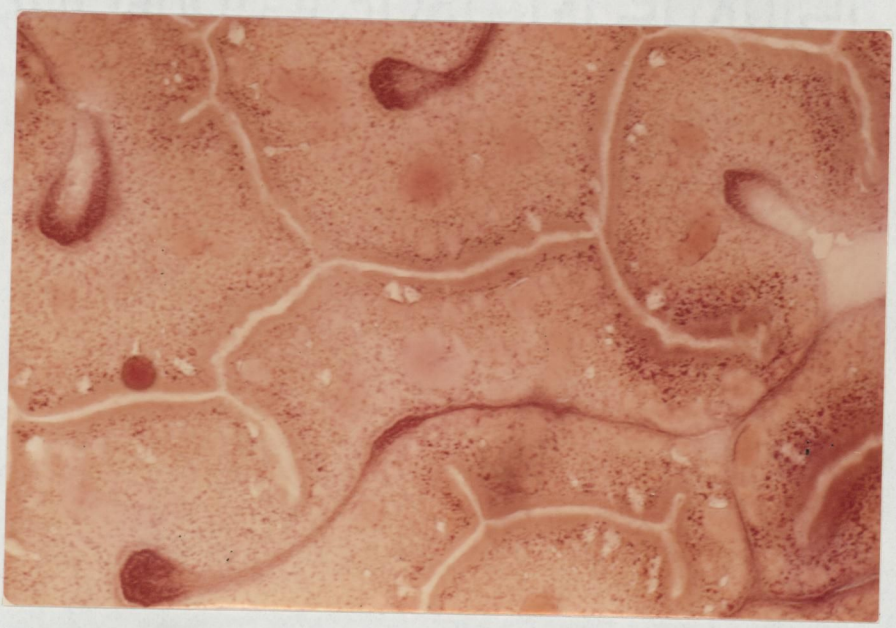
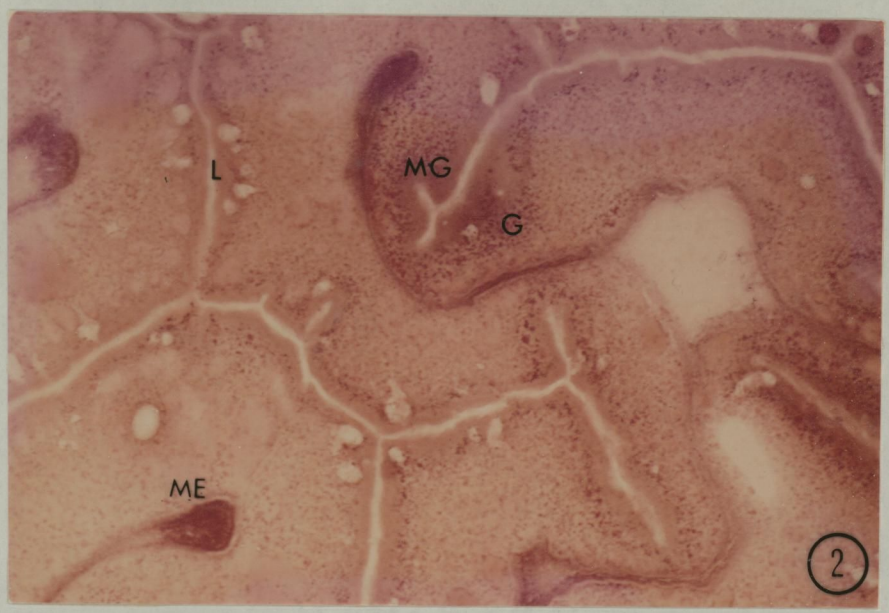
Gary David Caine
Name

Oct. 16/84
Date



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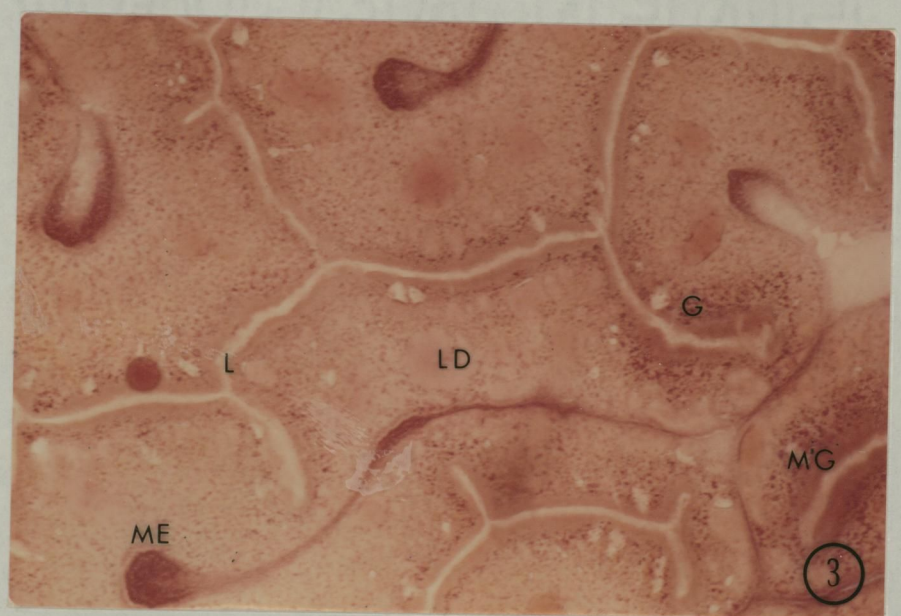
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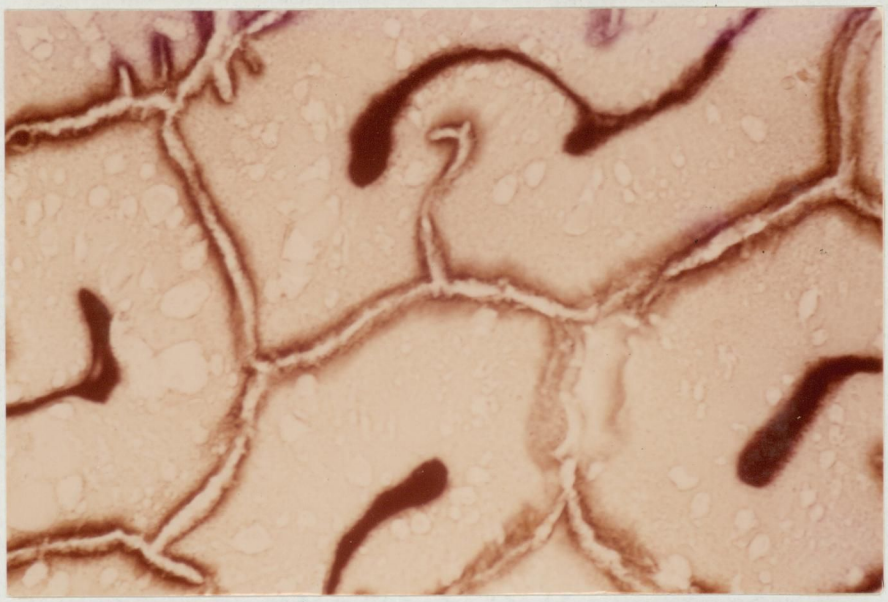

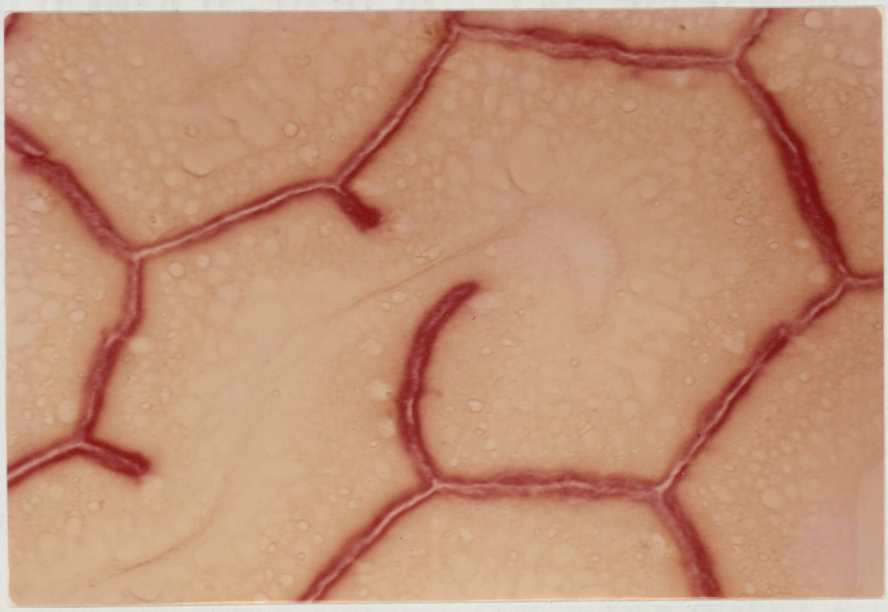

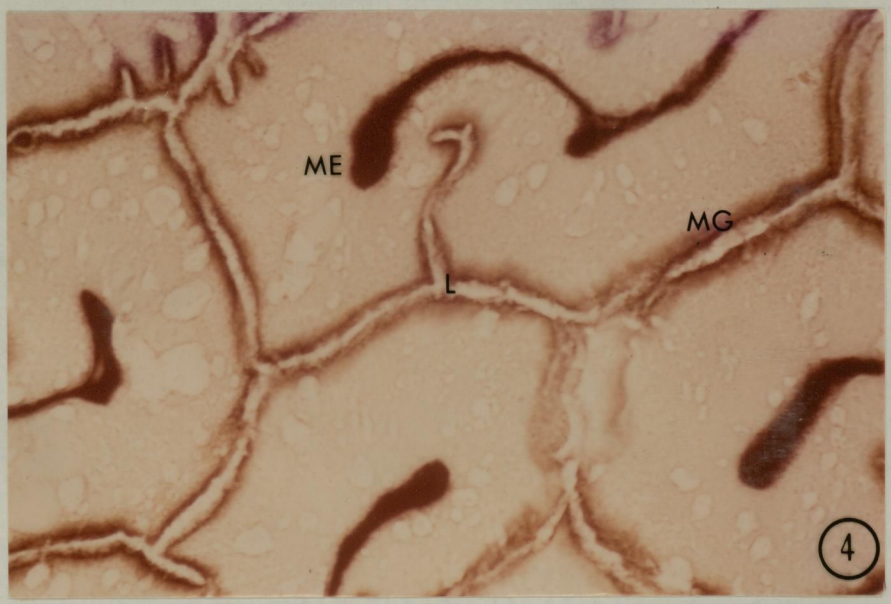
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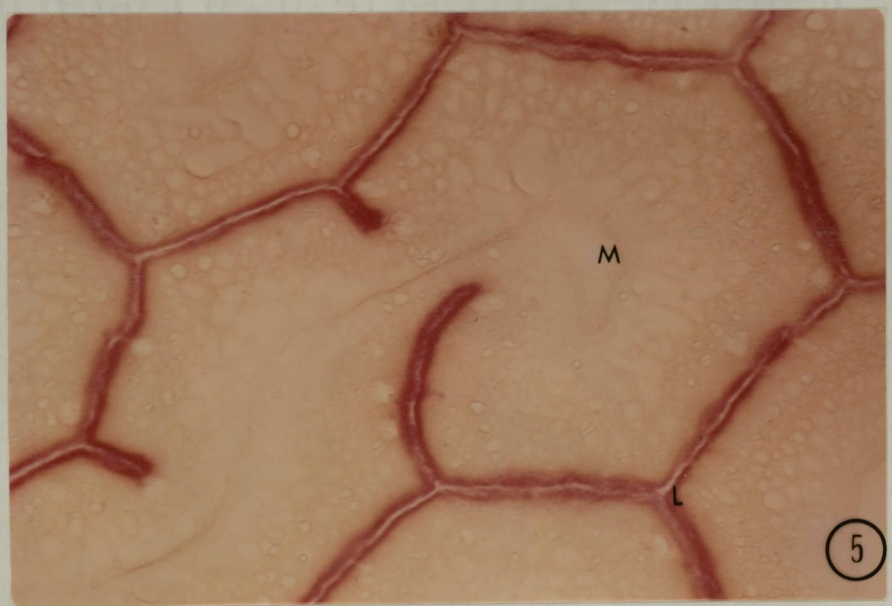
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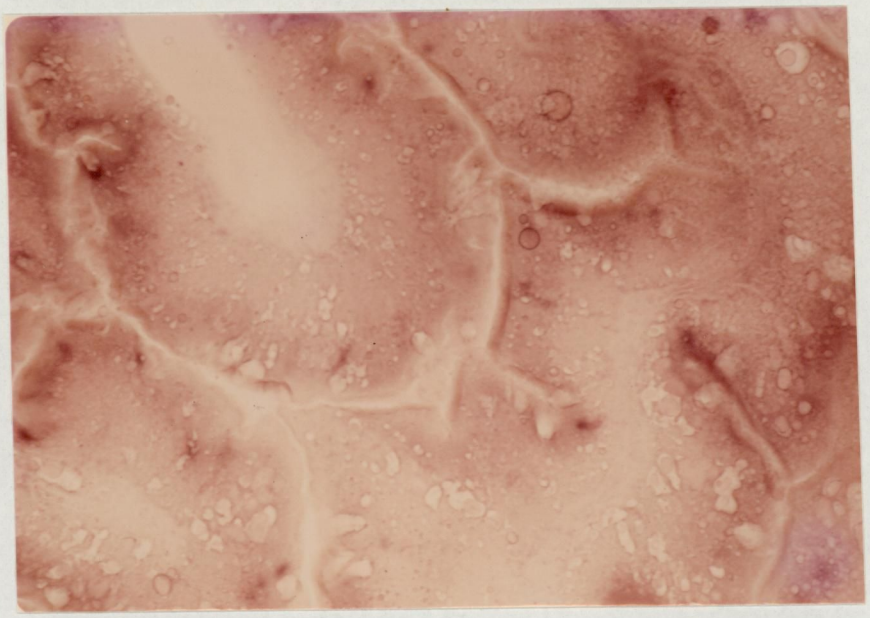

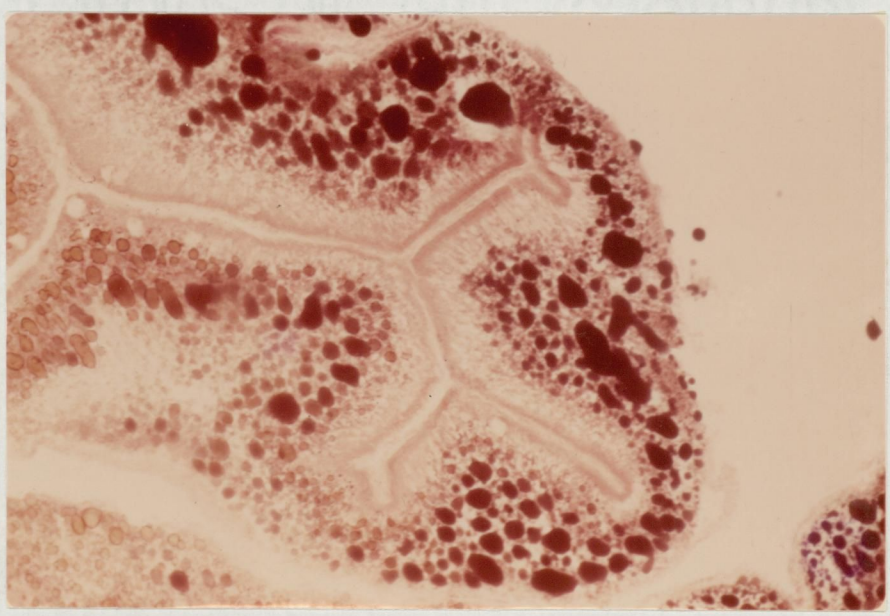

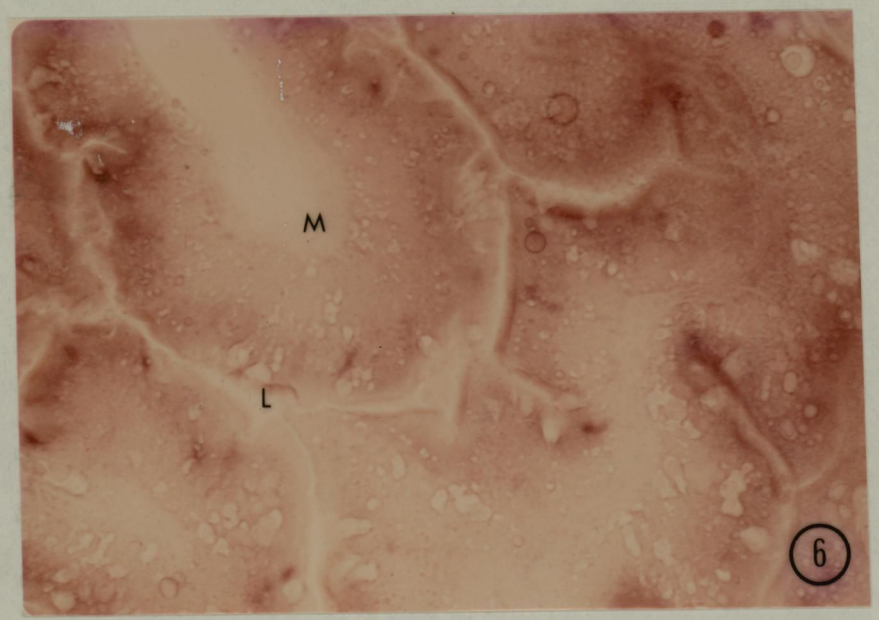
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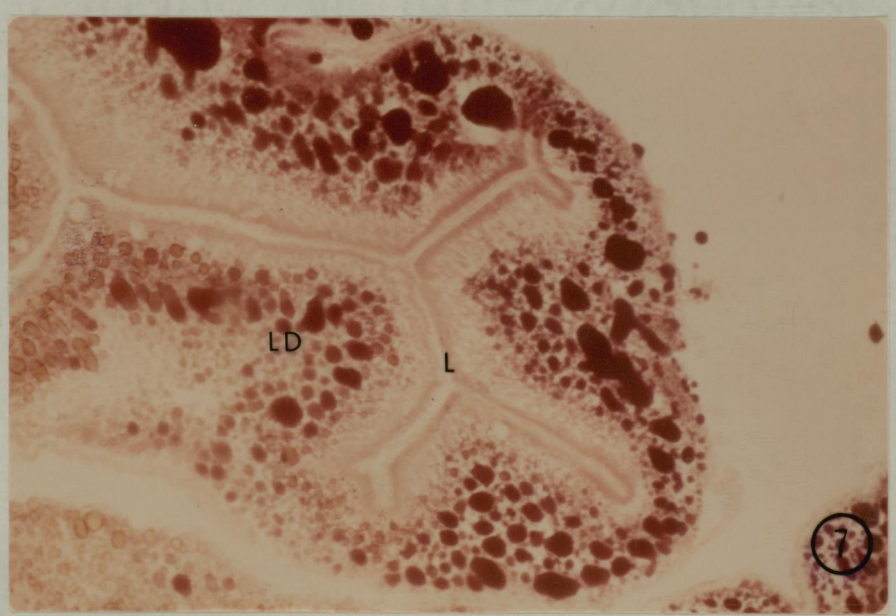
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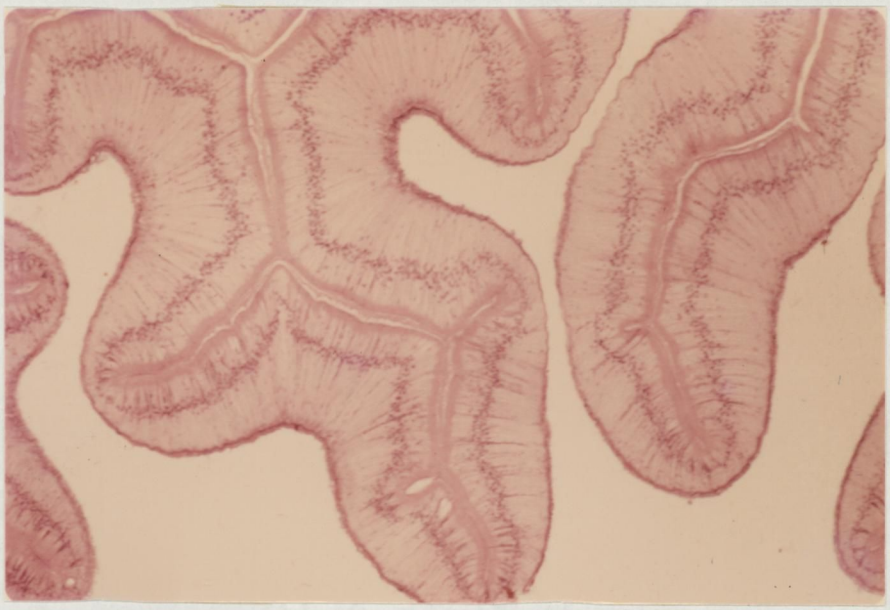

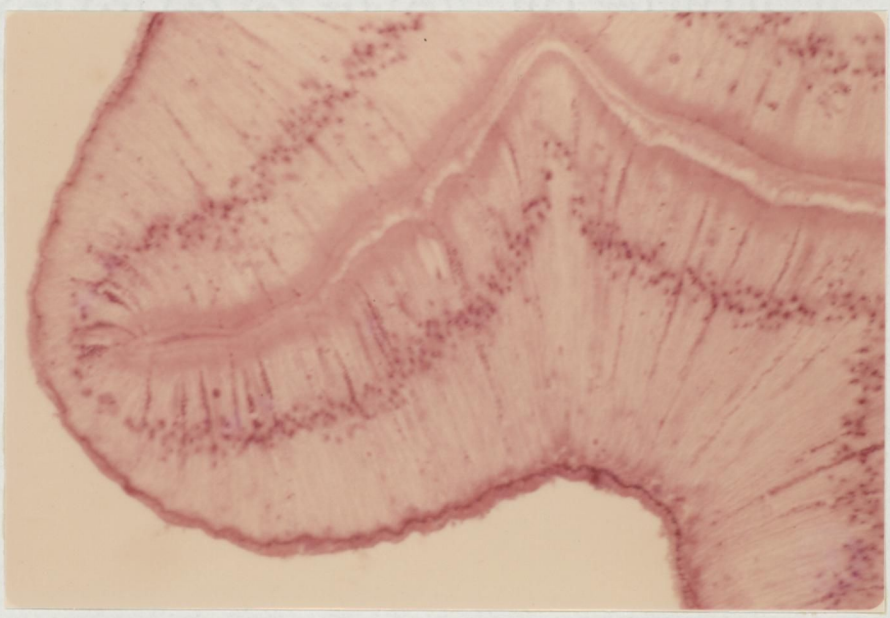

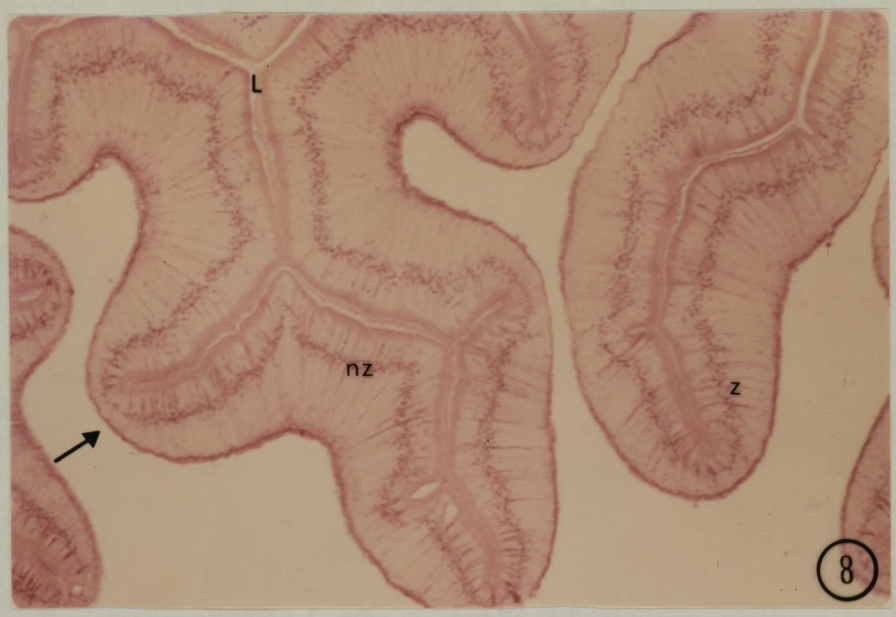
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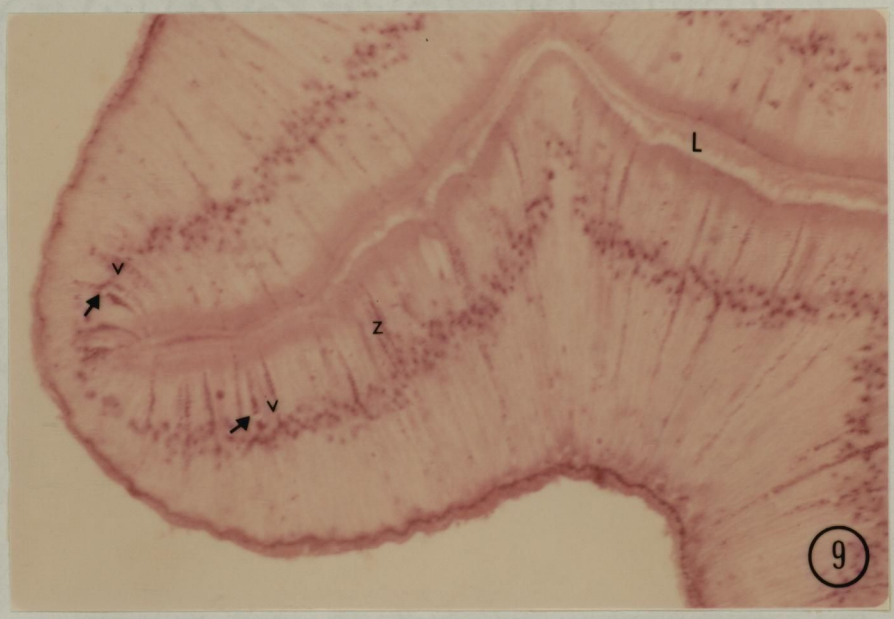
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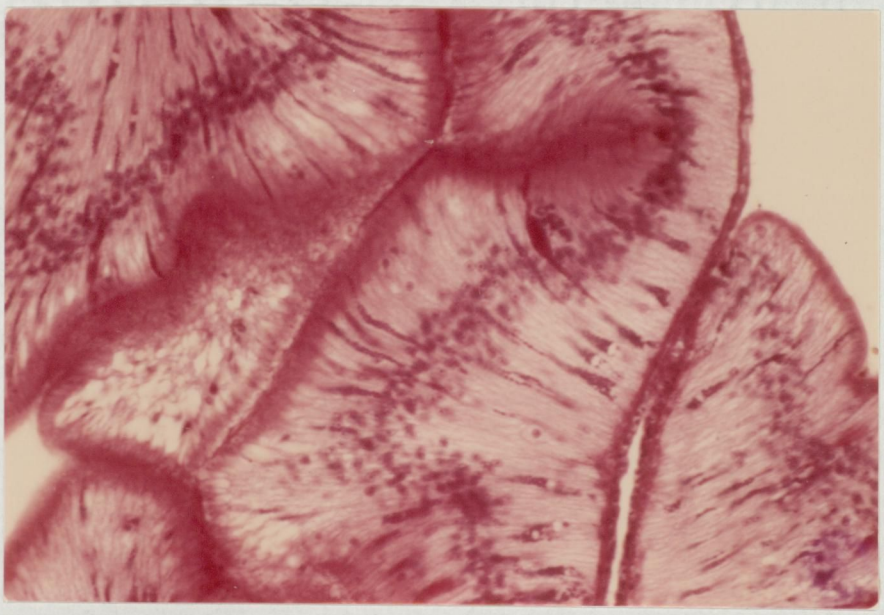


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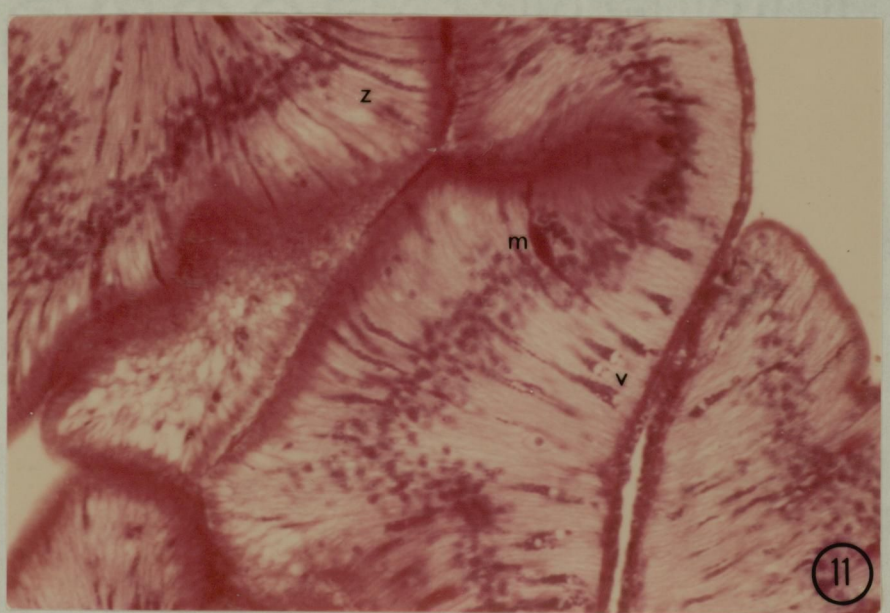
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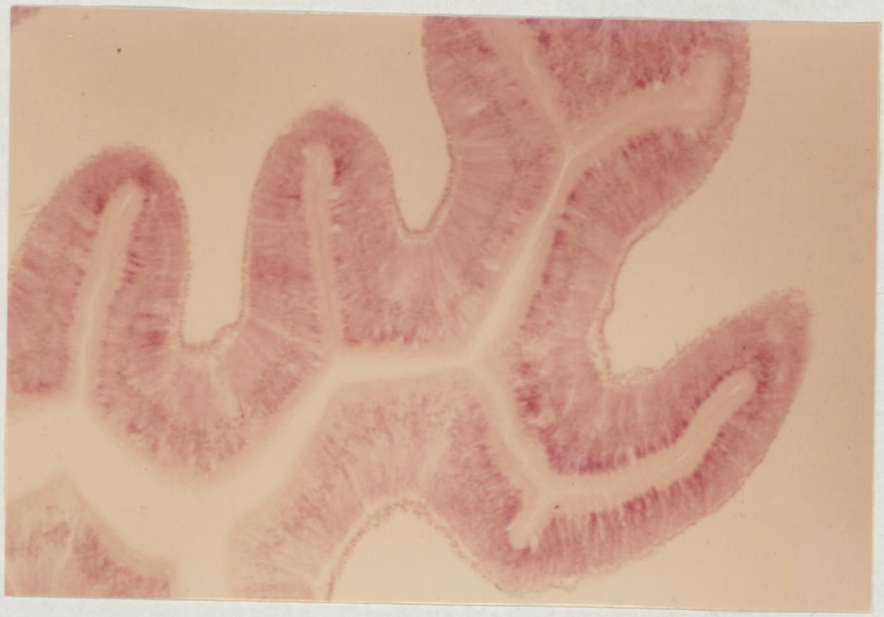

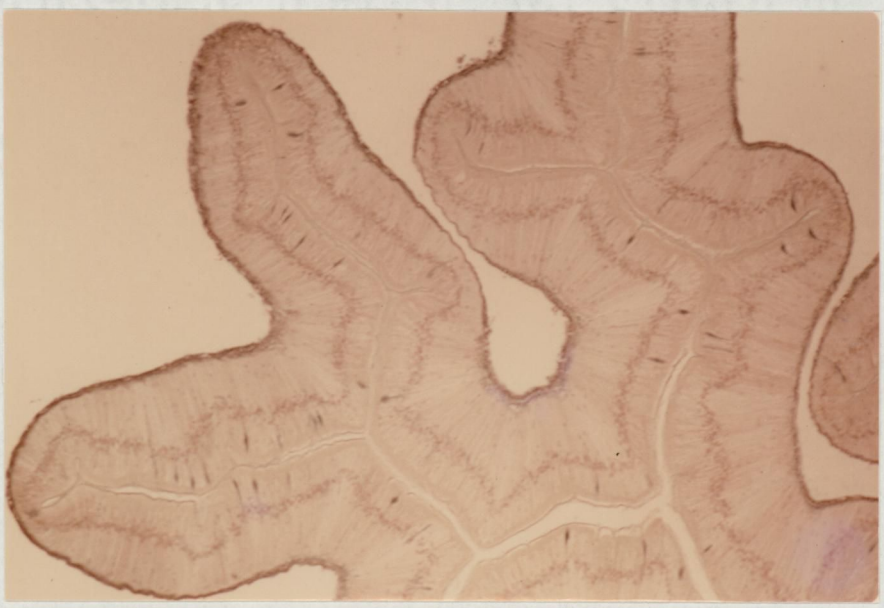

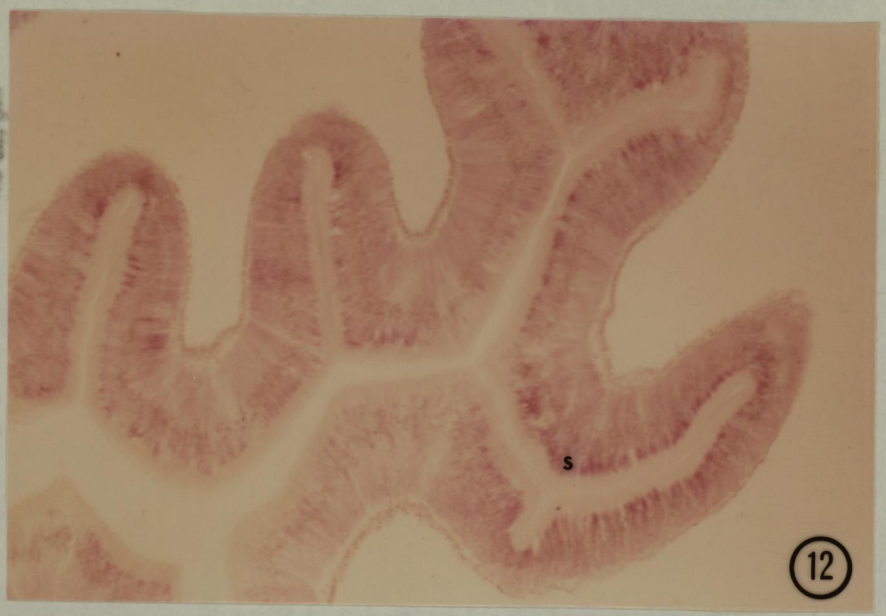
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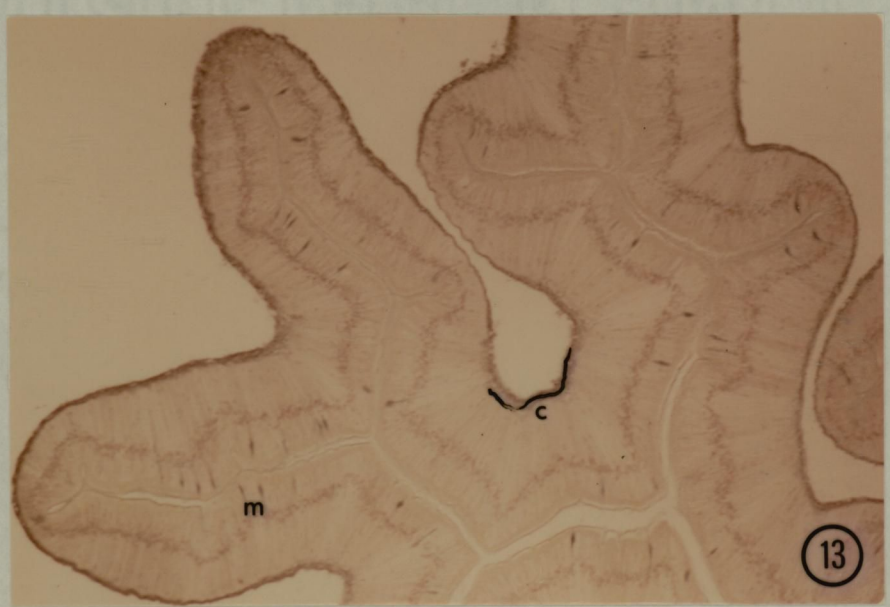
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