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During the past two years, we have carried out the following studies:(1) Ultrastructural study of Rift Valley fever (RVF) virions in the cardia. (2) Immunocytochemical studies of the salivary glands and other tissues in Anopheles sp. which had been shown to be non-transmitters when intrathoracically-infected as adults, but transmitters when infected as immatures. (3) Immunocytochemical study of Ockelbo virus in Culex pipiens. (4) Ultrastructural examination of various tissues for RVF virus in hemocoelically-infected Cx. pipiens. (5) Development of an immunogold procedure for in situ labelling of RVF virions in electron microscopic preps. (6) Worked toward the identification and isolation of the mosquito cell surface receptor molecule for RVF virus. (7) Developed and presented a pre-meeting workshop, "Mosquito Histology, Ultrastructure, and Methods for In situ Localization of Viruses," held in conjunction with the annual meeting of the American Society of Tropical Medicine & Hygiene. (8) Made progress in the development of a monograph on mosquito histology and ultrastructure.

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STUDIES OF INFECTION AND DISSEMINATION OF RIFT VALLEY FEVER VIRUS IN MOSQUITOES

FINAL REPORT

William S. Romoser

OCTOBER 15, 1991

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U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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SUMMARY

The information reported in this document represents a continuation of the research supported by a contract immediately preceding this one.

During the past two years, we have carried out or continued the following studies: (1) Ultrastructural study of Rift Valley fever (RVF) virions in the cardia. (2) Immunocytochemical studies of the salivary glands and other tissues in Anopheles sp. which had been shown to be non-transmitters when intrathoracically-infected as adults, but transmitters when intrathoracically-infected as immatures. (3) Immunocytochemical Study of Ockelbo Virus in Culex pipiens. (4) Ultrastructural examination of various tissues for RVF virus in intrahemocoelically- infected Culex pipiens. (5) Development of an immunogold procedure for in situ labelling of RVF virions in electron microscopic preparations. (6) Worked toward the identification and isolation of the mosquito cell surface receptor molecule for RVF virus. (7) Developed a pre-meeting workshop entitled "Mosquito Histology, Ultrastructure, and Methods for In situ localization of Viruses" to be held on Sunday, Dec. 1 in conjunction with the annual meeting of the American Society for Tropical Medicine & Hygiene in Boston, Massachusetts. (8) Made progress in the development of a monograph on mosquito histology and ultrastructure.

Our major findings and successes during the past two years are as follows: (1) We have added evidence in support of the predilection of RVF virus for the tissues which make up the proventriculus (cardia) in Culex pipiens. (2) We have found additional evidence of a "salivary gland infection barrier" in Anopheles sp. and have identified similar "infection" barriers in the pharyngeal pump muscles and the hindgut. Since the experiments were carried out "blind", the "infection barrier" study also allowed us to demonstrate the very high sensitivity and reliability of the ABC immunocytochemical technique for detecting RVF viral antigen in mosquito tissues. (3) We have successfully developed and applied a protocol for the immunogold-labelling of RVF virions in ultrathin sections and have by this means substantiated that the particles we have been interpreting in electron micrographs as RVF virons are, in fact, RVF virions. (4) We have demonstrated the presence of RVF virus in muscle and tracheal tissue, an issue that had been unresolved in our immunocytochemical studies. Further, we found a very high density of RVF virions, higher than that observed in any other tissue, in the salivary glands and what appeared to be paracrystalline arrays of RVF

virions. We also observed virions in fat body and nerves. We had previously reported virions in the ventral diverticulum and foregut epithelium.

(5) We have made some progress toward finding the mosquito cell surface receptor for RVF virus, especially by testing fractions of Aedes albopictus (C6/36) cells for inhibition of RVF virus infectivity on both Vero and C6/36 cell monolayers. (6) We made good progress toward to the development of a workshop on "Mosquito Histology, Ultrastructure, and Methods for the In situ Localization of Viruses" to be held Dec. 1991, in Boston immediately prior to the annual meeting of the American Society of Tropical Medicine & Hygiene. (7) We have made good progress toward the development of a monograph on mosquito histology and ultrastructure.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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_____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institute of Health.

William S. Romoser 28 July 1992

PI Signature Date

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ILLUSTRATIONS

I. Introduction

A. Background

The literature pertinent to arboviruses and mosquitoes and Rift Valley fever virus is extensively reviewed in Hardy et al. (1983), Hardy (1988), Meegan & Bailey (1988), Turell (1988), and in the original research contract proposal.

Since our research has involved an immunocytochemical study of Ockelbo-virus infected mosquitoes and the use of various biochemical and immunochemical procedures for identification of the mosquito cell surface receptor molecule, literature pertinent to these lines of research is briefly discussed below.

Cell Surface Receptors for Viruses

Cell surface receptors in general are discussed in Limbird (1986), while Lonberg-Holm & Philipson (1981) deal specifically with virus receptors. Several researchers have described various approaches to isolation of viral receptors. Prola (1979) developed a procedure to isolate receptor membrane components with a non-ionic detergent and further fractionate the extract with a lectin affinity column. Moldow (1977), concerned with the preservation of the biological activity of the membrane constituents, used a lithium diiodosalicylate extraction procedure to solubilize avian oncornavirus receptor. Butters and Hughes (1974) showed that non-ionic detergents and subsequent gel filtration resulted in a receptor-rich extract. Mischak (1988) described an extraction procedure for the minor group receptor from HeLa cells and a purification procedure using ion exchange and gel filtration and Western Blot assay using non-denaturing SDS gel electrophoresis. Kayanaraman (1978) described a centrifugation preparation of membrane fractions after osmotic swelling of cells and manual Dounce homogenization. Mapoles (1985) described a purification procedure extracting virus-receptor complex from cell lysates. Nemerow (1986) purified the Epstein-Barr virus-C3D receptor complex by immunoaffinity chromatography. Schneider (1982) described a one-step immunoaffinity matrix method of receptor purification to find the transferrin receptor. Marriott (1987) extracted polymavirus receptor moieties from mouse kidney cells using a non-ionic detergent. Kata and Akamatsu (1984) characterized glycoproteins using gel chromatography and Con A Sepharose lectin chromatography.

Tomassini and Colonno (1986) described an osmotic disruption of HeLa cells and subsequent crude separation by centrifugation, followed by a detergent solubilization. A

receptor monoclonal antibody affinity column was used to extract the receptor from the solubilized cell extract. Gel filtration fractionation of the solubilized membranes, affinity purification and subsequent RIA assay resulted in a possible receptor protein, which was used to generate rabbit polyclonal antiserum. Subsequent membrane binding and cell protection assays demonstrated that the protein was indeed the virus receptor.

Biochemical fractionation using the FPLC system has been used in the purification of the Human Rhinoviru Minor Group Receptor (Mischak et al., 1981).

The use of anti-idiotypic mimicry for the purification of cell surface receptors has become widespread in recent years because the technique does not require a purified receptor sample to produce antibodies specific for the receptor, but only the proper antibody with which to immunize the animal (Gaulton & Green, 1986). Co et al. (1985) have used anti-idiotypic antibodies to purify the Mammalian Reovirus type 3 cell surface receptor, and Marriot et al. (1987) have used anti-idiotypic antibodies to purify the polyomavirus receptor.

Immunocytochemical Study of Ockelbo Virus in Culex pipiens

This study represents a spinoff from the Rift Valley fever (RVF) virus project. Our objectives were two-fold. primary objective has been two-fold. Our primary objective was to see if the Avidin-Biotin-Peroxidase Complex or ABC immunocytochemical technique used successfully in studies of RVF virus in Culex pipiens and other mosquito species would work with a different arbovirus using a polyclonal antiserum. A second objective was been to determine the mosquito tissue tropisms of Ockelbo virus in Culex pipiens.

Ockelbo virus is a member of the Sindbis group of arboviruses, Sindbis being the prototype virus of the genus Alphavirus in the family Togaviridae. Sindbis virus was first isolated in 1952 from Culex mosquitoes collected near Cairo in the village of Sindbis. Occurrences of Sindbis virus infection have since been reported in Europe, Asia, Australia, and Africa, including epidemic outbreaks in South Africa in 1974.

Ockelbo (OCK) disease is characterized by symptoms of fever, arthralgia and rash. This disease is endemic in Sweden and was first recognized there in the late 1960's. OKB disease has been shown by serological studies to be caused by a Sindbis-like virus known as the OCK virus (Niklasson, 1990; Turell & Lundstron, 1990). Enzyme immunoassays have demonstrated two major antigenic subdivisions of Sindbis virus: (1) Those viruses from the

Palearctic region (which includes Europe, Africa, Arabia, and Asia north of the Himalayas); and (2) athose viruses from Oriental-Australian regions. There is no significant difference between the OCK virus of the Palearctic subdivision and the prototype strain of the Sindbis virus from Egypt (Niklasson, 1990).

B. Objectives

The overall objective of this research has been to contribute to our understanding of the epidemiology of Rift Valley fever. More specifically, our goals have been (1) to describe the dissemination, pathogenesis, and morphogenesis of RVF virus in vector competent and incompetent mosquitoes and (2) to identify the mosquito cell surface molecule (receptor) to which RVF virus binds prior to entering a cell.

Ultimately we have hoped to shed light on intrinsic factors which influence vector competence using RVF virus/mosquitoes as model systems.

An additional objective has been to contribute to studies of the dissemination, tissue tropisms and morphogenesis of selected arboviruses in ticks.

C. Topics Covered in This Report

1. Immunocytochemical & Ultrastructural Studies

    a. Continued study of RVF virus in the cardia.

    b. Continued studies of the putative salivary gland barrier in Anopheles.

    c. Immunocytochemical Study of Ockelbo Virus in Culex pipiens.

    d. Ultrastructural examination of various tissues for RVF virus in intrahemocoelically-infected Culex pipiens.

    e. Development of an immunogold procedure for the in situ labelling of RVF virions in electron microscopic preparations.

    f. Ultrastructural localization of CCHF virions in ticks

2. Progress in the Isolation and Characterization of the Mosquito Cell Surface Receptor Molecule for RVF Virus.

3. Development and presentation of a pre-meeting workshop entitled "Mosquito Histology, Ultrastructure, and Methods for In situ localization of Viruses" which was held on Sunday, Dec. 1, 1991 in conjunction with the annual meeting of the American Society for Tropical Medicine & Hygiene, Boston, Massachusetts.

4. Progress in the development of a monograph on mosquito histology and ultrastructure.

II. Materials and Methods

In Situ Localization of Viral Infection in Mosquitoes

A. Introductory Comments

We have applied several methods for localizing viral infection in mosquito tissues. Each method has inherent advantages and disadvantages. Therefore the best approach is to use more than one method (Hardy, et al., 1983). To detect infectious particles in dissected organs and tissues, we are using plaque assay on Vero cells. To detect viral antigen, we are using the avidin-biotin-peroxidase complex (ABC) immunocytochemical technique for light level microscopy (Faran et al., 1986) and are also applying immunocytochemical techniques at the electron microscope level. To detect viral genome, we are involved in the development of a protocol for the application of a peroxidase-labelled complementary DNA probe to paraffin sections of whole mosquitoes. To detect whole virions and nucleocapsids, we are using standard transmission electron microscopy.

B. Plaque Assay

During the past two years we have used viral plaque assay primarily to assess the potential inhibitory effects of various reagents (various fractions of cell surface preparations of C6/36 cells; anti-idiotypic antibodies directed against monoclonal antibodies which were in turn directed against the glycoprotein spikes associated with RVF virions (G_1 & G_2) on viral infectivity for Vero and Aedes albopictus, C6/36 cells. We also used plaque assay on Vero cells to determine the "dose" of RVF virus injected into mosquitoes and to determine the dissemination status of orally fed mosquitoes based on the presence of infectious particles in dissected legs.

A brief description of the plaque assay technique follows. Whole mosquitoes and mosquito parts are triturated in 1 ml of mosquito diluent (10% calf serum in Medium 199 with Hank's Salt and antibiotics) and tested for infectious

particles by plaque assay on 2- to 4-day-old Vero cell monolayers (Gargan et al., 1983). The mean amount of virus ingested by a sample of mosquitoes taken immediately following each infectious blood meal represents the viral "dose" for a given experiment. In order to determine whether or not virus is present in the body cavity (hemocoel), i.e. whether or not virus has disseminated from the midgut, legs are dissected and assayed.

C. ABC

Faran, et al. (1986) adapted the very sensitive ABC immunocytochemical technique (Hsu, et al., 1981) for use with serial paraffin sections of formaldehyde fixed, whole mosquitoes. The technique is based on the use of primary antibody directed against viral antigen, followed by biotinylated secondary antibody directed against immunoglobulin from the vertebrate in which the primary antibody was formed. Finally, a complex of avidin and biotinylated peroxidase is applied. This complex binds with the biotinylated secondary antibody due to the great affinity between biotin and avidin. The location of the primary antibody/secondary antibody/ABC complex is then rendered visible by the addition of diaminobenzidine tetrahydrochloride (DAB), the oxidative polymerization of which is catalyzed by peroxidase. The DAB polymer appears as a rusty brown precipitate. In our studies the "primary antibody" is actually a blend of monoclonal antibodies directed against RVF virus nucleocapsid protein and two envelope glycoproteins or a monoclonal antibody directed against RVF virus nucleocapsid protein. These antibodies are provided by Cdr. James Meegan and Dr. Jonathan Smith at USAMRIID.

D. Electron Microscopy

To prepare mosquito tissues for electron microscopy, tissues are (1) fixed in Karnovsky's solution (1.5% glutaraldehyde, 2.0 % formaldehyde in 0.1M phosphate buffer and 0.15M sucrose) for 1 1/2 to 2 hrs. at 0-4^{o}C; (2) placed in buffered sucrose (15 min. to 48 hours); (3) post-fixed in 1.0% buffered osmium at 0-4^{o}C for 1 hr.; (4) dehydrated by passing through an ethyl alcohol series; (5) placed in propylene oxide for 20 min., then 1:1 volumes of propylene oxide and resin (Epon 812; Araldite 502; DDSA; DMP-30) for 1 hr., the 1.0 ml of resin was added; (6) 3-24 hours later, tissues are embedded in aluminum foil pans and placed in an oven at 60^{o}C for 48 hrs. Blocks of embedded tissues are cut on an ultramicrotome using a diamond knife, mounted on copper grids, and stained with uranyl acetate and lead citrate. Specimen grids are studied and electron micrographs taken with a Zeiss 110 transmission electron microscope.

E. Immuno-electron microscopy Using Colloidal Gold

To verify that the particles we were observing in electron micrographs were RVF virions, we developed a post-embedding immuno-gold technique for the in situ labelling of RVF virions in ultrathin sections.

Mosquitoes were infected intrathoracically with RVF virus. Uninfected control specimens were IT-injected with diluent. All mosquitoes were then incubated for 5-7 days at 25 C, and then fixed overnight at 4 C in 2% paraformaldehyde/ 0.25 % glutaraldehyde in a sucrose and sodium cacodylate buffer. Fixed specimens were washed and rinsed in sodium cacodylate-sucrose buffer, dehydrated with 70% ethyl alcohol, and infiltrated in a mixture of the newly formulated acrylic LR white and 70% ethyl alcohol. This was followed by several changes of fresh LR white, and final transfer to gelatin capsules. Embedded specimens were held in an oven at 50-55 C for at least 48 hours. Ultrathin sections were cut and collected on uncoated nickel grids.

The following procedure which produces labelling of virus was developed as the result of many trail runs in which we varied reagent concentrations and times of exposure to tissues and reagents.

Immunostaining was done by floating specimen grids, section-side down, on drops of immuno-reagents on parafilm in a moisture chamber. Prior to exposing grids to various immuno-reagents, etching and/or deplasticization of ultrathin sections was required. Surface-etching was achieved by floating grids on drops of saturated sodium meta-periodate. Deplasticization was achieved by incubating grids on drops of saturated sodium hydroxide in absolute alcohol followed by rehydration through a graded alcohol series, distilled water, and filtered tris buffer solution (TBS).

Grids were then incubated in 1:10 normal goat serum in TBS in order to block non-specific sites, followed by incubation with a 1:100 dilution of primary antibody, i.e. a mixture of monoclonal antibodies directed against glycoprotein "spikes" on the envelope of RVF virions and agains RVF nucleocapsid protein. Grids were washed several times before being placed on drops of secondary antibody, which consisted of a 1:10 dilution of goat anti-mouse conjugated with colloidal gold. Grids were then washed with tris buffer and distilled water, post-stained with uranyl acetate and lead citrate and examined with a Zeiss ZM 109 electron microscope.

F. Specific Investigations Involving Plaque Assay, Immunocytochemistry, and Electron Microscopy

1. RVF virions in cardial tissues.

Several intra-hemocoelically infected were prepared for ultrastructural study of RVF virus in the cardia.

2. Anopheles salivary gland barrier

Turell (1988) reported that the ability of Anopheles albimanus to transmit RVF virus was determined by the developmental stage during which they were IT- infected. All adult mosquitoes which had been infected as larvae transmitted virus, but only a very small fraction of specimens IT-infected as adults and incubated for an equivalent period of time transmitted virus. Since "...Viral titers were similar for all groups tested...," he suggested the possibility that "...differences in transmission rates may have been due to site-specific (i.e., salivary gland) replication, rather than a generalized increase in viral replication in mosquitoes inoculated at an earlier age." He further suggested that "Perhaps inoculation of larvae allowed virus to enter cells in the primordial tissue that was destined to become adult salivary glands, which might otherwise become refractory to infection during metamorphosis to the adult stage."

On the basis of the above and additional data, Dr. Turell and personnel in our laboratory began a collaborative study to see if in fact the differences in transmission involved differences in the infection of the salivary glands. Further, we wanted to compare infection in various mosquito tissues as a function of the timing of introduction of virus into the mosquito. In addition, we tested the effectiveness of the ABC immunocytochemical technique in identifying the presence of RVF viral infection, in deducing the stage at which infection occurred, and finally "predicting" whether or not a given specimen had been a transmitter.

In order to produce a series of mosquitoes infected at different developmental stages, samples of fourth instar larvae, pupae of different ages post-pupation, and adults were intrahemocoelically-infected with RVF virus with the ZH- 501 strain of RVF virus. All specimens were incubated for equivalent lengths of time, time enough for all specimens to reach the adult stage and become ready to blood feed. All specimens were tested for their ability to transmit virus to a hamster. In addition, uninfected, negative control adult mosquitoes were given blood meals. Following the transmission tests, each specimen was assigned

a code number so the immunocytochemical study would be carried out blind. Specimens were fixed in 10% formaldehyde for approximately 5 hours and stored in 70% ethyl alcohol. Serial paraffin sections were prepared according to standard microtechnical methods. The ABC immunocytochemical procedure was applied to all slides. Neither the infection state nor the time of infection was known to the persons who would be interpreting the immunocytochemically stained slides.

3. Immunocytochemical Study of Ockelbo Virus in *Culex pipiens*

The strain of Ockelbo virus used in our study was 84-N-140 which had been isolated from *Culiseta morsitans* in Edsbyn, Sweden in 1984. Mosquitoes were infected by IT inoculation and incubated for six days at 25°C. At the time of fixation the titer of virus was approximately 10^6 plaque forming units per millliliter. Mosquitoes were fixed by intrathoracic-injection with 5.0 % formaldehyde diluted with PBS buffer, followed by immersion in the same formaldehyde solution for approximately 6 hours. Prior to preparation of paraffin section and application of the ABC procedure, specimens were stored in 70 % ethanol.

The anti-Ockelbo mouse polyclonal antiserum we used had been prepared by Bo Nicklasson and titered out to a dilution of 1: 1,280. We tested dilutions ranging from 1:25 to 1:500 in the ABC procedure. The results showed a clearly graded staining response as a function of dilution, the lower dilutions showing too much staining and the higher dilutions, no staining at all. The best specific signal resulted when a 1:800 dilution was used.

Several controls were used in one or more of the four ABC trials included in this study:

(a) As a check that the ABC technique was working, we included in all four trials specimens which had been intrathoracically (IT) -infected with RVF virus and specimens which had been injected with viral diluent only. Antibodies directed against RVF viral antigens were applied to these sections. Infected specimens were consistently antigen-positive and uninfected specimens, antigen-negative.

(b) In each run, we also included diluent-injected, uninfected specimens to which we applied the anti-Ockelbo antiserum. These specimens were Ockelbo viral antigen-negative in all four trials.

(c) In three of the four trials, we included uninfected specimens to which we applied non-specific mouse antiserum in order to check for non-specific binding by IgG. These specimens were consistently antigen-negative.

(d) In order to determine if there was non-specific binding to mosquito cells by the various immunoreagents, we included, in two of our ABC trials, infected controls in which the primary antibody or the biotinylated secondary antibody or the ABC complex was omitted. In the absence of any one of the reagents used in the ABC technique, specimens, Ockelbo antigen did not stain.

(e) Ultrastructural examination of various tissues for RVF virus in intrahemocoelically

Samples of Culex pipiens were infected intrahemocoelically with RVF virus and after a few days incubation, were killed and fixed for electron microscopy.

(f) Ultrastructural Study of CCHF virions in Tick Tissues

Several specimens of the tick, Hyaloma impeltatum were infected with CCHF and incubated for several days. Subsequently, they were dissected into gut, testes, salivary glands, etc. and prepared for electron microscopy.

G. Techniques applied in the search for the receptor

1. Introduction

Several techniques have been applied in an attempt to purify the cell surface receptor for the Rift Valley fever virus. These techniques have included hybridoma technology, biochemical fractionation of a crude cell extract, and the development of anti-idiotypic antibodies specific for putative receptor moieties.

We have had three potential sources of mosquito receptor molecules available to us: (1) Aedes albopictus cells (C6/36) in culture; (2) homogenized whole mosquitoes; and (3) specific tissues and organs which can be dissected from whole mosquitoes. For the research reported here, we have relied on the use of C6/36 cells.

Four virus preparations have been potentially available to us: (1) live Rift Valley fever virus; (2) cobalt irradiated live RVF virus; (3) a mouse liver extract of the HA antigen of RVF virus, BPL inactivated; and (4) a formalin-treated virus vaccine preparation, prepared in monkey lung cell culture. Since use of live RVF virus would require that all work be done in the P-3+ containment facility at USAMRIID and since no cobalt irradiated RVF virus was immediately available and although feasible to prepare would require extensive safety testing before release from Fort Detrick, only preparations #3 and #4 have

been used at Ohio University. All assays requiring the use of live virus were done in a P-3+ level biological containment facility at USAMRIID.

2. Cell Culture and Extraction

Aedes albopictus (C6/36) cells (ATCC CRL 1660) were grown in T-150 flasks in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum or fetal calf serum, 50 micrograms of gentamycin/ml, and 10% tryptose phosphate broth. Cells were collected by slapping the side of the tissue culture flask and pelleting the cells by centrifugation. Cells were washed three times in phosphate buffered saline (PBS) containing 0.14 M sodium chloride, 2.7 mM potassium chloride, 8.10mM dibasic sodium phosphate, and 1.50 mM monobasic potassium phosphate, and either extracted immediately or frozen in 10% glcerol-PBS.

C6/36 cells were extracted into a buffer consisting of 10 mM Chapso in 0.05% Tris-HCl, pH 8.0. Cells were suspended in extraction buffer at a ration of 1:5 and the enzyme inhibitors phenylmethylsulfonyl fluoride, pepstatin, and aprotinin were added to yield final concentrations of 0.174, 0.006, and 0.0025 mg/ml respectively. Cells were extracted by vigorously voretxing them in extraction buffer for 40 minutes in a tube jacketed with an ice-water bath. The extraction mixture was centrifuged at 35,000 x g for 1.5 hours at 4°C. The supernatant was collected and dialyzed overnight at 10°C against PBS. The extract was collected and stored in aliquots at -20°C.

3. Chromatography and Protein Precipitation

Anion exchange chromatography was performed on a Mono Q 5/5 column on the FPLC system. Three milliliters of the crude C6/36 cell extract were dialyzed against 20 mM Tris-HCl, pH 7.5, and applied to the column. The column was eluted with a NaCl gradient in 20 mM Tris-HCl, pH 7.5, going from 0.0 to 0.35 M NaCl. The flow rate was set at 0.5 ml/minute, the recorder chart speed was set at 0.5 cm/minute, and the fraction collector was set to collect one fraction every two minutes. Twenty-three 1 ml fractions were collected.

Gel filtration chromatography was performed on a Superose 12 column on the FPLC system. Five-hundred microliters of crude C6/36 extract, or pooled anion exchange fractions concentrated in Centricon 10 Microconcentrators, was applied to the column and eluted with 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 M NaCl. The flow rate was set at 0.5 ml/minute, the recorder chart speed was set at 0.5 cm/minute, and the fraction collector was set to collect one fraction every two minutes. Thirty 1 ml fractions were

collected. The gel filtration column was calibrated with a MW-GS-1000 gel filtration calibration kit from Sigma.

4. Ammonium Sulfate Precipitation

The solution used for protein precipitation was prepared by stirring 200 g of ammonium sulfate in 200 ml of distilled water, allowing the the mixture to equilibrate overnight at room temperature, and adjusting the pH of the solution to 7.2 with NaOH. The crude C6/36 extract was precipitated by mixing the extract and the saturated ammonium sulfate solution at a 1:1 ratio, incubating the mixture for 30 minutes at room temperature, and centrifuging the mixture at 1,000 x g for 15 minutes at room temperature. The supernatant solution was collected, dialyzed extensively against PBS at 10 C, and frozen at -20°C for future use. The precipitated pellet was solubilized in PBS and reprecipitated again under the same conditions. The pellet was solubilized in PBS and stored at -20°C for future use.

5. Polyacrylamide Gel Electrophoresis (PAGE)

Discontinuous PAGE in the presence of sodium dodecylsulfate (SDS) was performed according to the method of Laemmli (1970). Samples were either mixed 1:1 with sample buffer, concentrated in Centricon 10 microconcentrators before mixing 1:1 with sample buffer, or precipitated with cold acetone and dried, and dissolved directly in sample buffer. Acetone precipitation was performed by mixing the sample 1:5 with acetone at -20°C, incubating the sample for 10 minutes at -20°C, centrifuging the sample at 10,000 x g for 5 minutes, and drying the resultant pellet. All gels were stained for protein with Coomassie Brilliant Blue.

6. Immunization for Polyclonal Antibody Production

In an attempt to obtain polyclonal antibody specific for Rift Valley fever virus (RVFV), mice were immunized with either whole C6/36 cells, or a neutralizing monoclonal antibody specific for the hemagglutinating antigen (HA) of the RVFV. To immunize mice against whole cells, Balb/c mice were injected with approximately 100 microliters of C6/36 cells in PBS at two week intervals for eight weeks. Blood samples were colected from the mice by ocular bleed.

To immunize mice against the neutralizing monoclonal antibodies (directed against RVFV spike glycoproteins), Balb/c mice were injected with an emulsion consisting of 1 part Freund's Adjuvant and one part PBS containing eight parts specific ascites per thousand parts emulsion. The emulsions for the initial emulsions were prepared with Freund's Complete Adjuvant, while subsequent emulsions were

prepared with Freund's Incomplete Adjuvant. The specific ascites samples used were R1-5G2, R1-1G6, R1-4B6, and R1-4D4, and were supplied by Dr. Jonathan Smith, USAMRIID. These antibodies had plaque reduction titers of 25,600, 1,600, 25,600, and greater than 10,240 at 80% plaque reduction. Blood samples from these mice were obtained by ocular bleed.

7. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates for the detection of anti-idiotypic antibodies specific for RVFV receptors were prepared by coating plates with either whole C6/36 cells or C6/36 cell crude extract. The plates coated with whole cells were prepared by growing C6/36 cells to confluency in 96-well tissue culture plates, washing the cells gently three times with PBS, fixing the cells for 15 minutes in 0.25 % glutaraldehyde in PBS, washing the plate two times with PBS, and drying the plates. The plates were stored at 4°C for future use.

Plates prepared using the crude extract were made by coating the wells of a 96-well microtiter plate with 50 microliters of a 1:50 dilution of the crude extract in PBS, and incubating the plate eitehr two hours at room temperaturre, or overnight at 4°C. The coated wells were washed two times with PBS and used immediately. The plates, either cell-coated or extract-coated, were blocked with 1 % bovine serum albumin (BSA) for one hour at room temperature. The serum samples to be tested were diluted in 1 % BSA-PBS-0.05% Tween-20 and applied to coated wells and bland wells in 50 microliter aliquots and incubated for 1.5 hours or overnight at 4°C. All wells were washed three times with PBS containing 0.05% Tween-20. Horseradish peroxidase-labelled goat anti-mouse IgG, obtained from Tago Immunochemicals, was diluted 1:10,000 in 1% BSA-PBS-0.05% Tween-20, applied to the plates in 50 microliter aliquots and incubated for 1.5 hours. All wells were washed three times with the PBS-Tween wash solution, tapped dry, and filled with 50 microliters/well of substrate solution. The substrate solution was prepared by adding 5 microliters of 30% hydrogen peroxide, and 0.02 g of O-phenylenediamine to citrate-phosphate buffer made by titrating 34 mM citric acid to pH 6.0 with 130 mM dibasic sodium phosphate. The plates were allowed to incubatet for 15 minutes in the dark at room temperature, and the absorbance of all of the well at 490 nanometers was read and recorded by an automated ELISA plate reader.

H. Workshop in Mosquito Histology, Ultrastructure, and Methods for In situ Localization of Virus

Following is the description of the workshop which appeared in the American Society of Tropical Medicine & Hygiene Newsletter:

"The American Committee of Medical Entomology and the Ohio University Tropical & Geographical Disease Institute are co-sponsoring a pre-meeting workshop entitled "Mosquito Histology, Ultrastructure and Methods for the In situ Localization of Viruses" on Sunday, December 1, 1991 in conjunction with the ASTMH meeting in Boston. This workshop is being supported in part with funds from the U.S. Army Medical Research & Development Command."

"This workshop will be presented by personnel associated with the O.U. Tropical & Geographical Disease Institute and tentatively will include the following topics: (1) An introduction to the internal anatomy & histology of mosquitoes; (2) An introduction to mosquito ultrastructure; (3) The use of immuno- and nucleic acid probes for in situ localization; and (4) Specific examples of the application of immuno- and nucleic acid probes for the in situ detection of viral antigens and genomic material. This workshop is intended for persons with minimal background in the topics to be presented."

"The fee for participation will be $50.00. Materials for particpants to keep will include a box of slides of selected histological preparations, a packet of electron micrographs, and various protocols for in situ studies. Enrollment in this workshop will be limited to 15. For further information, contact Dr. William S. Romoser, Tropical & Geographical Disease Institute, Department of Zoological & Biomedical Sciences, Ohio University, Athens, Ohio 45701. Phone: (614) 593-2372 FAX: (614) 593-0300."

Sponsorship: Co-sponsored by The American Committee of Medical Entomology and the Ohio University Tropical & Geographical Disease Institute. Supported in part by the U.S. Army Medical Research & Development Commmand.

I. Monograph on Mosquito Histology & Ultrastructure

During the course of our histological and ultrastructual studies, we have accumulated a large number of light- and electron photomicrographs, many of which are suitable for inclusion in a monograph.

III. Results and Discussion

A. RVF Virus in the Cardia

Aspects of the histology and ultrastructure of the cardia were described in the last final report (DAMD17-86-C-6133). We have continued to study the occurrence of RVF virus in the tissues which compose the cardia. It is clear that RVF virus has a strong predelection for the tissues of the cardia. One can consistently find RFV virions in these tissues in intra-hemocoelically-infected tissues (Figs. 1-3).

B. Immunocytochemical & Ultrastructural Studies

Anopheles Salivary Gland Barrier

The ABC technique proved to be very effective in determining whether or not a specimen was infected with virus. Antigen was detected in 100% (60/60) of the IT-infected specimens, i.e. there were no false negatives and the ABC technique produced negative results in 93.3% (14/15) of the uninfected specimens, i.e. there was a single false negative (Table 1). The overall predictive value of the ABC technique, i.e. correct identification of infected and non-infected specimens, was 98.7%

As with the first run of this experiment, our results were consistent with those of Turell, that is the more advanced the developmental stage (larva, pupa, adult) when infected with RVF virus, the lower the rate of transmission by the adult stage (Table 2). In our study, among adults produced from IT-infected larvae, 100% (12/12) of the larvae infected more than 24 hours before pupation and 93.3% (14/15) of larvae infected less than 24 hours before pupation transmitted virus to hamsters; among those infected as less than 4 hour old pupae, 25% (3/12); among those infected as more than 24 hour old pupae, 22.2% (2/9); and among adults which were IT-infected as adults and incubated a period of time equivalent to those infected as larvae and pupae, none transmitted.

The development of a salivary gland infection barrier is apparent when the frequency of salivary gland infection as a function of developmental stage at infection (Table 3), that is the later the developmental stage infected, the lower the frequency of salivary gland infection. No mosquitoes infected as adults transmitted virus and as shown in this Table 3, none had detectable antigen in the salivary glands. It, therefore, appears that there is a salivary gland barrier operating.

Figure 1. RVF virions in the cardia. Arrow indicates virion in spongy basal lamina.

Figure 2. RVF virions in the cardia. Virions can be seen both on the spongy basal lamina-side and on the foregut intima-side of the intussuscepted foregut epithelium (arrows).

Figure 3. RVF virus assembly in the cardia (arrow).

TABLE 1

VALIDITY OF THE ABC IMMUNOCYTOCHEMICAL "TEST" TO DETECT

ANOPHELES STEPHENSI

INFECTED WITH RIFT VALLEY FEVER VIRUS

		ACTUAL	
		+	-
PREDICTED	+	60	0
	+	1	14

SENSITIVITY = 98.4%

SPECIFICITY = 100 %

PREDICTIVE $VALUE_{pos}$ = 100 %

PREDICTIVE $VALUE_{neg}$ = 93.3 %

PREDICTIVE $VALUE_{overall}$ = 98.7 %

TABLE 2

Transmission of Rift Valley fever virus by

Anopheles stephensi

as a function of developmental stage at infection

Stage Infected	Sample Size	Number that Transmitted	Percent Transmission
larvae >24 h	12	12	100
larvae <24 h	15	14	93.3
pupae <4 h	12	3	25
pupae >24 h	9	2	22.2
adult	7	0	0

TABLE 3

Distribution of RVF viral antigen in the salivary glands of *Anopheles stephensi* as a function of developmental stage at infection.*

Developmental Stage Infected	Antigen in Salivary Glands		
	Entire	Proximal lobes	Distal lobes
larvae <24 h	100 (14/14)	85.7 (12/14)	57.1 (8/14)
larvae >24 h	100 (16/16)	81.8 (9/11)	54.6 (6/11)
pupae <4 h	54.6 (6/11)	27.3 (3/11)	45.5 (5/11)
pupae >24 h	0 (0/9)	-	-
adult	0 (0/7)	-	-

* Percent (number RVF viral antigen positive/number examined.

Since transmission by the adult decreases the more advanced the developmental stage and since some adults infected as pupae do not transmit, it appears that the salivary gland barrier develops some time during the pupal stage.

None of the non-transmitting adults contained antigen in their salivary glands. One might argue that virus may replicate at different rates depending on the timing of injection relative to developmental stage. However, Turell (1988), studying Anopheles albimanus, found, that when incubation times were equivalant, similar viral titers were produced regardless of the timing of infection.

As is evident in Table 3, in a large percentage of mosquitoes with salivary gland infections antigen was detected in both the proximal and distal regions. However, there were transmitters transmitters in which only the distal regions or only the proximal regions of the salivary gland lobes are infected is evidence that transmission can occur via either of these regions. However, among all transmitters, the proximal region showed the highest frequency of infection.

Infection barriers appear to be operating in tissues other than the salivary glands (Table 4). This is particularly evident in the pharyngeal pump muscles which showed a high frequency of infection in adults infected as larvae or early pupae, but decreased to a frequency of 0 in those infected as old pupae or as adults. Similar effects appear to be operating in association with the intussuscepted foregut (IF) and the hindgut epithelium, although in both of these tissues the infection barriers appear to form later in the pupal or even adult stage in the case of the IF.

On the basis of examining the pattern of infection of various tissues, and since our experiment was blind, we attempted to deduce the developmental stage at which infection occurred and to "predict" which specimens in our samples were transmitters. As shown in Table 5, we were correct in our deduction of stage infected relative to 76.2 % of the specimens examined.

We were correct in our assessment of transmitters relative to 88.3 % of the specimens (Table 6).

TABLE 4

Distribution of RVF viral antigen in various tissues of *Anopheles stephensi* as a function of developmental stage at infection

Stage Infected	Pharyngeal Pump Muscle	Intussus. Foregut	Hindgut
larvae <24 h (n = 14-16)	93.8	100	100
larvae >24 h (n = 13-14)	85.7	92.9	100
pupae <4 h (n = 11-12)	58.3	72.7	91.7
pupae >24 h (n = 8-9)	0	22.2	75
adult (n = 7)	0	14.3	0

TABLE 5

VALIDITY OF THE ABC IMMUNOCYTOCHEMICAL "TEST" IN PREDICTING TRANSMISSION OF VIRUS BY ANOPHELES STEPHENSI INFECTED WITH RIFT VALLEY FEVER VIRUS

		ACTUAL	
		+	-
PREDICTED	+	28	4
	+	6	4

SENSITIVITY = 82.4%

SPECIFICITY = 50 %

PREDICTIVE $VALUE_{pos}$ = 87.5 %

PREDICTIVE $VALUE_{neg}$ = 40 %

PREDICTIVE $VALUE_{overall}$ = 76.2 %

TABLE 6

VALIDITY OF THE ABC IMMUNOCYTOCHEMICAL "TEST" TO IDENTIFY THE STAGE AT WHICH ANOPHELES STEPHENSI HAS BECOME INFECTED WITH RIFT VALLEY FEVER VIRUS

		ACTUAL	
		+	-
PREDICTED	+	35	0
	+	7	18

SENSITIVITY = 83.3 %

SPECIFICITY = 100 %

$PREDICTIVE\ VALUE_{pos}$ = 100 %

$PREDICTIVE\ VALUE_{neg}$ = 72 %

$PREDICTIVE\ VALUE_{overall}$ = 88.3 %

Immunocytochemical Study of Ockelbo Virus in *Culex pipiens*

As previously shown in RVF virus studies, the Malpighian tubules, the pericardial cells, and the nephrocytes stained non-specifically.

The tissues that were found to stain specifically for Ockelbo viral antigens included the following: fat body, all foregut tissues (i.e. the esophagous, the diverticula, and the intussuscepted foregut); the salivary glands; the cell bodies of the cephalic, thoracic and abdominal ganglia; and Johnston's organ.

Tissues in which antigen was not detected were: the ommatidia of the compound eyes; skeletal muscles; neuropile of the various ganglia; the midgut epithelium; the common oviduct, follicular epithelia, and oocytes & nurse cells. With regard to thje midgut, it is important to note that these specimens were IT-infected and it seems likely, as is the case with RVF virus, that these tissues would become infected with longer incubation periods.

We noted two particularly significant differences between Ockelbo virus mosquito tissue tropisms and Rift Valley fever tropisms: (1) Although we did see evidence of intussuscepted foregut infection with Ockelbo virus, it was much less extensive than with RVF virus. (2) The ommatidia of the compound eyes were found to be extensively infected with RVF virus whereas they do not appear to be susceptible to infection with Ockelbo virus.

Ultrastructural Examination of Various Tissues

The results of applying the ABC immunocytochemical technique to mosquitoes infected with RVF virus were equivocal in regard to muscle and tracheal tissues. Ultrastructural study revealed that putative RVF virions can be found in both of these tissues (Figs. 4-7)

Ultrastructural examination of the salivary glands revealed the greatest density of virions we have observed in any tissue. RVF virions are clearly assembled in association with smooth membrane (Figs. 8 & 9). We observed virions tightly packed in apparent paracrytalline arrays in the salivary glands (Figs. 10 & 11).

In addition to the above-mentioned tissues, we have observed RVF virions in fat body (Fig. 12) and nerves (Fig. 13).

Figure 4. RVF virions in muscle tissue (arrows).

Figure 5. RVF virions in muscle (arrows)

Figure 6. RVF virions in trachea (arrows).

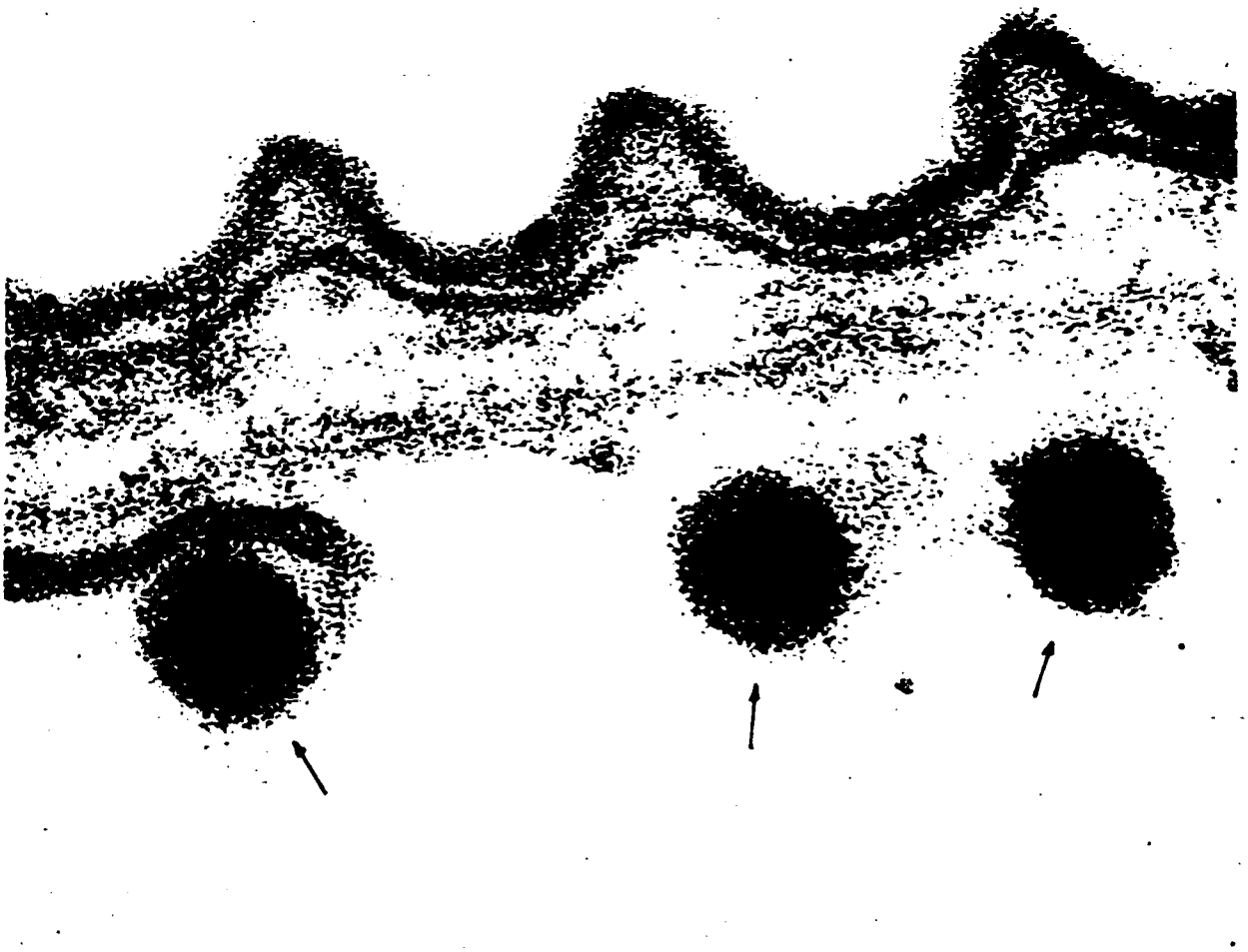

Figure 7. RVF virions in trachea (>250,000 X) (arrows)

Figure 8. RVF virions in salivary gland cell.

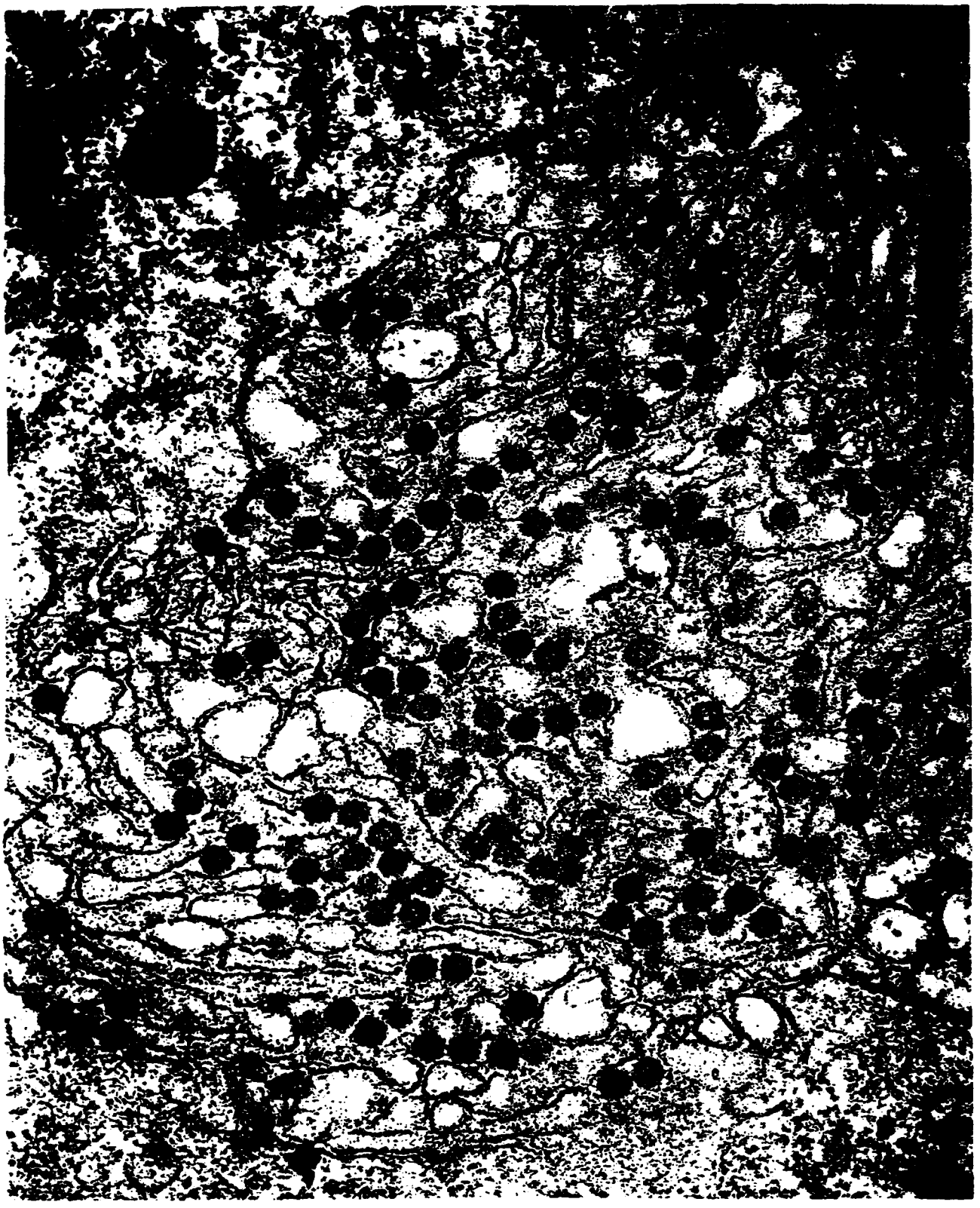

Figure 9. RVF virions in salivary gland cell; assembly in asssociation with smooth membrane.

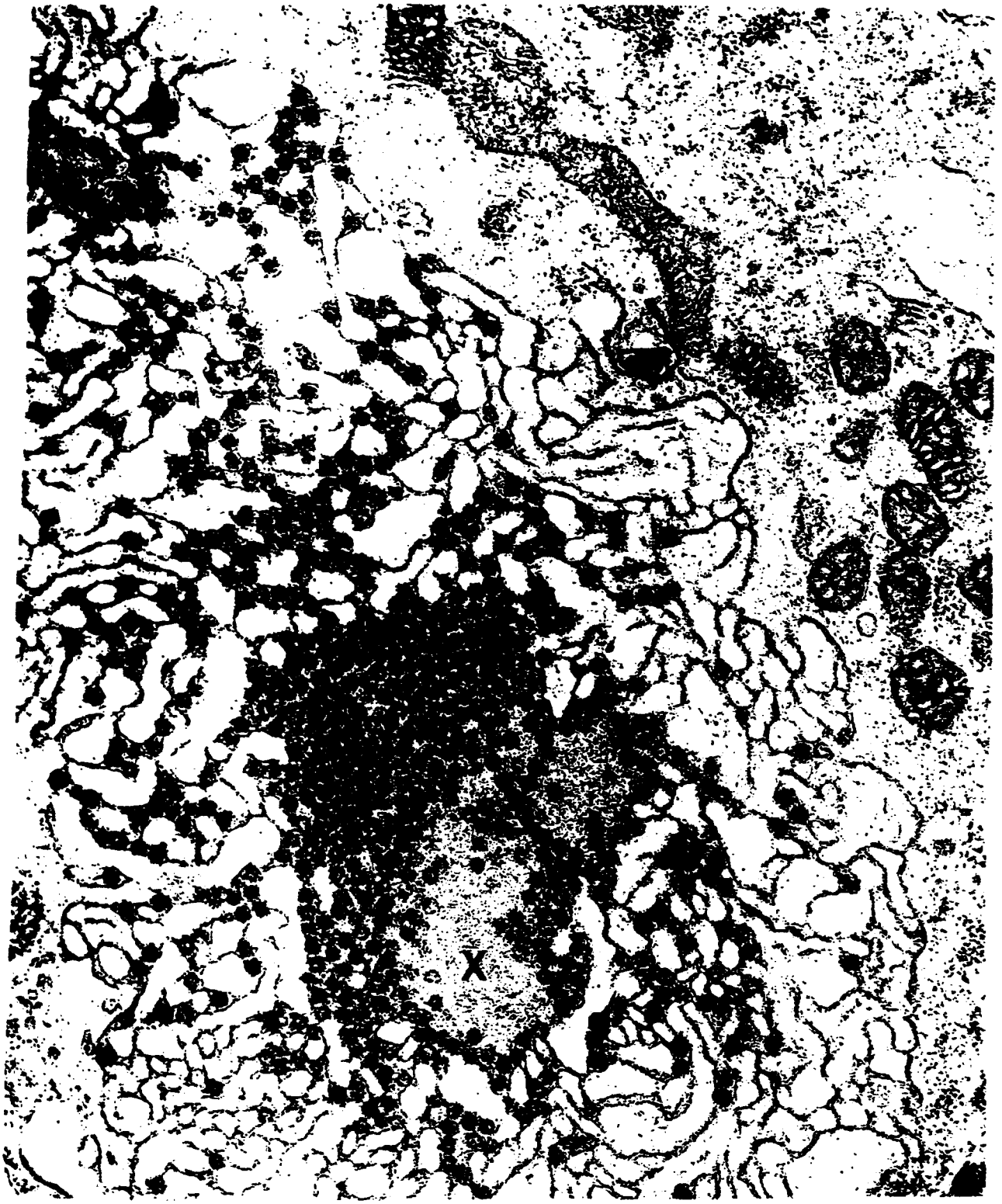

Figure 10. An array of RVF virions in salivary gland cell; appear to be entering apical cavity (x).

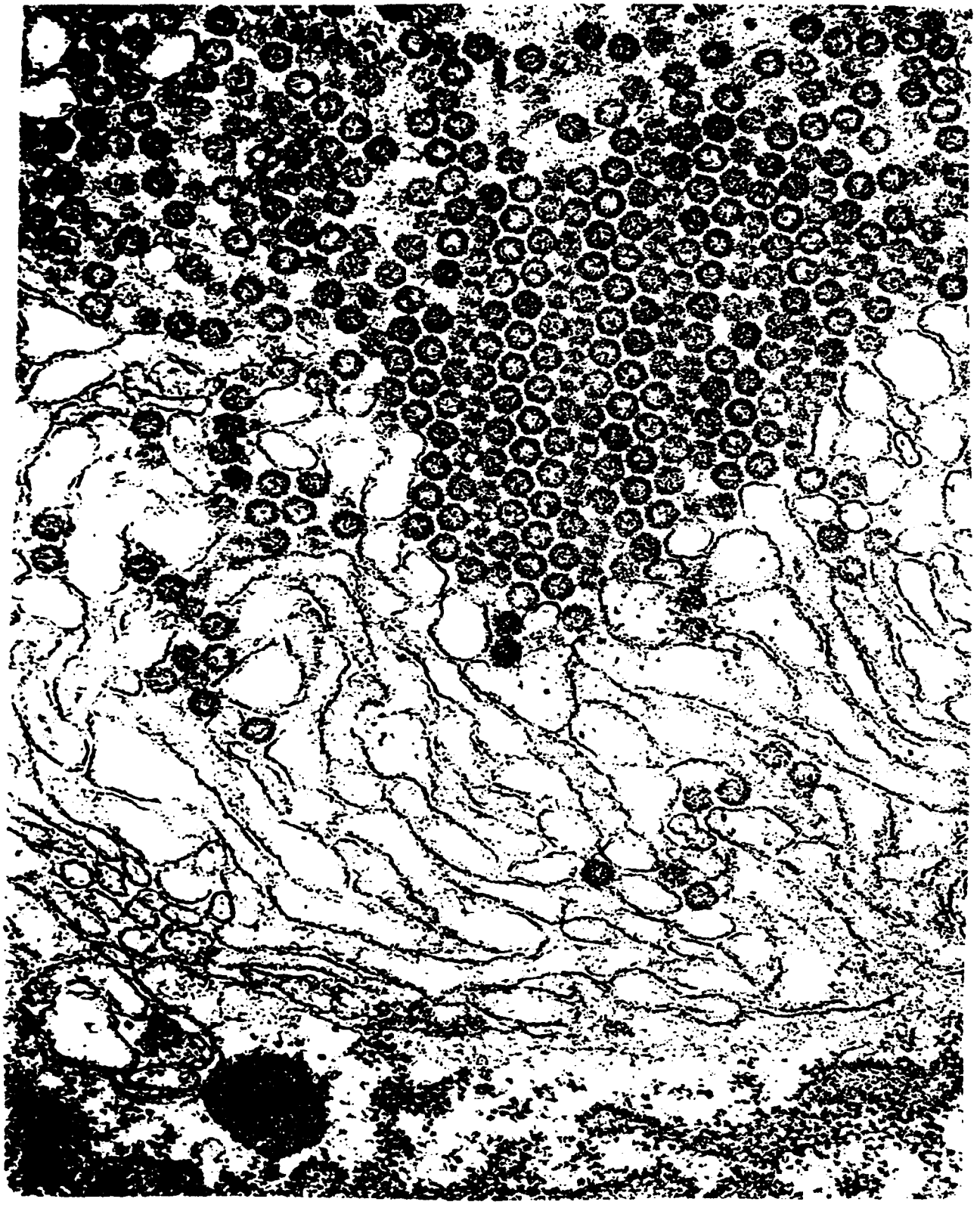

Figure 11. Array of RVF virions in salivary gland cells.

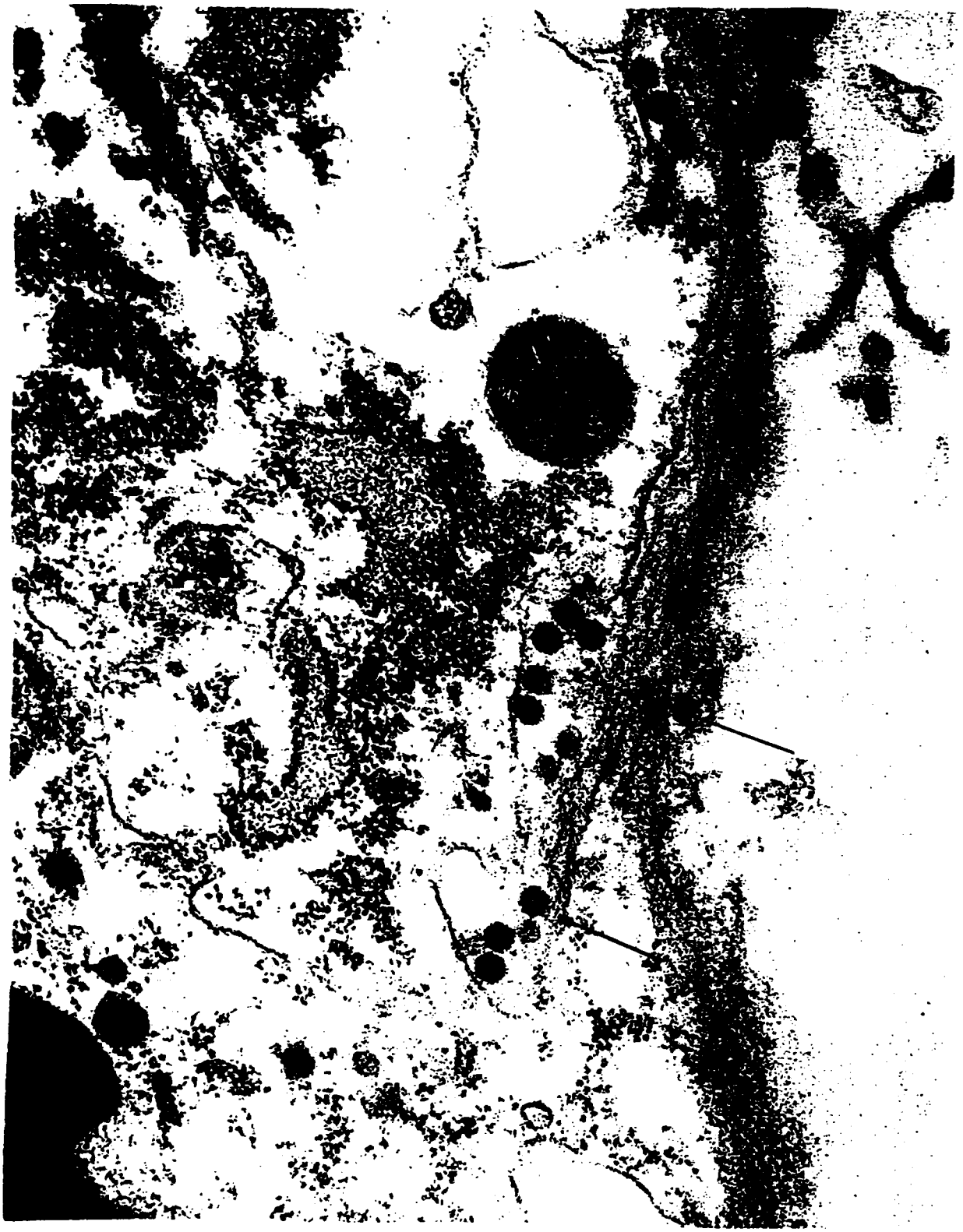

Figure 12. RVF virions in fat body (arrow)

Figure 13. RVF virions in neuron (arrow)

Immunogold

Once we were able to achieve consistent labelling with gold-conjugated antibody (Figs. 14 & 15), we determined the following: (1) Examination of the control ultrathin sections did not reveal non-specific labelling. (2) The particles, both intracytoplasmic and extracellular, which we had been interpreting as RVF virions were labelled. (3) For extracellular particles, most of the colloidal gold labeling was found to be on the envelopes of the virions. (4) Labelling of colloidal gold on the RVF virions was less dense than would be expected had we used a pre-embedding procedure.

Using RVF virus-specific and goat anti-mouse IgG and/or IgM conjugated to colloidal gold as markers, we successfully achieved *in situ* labelling of putative virus particles providing a degree of assurance that these particles were indeed RVF virions. In our opinion, that the labelling was less dense than would be expected had we used a pre-embedding procedure is due to the difficulties inherent in exposing antigenic sites to gold-labelled antibodies.

Ultrastructural Study of CCHF-Infected Tick

Although our results are preliminary, we found putative CCHF virions in the salivary glands (Figs. 16 & 17), testes (Figs. 18 & 19), and attached to the surface of a tracheolar cell (Fig. 20).

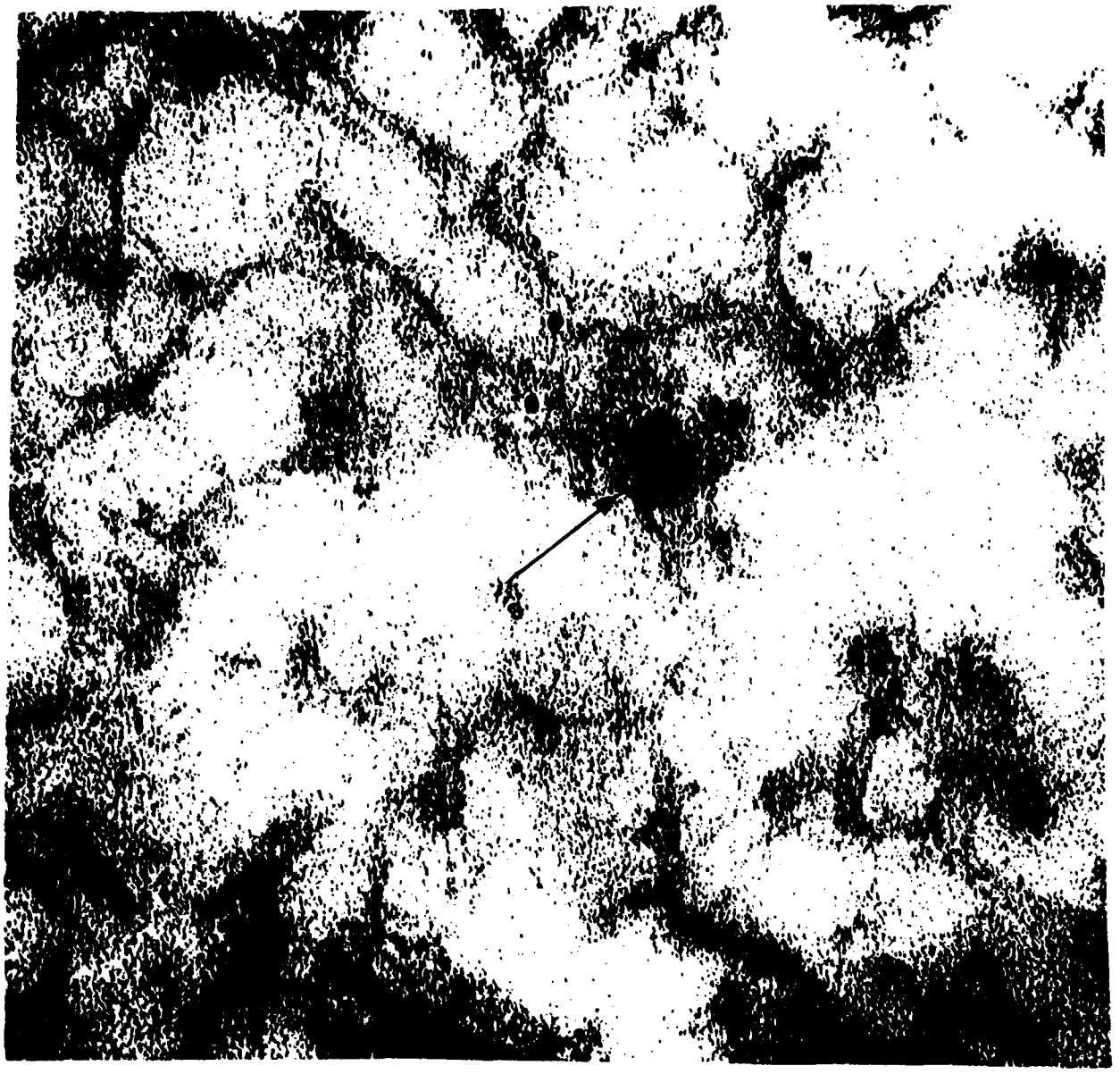

Figure 14. Immunogold-labelled RVF virion (arrow); black dots are gold particles.

Figure 15. Immunogold-labelled RVF virion (arrow).

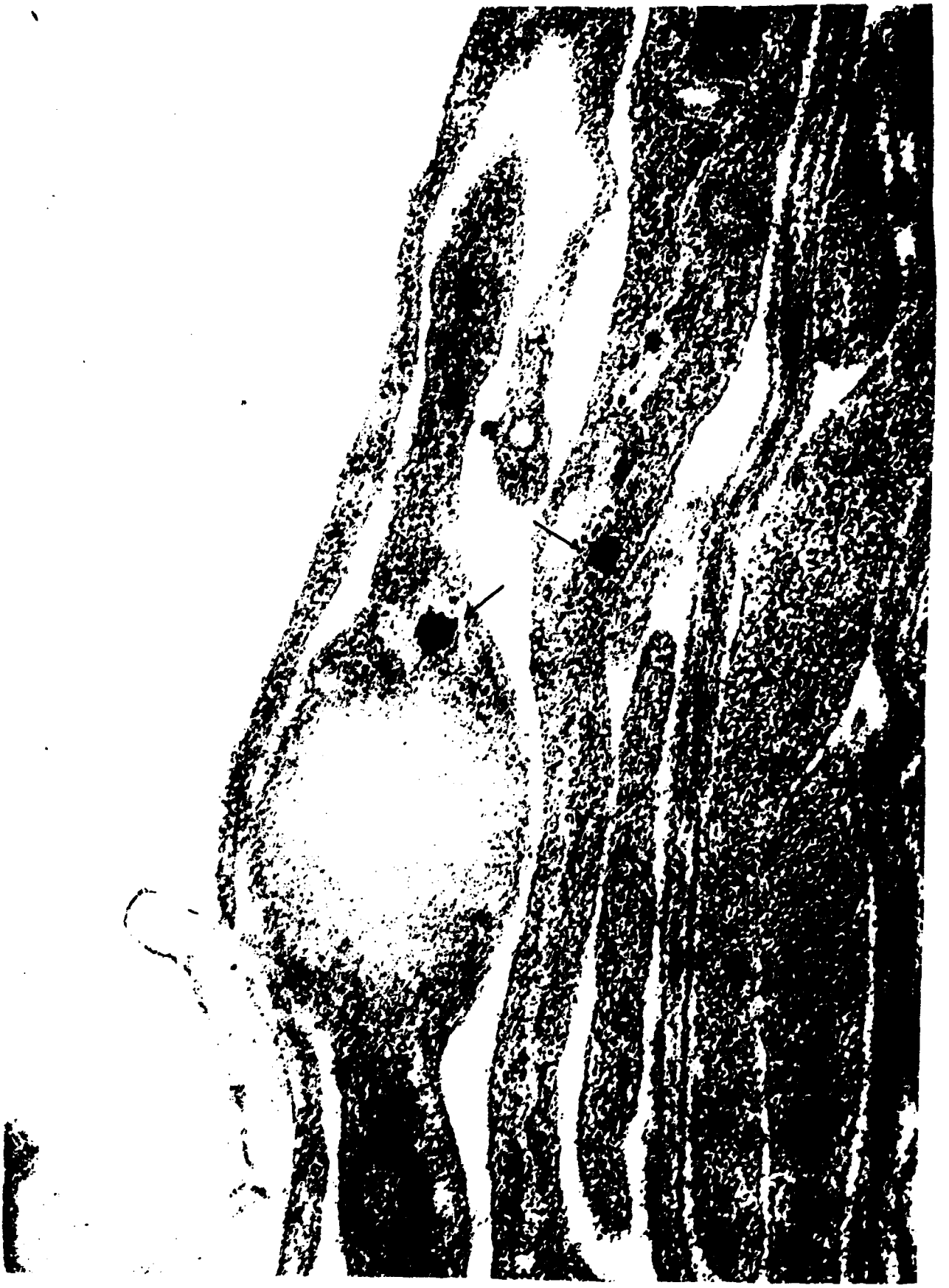

Figure 16. Putative CCHF virions (arrows) in salivary gland cell of the tick H. impeltatum.

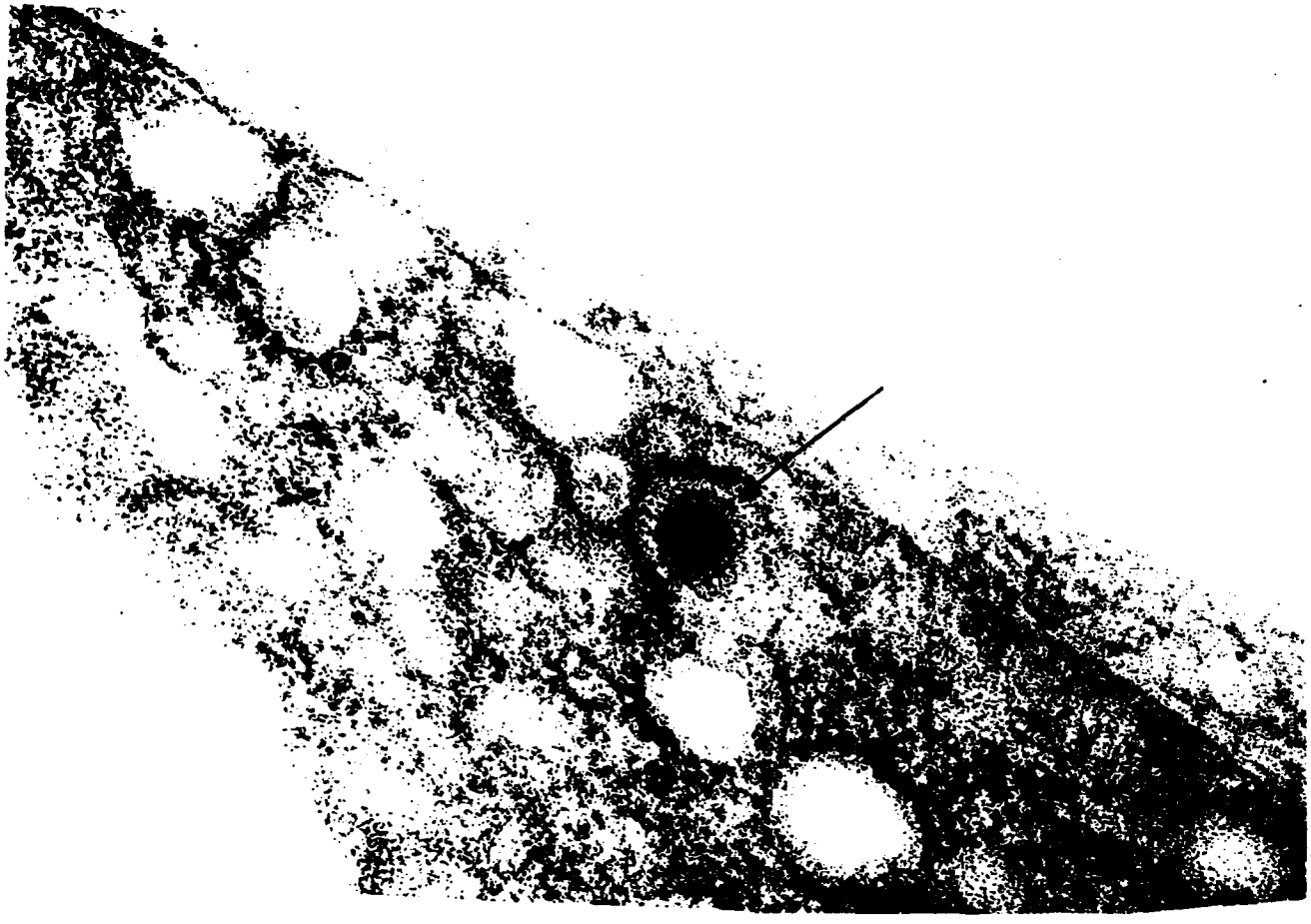

Figure 17. Putative CCHF virion (arrow) in salivary gland cell of the tick H. impetatum.

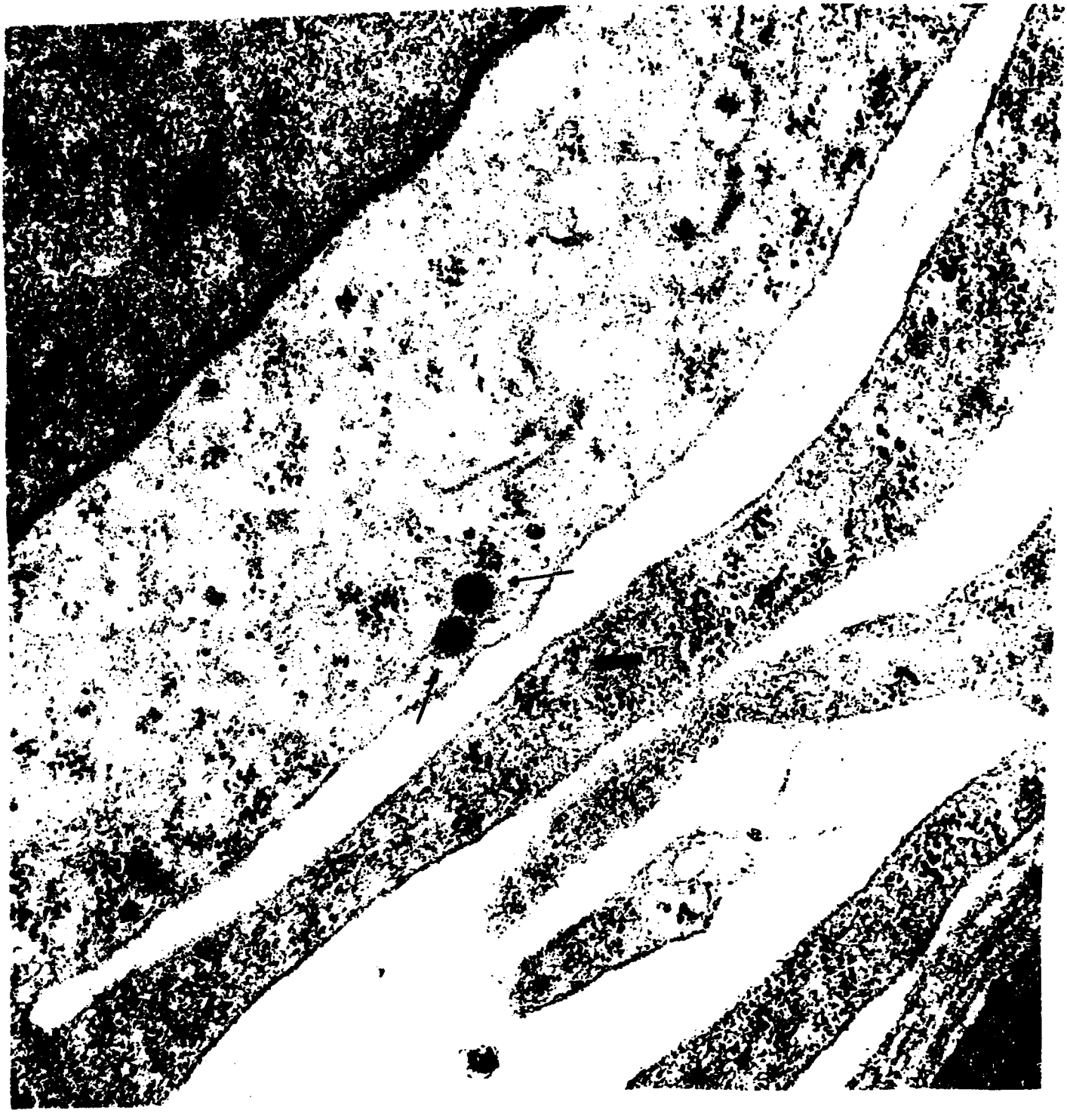

Figure 18. Putative CCHF virions (arrows) in testicular cell of the tick H. impeltatum.

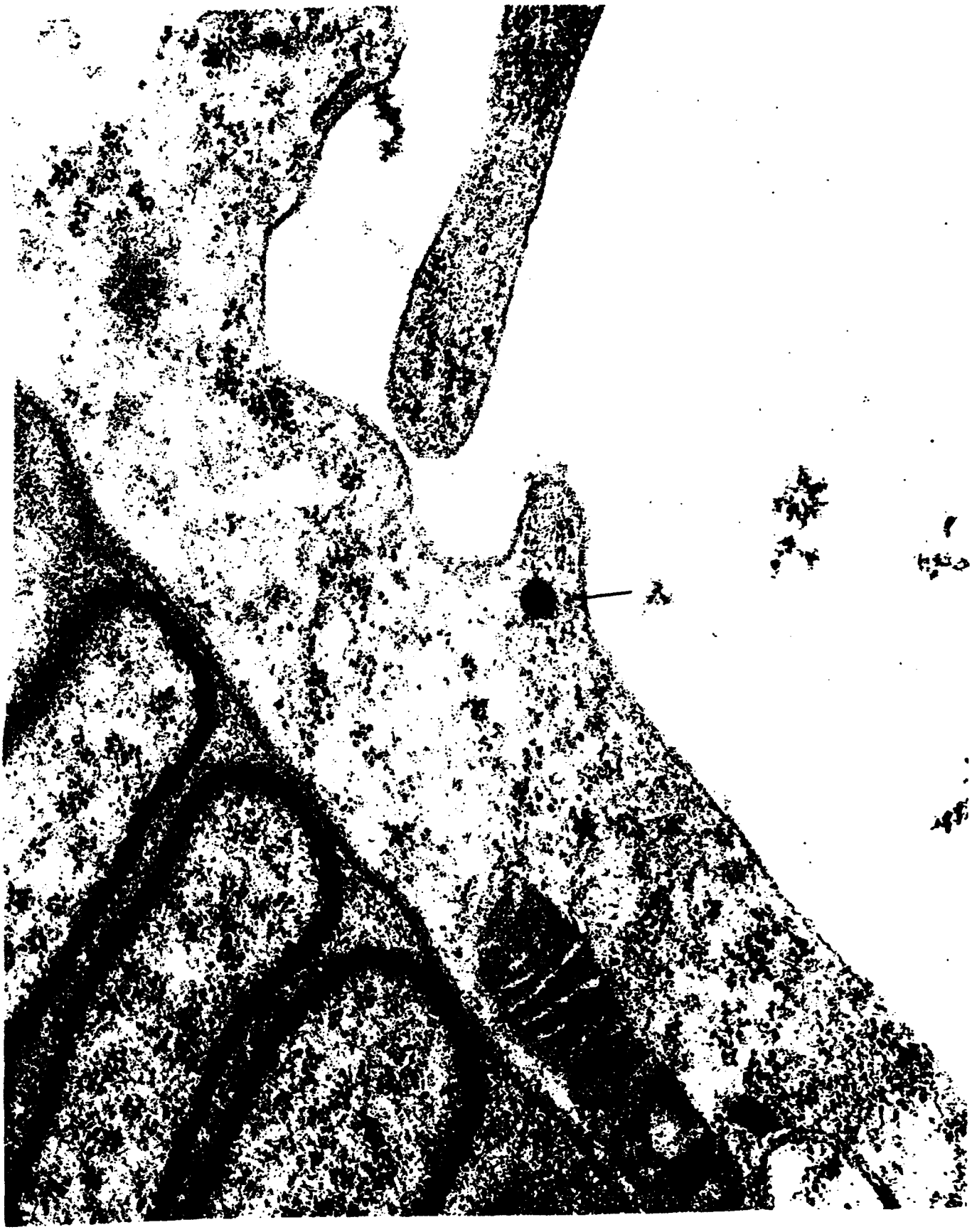

Figure 19. Putative CCHF virion (arrow) in testicular cell of the tick H. impeltatum

Figure 20. Putative CCHF virion (arrow) attached to a tracheal cell in a female tick, H. impeltatum.

Search for the RVF Virus Mosquito Cell Surface Receptor

Although our first attempt with mouse polyclonal antisera directed against C6/36 cells was promising (see Final Report DAMD17-86-C-6133), monoclonal antibodies produced with cells from these mice and additional polyclonal antisera developed in other mice failed to produce inhibition of viral infectivity in cell monolayers. Likewise, tests of a variety of lectins, HA antigen, and anti-idiotypic antibodies directed against monoclonal antibodies against the surface glycoprotein spikes of RVF virus produced negative or at best equivocal results. However, the anti-idiotypic antibody approach deserves further testing. We also obtained some interesting results using biochemical extraction and purification techniques as explained below.

Chromatography

Attempts to fractionate the crude C6/36 extract using anion exchange chromatography revealed that only a small proportion of the total protein content of the crude extract was not adsorbed to the column in 20 nM Tris-HCl, pH 7.5, indicating that the majority of the extracted proteins have a net negative surface charge under these conditions. Separation of the anion exchange fractions using SDS-PAGE gel (Fig. 21) showed that most of the column fractions were still complex mixtures of proteins.

We also fractionated the crude C6/36 extract using gel filtration chromatography. This procedure revealed a large sharp peak at the beginning of the chromatogram which was most likely micelles which were above the exclusion limit of the column. This was corroborated by an SDS-PAGE gel of gel filtration fractions of the crude extract, which showed that fraction 7 contains proteins over a wide range of molecular weights (Fig 22). Figure 22 also shows that a rough fractionation according to molecular weight was achieved. Fractions 12 and 13 showed possible inhibition of viral plaque formation (see "Plaque Assay" below). Since anion exchange fractions 15-22 (Fig. 21) of the crude extract showed possible inhibition of viral plaque formation (see "Plaque Assay" below) , these fractions were pooled and concentrated in a Centricon 10 microconcentrator and separated on a gel filtration column. The microsomal fraction was significantly reduced. Figure 23, a SDS-PAGE gel of the gel filtration of anion exchange fractions 5-22 shows that a significant purification of the extract was been achieved and that the majority of the protein elutes in fractions 11-15.

Figure 24 is a SDS-PAGE gel of the samples produced in the ammonium sulfate precipitation of the crude C6/36

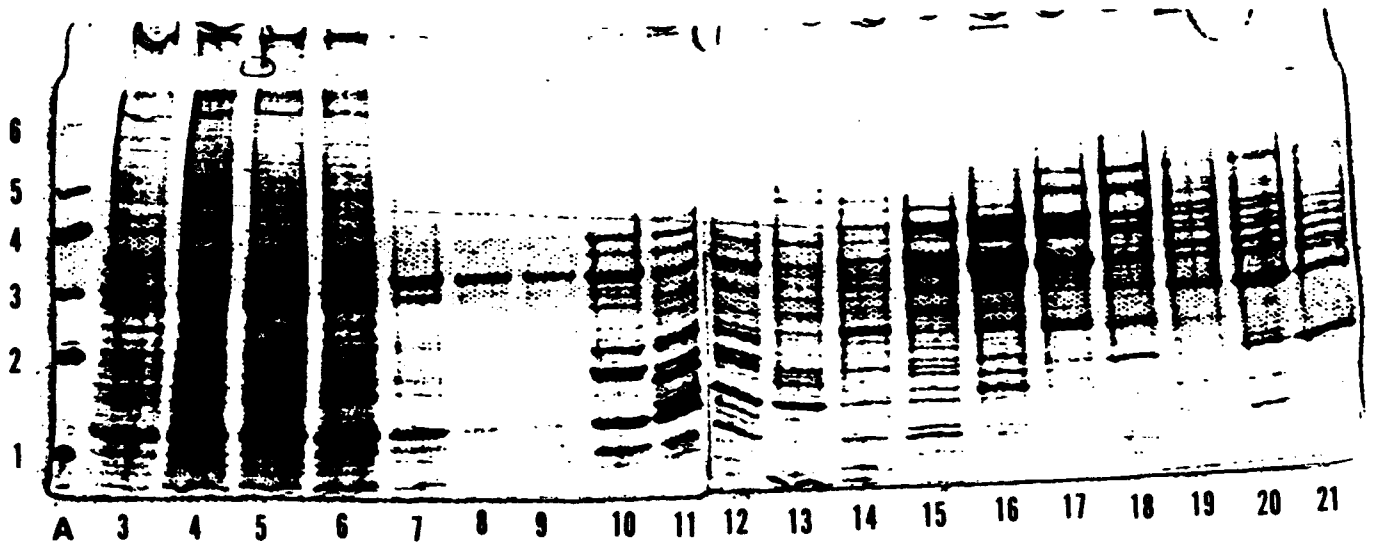

Figure 21. SDS-PAGE gel of anion exchange chromatography fractions (3-21) of the C6/36 cell crude extract. A = molecular weight standards (1, -lactalbumin, 14,200 daltons; 2, carbonic anhydrase, 29,000; ovalbumin, 45,000; BSA, 66,000; urease-monomer, 240,000; urease-dimer, 480,000).

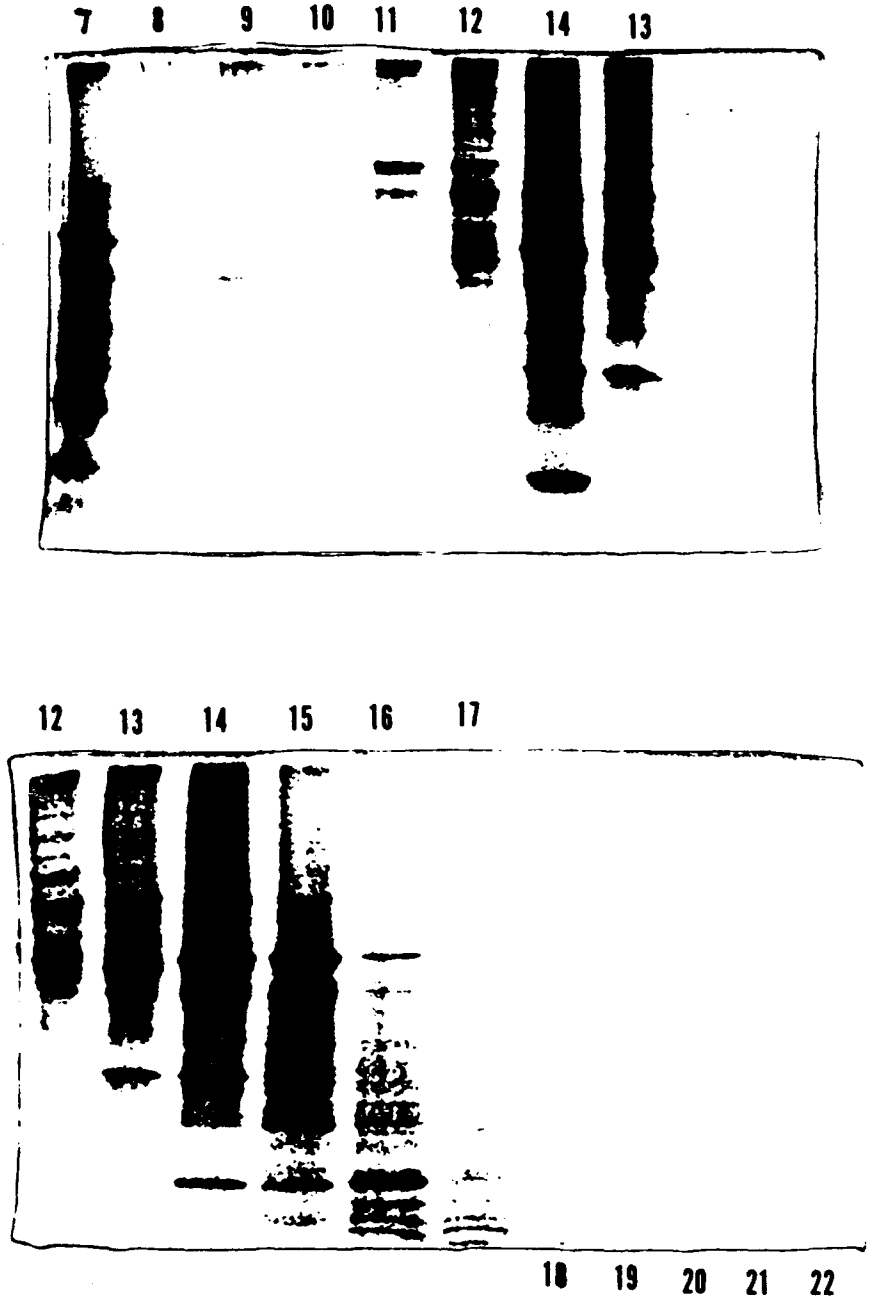

Figure 22. SDS-PAGE gel of gel filtration fractions (7-22) of the C6/36 cell crude extract.

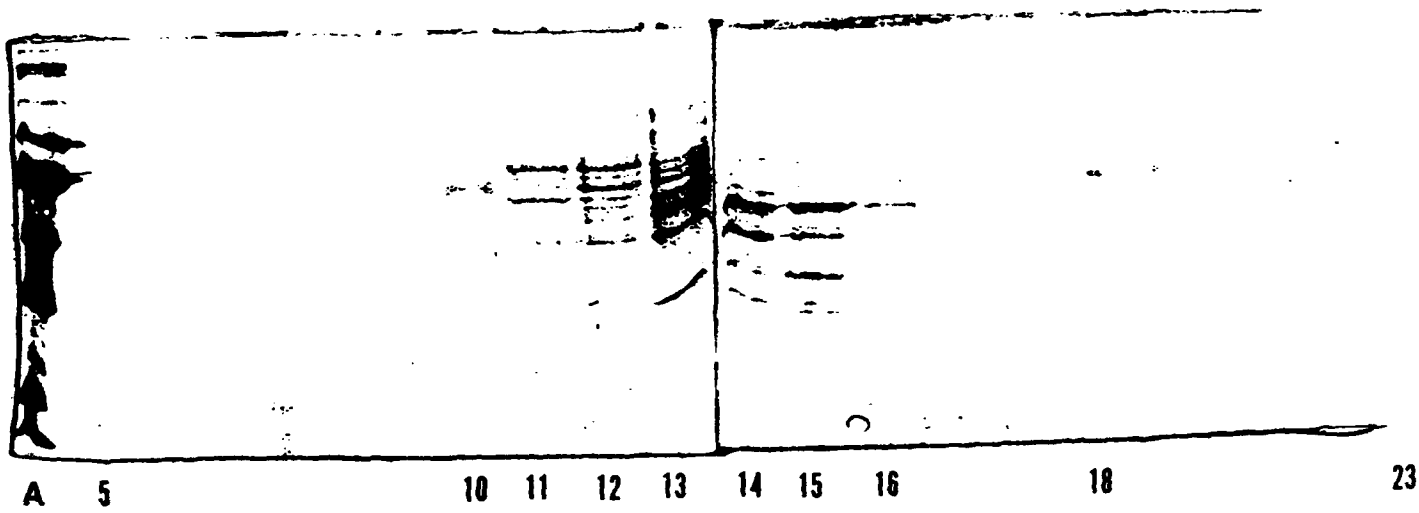

Figure 23. SDS-PAGE gel of gel filtration separation of pooled, concentrated anion exchange fractions 5-23. A, molecular weight markers.

Figure 24. SDS-PAGE gel of the fractions produced by ammonium sulfate precipitation of C6/36 cell crude extract. 1, molecular weight markers; 2 & 3, C6/36 extract; 4 & 5, supernatant from 1st precipitate; 6, insoluble precipitate; 7 & 8,; resolubilized percipitate; 9 & 10, flowthrough of HA column.

extract. It is apparent from lanes 4 and 5 that two proteins are left in the supernatant, and that the rest of the extract seems to be efficiently precipitated and solubilized.

Proteins in a calibration kit plus blue dextran were separated using a gel filtration column. According to the plaque assays performed at USAMRIID, Fort Detrick, Maryland, gel filtration fractions 12 and 13 displayed possible inhibitory effects on the formation of plaques by RVF virus. These fractions corresponded to the molecular weight range of 54,000 to 83,000 daltons in the gel filtration column separation of the calibration proteins. Nearly all of the protein bands revealed by gel filtration chromatography from pooled and concentrated anion exchange chromatography fractions 15-22 fell between BSA and Carbonic Anhydrase, which have molecular weights of 66,000 and 29,000 daltons respectively.

Plaque Assays

Several sets of viral plaque inhibition assays were performed at Fort Detrick, which produced several interesting results. Samples which consistently caused significant inhibition of plaque formation, and are possibly rich in the putative receptor molecule, were gel filtration fractions 12 and 13, and anion exchange chromatography fractions 15-22 of the crude C6/36 cell extract consistently inhibited plaque formation in plaque assays. Also of interest was the plaque inhibition caused by the C6/36 crude extract which has been ammonium sulfate precipitated and solubilized in PBS, and the supernatant from this precipitation. Even more intriguing, was the observation that only two bands were apparent in this supernatant as shown in Fig. 24. Finally, it should be noted that the highest percentage of plaque inhibition obtained with C6/36 extract preparations or fractions occurred when the samples were pre-incubated with the virus as opposed to being added to the cells at the same time that the virus is added. This implies that the putative receptor fraction is having some direct effect on the virus. Hopefully, this extra pre-incubation time allows receptor molecules to block binding sites on the virus. This hypothesis, however, has yet to be confirmed. There are several other possible mechanisms which may be responsible for the plaque inhibition which must be ruled out through the application of appropriate controls.

ELISA

The ELISAs performed for the detection of anti-idiotypic antibodies specific for C6/36 cells in combination with the plaque assays of the same samples yielded dubious results. The serum from mice immunized with R1-4B6 ascites consistently produced strong signals of ELISA relative to the normal mouse serum, thje injected ascites, and serum from the mice immunized with the other specific ascites samples. These same samples, however, did not cause any significant reduction of plaque number in plaque assays. Western blots using the C6/36 extract as the antigen were also performed and no consistent specific staining was observed.

C. Workshop & Monograph

The workshop was successfully presented on December 1, 1991 to 17 persons who attended formally and to several additional persons who sat in on parts of the lecutres. We were fortunate in being able to add two additional instructors to the workshop, Dr. Mark Brown, University of Georgia and Dr. Claudia Golenda, Walter Reed Army Institute for Research. The workshop evaluation summary sheet that was submitted to the American Society of Tropical Medicine and Hygiene and a copy of the workshop manual are included in this report as Appendix I and Appendix II respectively.

The histological and ultrastructural portions of the manual we prepared for and used in the workshop (Appendix II) will serve as a model upon which we will expand considerably in the development of our monograph.

As our financial support by the U.S. Army Medical Research and Development Command has terminated and Dr. K. Lerdthusnee, who will be the co-author of the monograph, has returned to his home in Thailand, we anticipate some delay in completing our monograph, but are fully committed to this project. The fact that two colleagues have agreed to provide us with additional electron photomicrographs will be a great help. Our hope is that the monograph will be ready for publication within the next two years.

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V. Publications, Manuscripts in prep, and Presentations

Publications

Romoser,W.S., M.E. Faran, C.L. Bailey & K. Lerdthusnee. 1992. An immunocytochemical study of the distribution of Rift Valley fever virus in the mosquito Culex pipiens. Am. J. Trop. Med. Hyg., 46(4):489-501.

Dohm, D.J., W.S. Romoser, M.J. Turell & K.J. Linthicum. 1991. Impact of stressful conditions on the survival of Culex pipiens. J. Am. Mosquito Control Assn., 7(4):621-623. (D.J. Dohm at USAMRIID did most of the work and writing, but we carried out preliminary studies and contributed to the development of the manuscript).

Manuscripts submitted or to be submitted

Two manuscripts, "Rift Valley fever virus in the cardia of Culex pipiens: an immunocytochemical & ultrastructural study" (by K. Lerdthusnee & W.S. Romoser) and "Tissue tropisms displayed by Ockelbo virus in Culex pipiens: an immunocytochemical study" (by A. Moncayo, K. Lerdthusnee, J. Lundstrom, M. Turell and W.S. Romoser), are nearly ready for submission to a journal.

We anticipate submission of the following manuscripts during 1992-1993:

"Rift Valley fever virus in tissues of Culex pipiens: an ultrastructural study" (by K. Lerdthusnee & W.S. Romoser)

"Rift Valley fever virus in an African floodwater mosquito, Aedes mcintoshi: tissue tropisms & dissemination dynamics" (by W.S. Romoser, K. Lerdthusnee, K.J. Linthicum, D. Dohm, and C.L. Bailey)

"The potential for vertical transmission of Rift Valley fever virus in an African floodwater mosquito, Aedes mcintoshi: immunocytochemical evidence" (by W.S. Romoser, K. Lerdthusnee, L. Patrican, K.J. Linthicum & C.L. Bailey)

"Immunocytochemical evidence for a salivary gland infection barrier to Rift Valley fever virus in Anopheles stephensi" (by K. Lerdthusnee, M. Turell and W.S. Romoser)

"Immunocytochemical studies of Rift Valley fever virus in selected African mosquitoes" (by W.S. Romoser, K. Lerdthusnee, K.J. Linthicum, D. Dohm & C.L. Bailey)

"Detection of Rift Valley Fever Virus RNA in Paraffin Sections of Mosquitoes by In Situ Hybridization" (by L.A. Patrican, T.A. Hoover, D.J. Dohm, and W.S. Romoser)

Presentations

Rift Valley fever and mosquito vector competence: a look on the inside. Invited lecture presented to the Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD., June, 1989.

Multimethod studies of arbovirus infection and spread within individual mosquitoes: Rift Valley fever virus as a model (with K. Lerdthusnee, L.A. Patrican, K.J. Linthicum, D. Dohm & C.L. Bailey). The First Asia-Pacific Conference of Entomology, Chiangmai, Thailand, Nov.8-13, 1989.

Detection of Rift Valley fever virus RNA in paraffin sections of mosquitoes by in situ hybridization (L.A. Patrican presented). Poster presented to the American Society of Tropical Medicine and Hygiene, Honolulu, Hawaii, Dec. 10-14, 1989.

An inside look at vector competence: in situ studies of Rift Valley fever virus in mosquitoes. Invited presentation as part of a symposium on vector competence, American Society of Tropical Medicine and Hygiene, Honolulu, Hawaii, Dec. 10-14, 1989.

Tissue tropisms displayed by Ockelbo virus in Culex pipiens: an immunocytochemical study (A. Moncayo presented; with K. Lerdthusnee, J. Lundstrom & M. Turell). Presented to the American Mosquito Control Association, Lexington, Kentucky, April, 1990.

Immunogold labelling of Rift Valley fever virions in ultrathin sections of mosquito tissue (with K. Lerdthusnee). Presented to the American Mosquito Control Association, Lexington, Kentucky, April, 1990.

Impact of stressful conditions on the survival of Culex pipiens infected with Rift Valley fever virus (D. Dohm presented; with M. Turell, K. Linthicum & J.P. Kondig). Presented to the American Society of Tropical Medicine & Hygiene, New Orleans, Louisiana, Nov., 1990

Rift Valley fever virions in the cardia of Culex pipiens: ultrastructural localization on the basis of morphology and immunogold labelling (with K. Lerdthusnee). Presented to the American Society of Tropical Medicine & Hygiene, New Orleans, Louisiana, Nov. 1990.

In situ studies of arbovirus dissemination in mosquitoes and ticks: an overview. Invited lecture presented to the Dept. of Arboviral Entomology, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, January, 1991.

Immunocytochemical evidence for a salivary gland infection barrier to Rift Valley fever virus in *Anopheles stephensi*. (with K. Lerdthusnee & M.J. Turell). Presented to the American Mosquito Control Association, New Orleans, Louisiana, March, 1991.

Detection of Rift Valley fever virus nucleic acid in mosquitoes by *in situ* hybridization with a digoxigenin cDNA probe. (L. Patrican presented; with T.A. Hoover, D.J. Dohm, & K. Lerdthusnee). Presented to The American Soceity of Tropical Medicine and Hygiene, Boston, MA, December, 1991.

In situ Localization of Viral Infection in Mosquitoes: Rift Valley Fever Virus as an Example. Presented as part of "Workshop on Mosquito Histology, Ultrastructure and Methods for the *In situ* Localization of Viruses," December, 1991. [A pre-meeting workshop held in association with the American Society of Tropical Medicine & Hygiene]

Rift Valley fever virus in *Culex pipiens*: an ultrastrutural study (with K. Lerdthusnee). Presented to the American Mosquito Control Assn., March, 1992, Corpus Christi, Texas.

D. Personnel

1. Principal Investigator: William S. Romoser, Ph.D.

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4. Technician:

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Brian Law, B.S. (Chemist)

APPENDIX I

Workshop on Mosquito Histology, Ultrastructure and Methods for the *In situ* Localization of Viruses

Evaluation of Workshop – SUMMARY

Name _____ Date _____

Address _____

Number of Participants = 17+

Number of Respondents to this evaluation = 11

Primary Professional Activities:

Research 6

Teaching + Research 1

Other: Graduate Students 2 ; Administration 2

Please Rate the Workshop (0 = poor; 5 = outstanding):

Organization 4.6 Quality of Instruction 4.3

Audiovisuals 4.5 Facilities 4.1 Lab Materials 4.7

Overall Quality of Workshop 4.4

Value of Workshop to You 3.9

Instructors/Speakers (0 = poor; 5 = outstanding):

	Clarity	Relevance	Comments
Brown	4	3.3	
Golenda	4.8	4.5	"most applicable to my work; could have been a bit shorter."
Lerdthusnee	4.0	4.3	
Mikhail	3.7	4.1	
Patrican	4.3	4.2	
Romoser	4.8	4.2	

Additional Comments

"Very interesting
Often was a bit too specialized; could have been more general info.
Perhaps too many speakers per short time, but excellent job.
Thanks, I learned quite a bit.
Very good workshop – Best I have attended"

Additional Comments

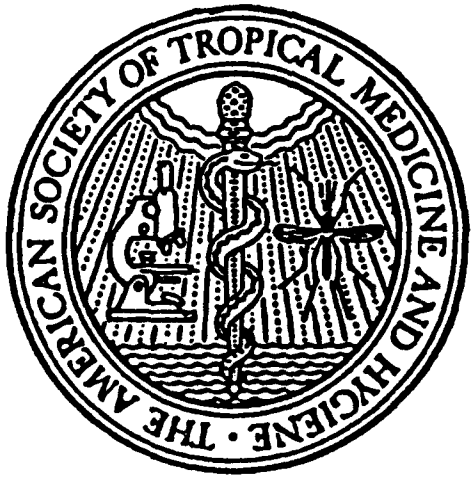
"Sorry, but I don't recall the individual presentations well enough to evaluate them. Personally, I was a bit disappointed with the course. It was mainly theoretical; whereas, I had expected a 'hands-on' type of course where I could actually learn how to do some of the techniques. For example, I know how to and have made monoclonal antibodies. But in attempting to use them in immunoperoxidase tests in insects, my results are unsuccessful. Thus I had hoped to work with you and learn how."

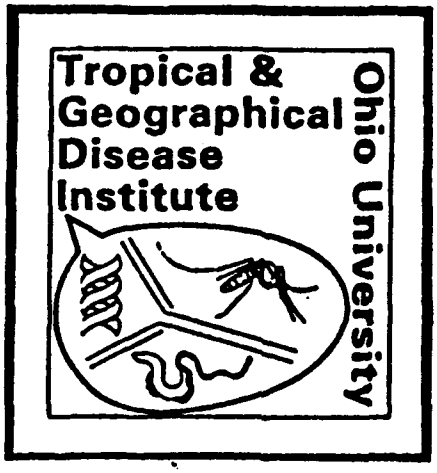
"Thanks again for a well-done workshop. I'm filling this out on New Year's Day without benefit of notes, so my memory on one of the speakers may be a bit hazy. My only constructive criticism was that I would have liked a litle more integration of structure and function on the ultrastructure part of the workshop."

"At this point, I don't remember specifics, but I was happy with all the speakers. Some talked about applications in their research a bit much...I was very happy with the quality of slides, handouts, and the pleasantness of the people. I learned a lot, but some of the information I already knew (thus value of workshop to me 3-4). I enjoyed it. Thanks."

WORKSHOP ON MOSQUITO HISTOLOGY, ULTRASTRUCTURE, & METHODS FOR THE IN SITU LOCALIZATION OF VIRUSES

Sunday, December 1, 1991
Sheraton Boston Hotel, Hampton Room





Sponsored by
The American Committee of Medical Entomology,
American Society Of Tropical Medicine & Hygiene &
The Ohio University Tropical & Geographical Disease Institute.
Supported in part by the U.S. Army Medical Research & Development Command.

CONTENTS

THE AMERICAN COMMITTEE OF MEDICAL ENTOMOLOGY

The American Committee of Medical Entomology was organized under the auspices of the American Society of Tropical Medicine and Hygiene (ASTMH). The objectives of this committee are: (1) To promote medical entomology in the ASTMH and in organizations whose scope of activities include the area of human diseases transmitted by arthropods. (2) To organize symposia or workshops annually that emphasize, but are not limited to, the contributions of medical entomology to tropical medicine. (3) To encourage active participation of medical entomologists in the ASTMH. (4) To recognize outstanding contributions by medical entomologists through The Harry Hoogstraal Medal for Outstanding Achievement in Medical Entomology.

Membership in the American Committee of Medical Entomology is open to all medical entomologists in ASTMH. Currently there are approximately 200 members. This committee is represented by an Executive Council.

THE OHIO UNIVERSITY TROPICAL & GEOGRAPHICAL DISEASE INSTITUTE

The Ohio University Tropical & Geographical Disease Institute was created in 1989 in order to contribute to the U.S. effort in solving tropical infectious disease problems. Although our primary focus is tropical disease, our research interests also include conditions indigenous to Ohio and surrounding states.

The specific objectives of our institute are as follows: (1) To establish and carry out collaborative research projects on the biology of tropical infectious diseases. (2) To facilitate training in the biology of tropical infectious diseases for graduate and post-doctoral students. (3) To promote basic and applied research on tropical infectious diseases through public relations activities (guest lectures, films, etc.), through training and educational activities (workshops, conferences, etc.), and through contributions to International Studies at Ohio University.

At present our major research/ training interests are: (1) studies of host-parasite interactions at the histological, cellular and molecular levels; (2) immune regulation in parasitic infection; (3) rapid identification of parasites for diagnostic and surveillance purposes; and (4) histology/ ultrastructure and physiology of mosquitoes and other arthropod vectors.

The membership of our institute includes two immunoparasitologists, two molecular biologists, an infectious disease specialist, a virologist, a pharmacologist and a medical entomologist. Adjunct members include a telecommunications specialist, an immuno-parasitologist and a medical entomologist.

Workshop Schedule

8:30 - 9:00: Doughnuts & Coffee

9:00 - Noon: Basic Mosquito Histology & Ultrastructure: Lecture & Laboratory **(Romoser, Lerdthusnee, Costello)**

Noon - 1:15: Lunch*

1:15 - 1:20: Introduction **(Romoser)**

1:20 - 1:50: In situ Localization of Viral Nucleic Acid in Mosquitoes **(Patrican)**

1:50 - 2:30: Principles of In Situ Immunolocalization **(Mikhail)**

2:30 - 3:00: In situ Localization of Viral Infection in Mosquitoes: Rift Valley Fever Virus as an Example **(Romoser)**

In Situ Immunogold Labelling of Rift Valley Fever Virions in Mosquitoes at the Ultrastructural Level **(Lerdthusnee)**

3:00 - 3:15: Break

3:15 - 3:45: The Distribution of Circumsporozoite (CS) Protein of Plasmodium falciparum in Anopheles stephensi mosquitoes **(Golenda)**

3:45 - 4:15: Localization of Peptide Hormones in the Nervous System and Midgut of Mosquitoes **(Brown)**

4:15 - 4:45: Immunocytochemistry in Insect Development **(Costello)**

* Not Provided

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THE ADULT MOSQUITO

Integumentary System

(Lm. 1-6 & 7; Em. 1 & 2)

- cuticle
  - epicuticle
  - exocuticle
  - endocuticle (lamellae)
- epidermis (one-cell thick)
- basal lamina (basement membrane)
- sclerite
- arthrodial membrane
  - intersegmental membrane
  - pleural membrane
  - cervix
- tentorium
- apodemes
- setae (hairs)
- scales (Lm. 13)
- spines
- microtrichae
- cornea of compound eyes

Skeletal Muscle System

(Lm. 1, 3, 5, 6 & 8; Em. 27 - 29)

- indirect flight muscles
  - longitudinal
  - tergosternal
- leg muscles
- intersegmental muscles
- extrinsic & intrinsic muscles of appendages
- muscle fiber
- myofibril
- sarcomere (A-band)
- microfilaments
- actin & myosin

(Note: all insect muscles are striated; visceral muscles will be mentioned in association with the various systems)

Central Nervous System

(Lm. 1, 2, 3, 4 & 8; Em. 30, 31 & 32)

brain
subesophageal ganglion
thoracic ganglion (a fusion of 3 segmental ganglia)
abdominal ganglia (6 in number, the last, the caudal ganglion, being in the 7th abdominal segment)
interganglionic connectives (ventral nerve cord)
nerves
cell bodies
neuropile
neurites (axons & dendrites)
ganglion sheath
external lamina (neural lamella)
axon
glial cells

Alimentary & Excretory System

(Lm. 1-6 & 8; Em. 3 - 22)

mouthparts
foregut, midgut, hindgut
zonula continua
intima (fore- & hindgut)
visceral muscles: circular, sphincter & longitudinal
skeleto-visceral muscles: pump & suspensory muscles
foregut
    cibarium
    cibarial pump muscles
    pharynx
    pharyngeal pump muscles
    esophagous
    dorsal diverticula
    ventral diverticulum
proventriculus (cardia)
    intussuscepted foregut (=foregut tissue)
    reflected wall of esophagous (=foregut tissue)
    anterior cardial epithelium (=midgut tissue)
    cardial epithelium (=midgut tissue)
    regenerative cell
    microvilli of cardial epithelium
    basal lamina
midgut
    anterior midgut
    posterior midgut
    microvilli of midgut epithelium
    regenerative cell
    basal labyrinth (basolateral membrane)
    basal lamina
    blood bolus

peritrophic membrane
plug
Malpighian tubules
microvilli
sphero-crystals
hindgut
pyloric valve
pyloric ampulla
ileum (anterior intestine)
rectum
rectal papillae

Salivary Glands

(Lm. 6 & &; Em. 23)

lateral lobes
proximal zone
intermediate zone
distal zone
median lobe
proximal zone
distal zone
lateral salivary duct
common salivary duct
salivary pump

Circulatory System

hemocoel
dorsal vessel
heart
alary muscles
pericardial cells
aorta
accessory pulsatile structures
fat body (Lm. 1, 2, 5, 7 & 13; Em. 2, 3, 24)
oenocytes
nephrocytes

Ventilatory System

(Em. 14, 17, 21, 25, 26, 27, 28, 36)

tracheae
taenidia
tracheoles
tracheal end cell
spiracles
atrium
spiracular closure mechanism

Stomatogastric/Endocrine System

- frontal ganglion
- recurrent nerve
- hypocerebral ganglion
- corpus cardiacum
- corpora allata
- median neurosecretory cells
- neurosecretory granules (Em. 33)
- ventricular nerve
- ventricular ganglion

Peripheral Nervous System

- antenna
  - scape
  - pedicel
  - flagellum
  - Johnston's organ (Lm. 1 & 5)
- compound eyes (Lm. 1, 3 & 5)
  - ommatidia
  - cornea
  - corneal lens
- halteres
  - campaniform sensilla

Female Reproductive System

(Lm. 2, 4, 6, 8, 11 - 14; Em. 34, 35, 36)

- ovary
- ovarian sheath
- terminal filament
- ovariole
- ovarian follicle
- follicular epithelium
- lateral oviduct
- common oviduct
- genital chamber
- oocyte
- nurse cell
- chorion
  - endochorion
  - exochorion
- vitellogenesis
- yolk granules
- lipid droplet
- protein granule
- zonula pellucida

Male Reproductive System

(Lm. 9, 10; Em. 37 & 38)

testes
follicular epithelium
spermatogonia
spermatozoa
vas efferens
vas deferens
accessory glands
seminal vesicles
ejaculatory duct

SPECIAL TOPICS

The Larva

The Pupa & Metamorphosis

Detection of Multiple Blood Meals

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Mosquitoes--Specific Research Papers

A treatment of the serial literature on mosquito histology & ultrastructure is beyond the scope of this workshop manual. However, for nearly comprehensive access to the literature on mosquito histology & ultrastructure, see the "Anatomy and Morphology" section of "Literature References for Mosquitoes and Mosquito-borne Diseases" published in each issue of the Journal of the American Mosquito Control Association. A search of the JAMCA literature references back to the 1950s and subsequent consultation of the literature cited in Clements (1963) and Christophers (1960) should provide reasonably comprehensive access to the major literature on mosquito histology & ultrastructure.

CAPTIONS FOR LIGHT PHOTOMICROGRAPHS

Lm. 1. Saggital section of *Culex pipiens* head & thorax. (stain = hemotoxylin)

Lm. 2. Saggital section of *Cx. pipiens* abdomen. (stain = hemotoxylin)

Lm. 3. Saggital section of female *Aedes aegypti*. (stain = modified Azan, Hubschman, 1962, Stain Technol., 37:379-80)

Lm. 4. Saggital section of female *Ae. aegypti* abdomen. (stain = modified Azan)

Lm. 5. Section in horizontal plane of *Ae. aegypti* (stain = modified Azan)

Lm. 6. Saggital section of blood-fed female *Cx. nigripalpus*. (Note the double blood meal; stain = modified Azan)

Lm. 7. Section of salivary gland in female *Cx. nigripalpus*. (stain = modified Azan)

Lm. 8. Saggital section of *Ae. aegypti* 72 hours after a blood meal. (stain = modified Azan)

Lm. 9. Section of testis in male *Cx. pipiens*. (stain = hemotoxylin as counterstain in application of the ABC immunocytochemical technique)

Lm.10. Section of seminal vesicle and accessory gland in male *Cx. pipiens*. (stain = hemotoxylin as counterstain in application of ABC immunocytochemical technique)

Lm.11. Saggital section of posterior abdominal region in sugar-fed female *Cx. pipiens*. (stain = hemotoxylin)

Lm.12. Section of ovariole in female Ae. aegypti 24 hours after a blood meal. (stain = modified Azan)

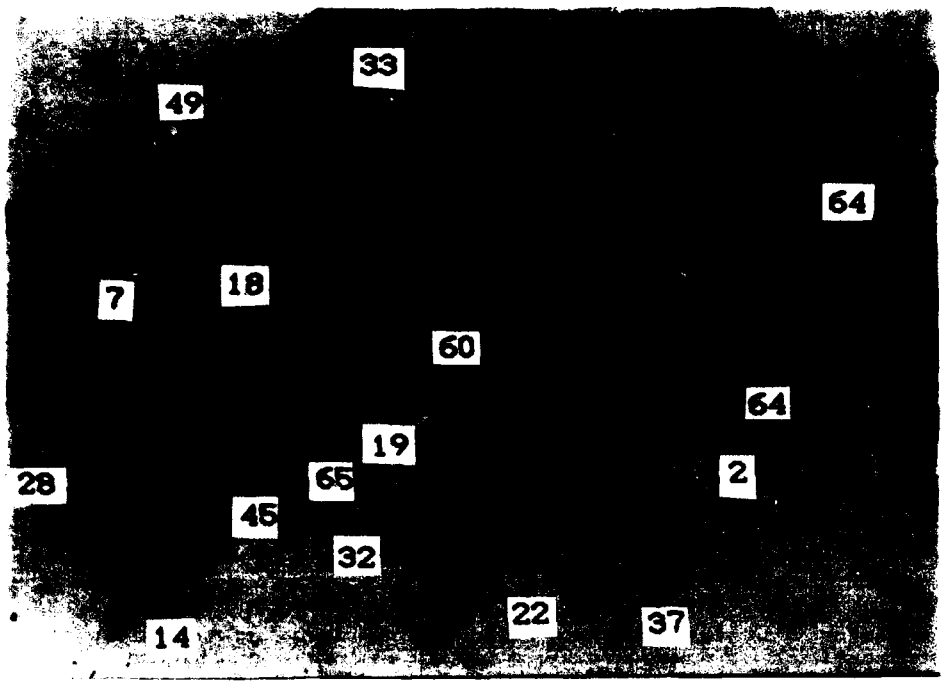
Lm.13. Saggital section of posterior abdominal region in female *Ae. aegypti*. (stain = modified Azan)

Lm.14. Section of ovarioles with chorionated eggs in female *Ae. aegypti*. (stain = modified Azan)

LIGHT PHOTOMICROGRAPH LABEL CODE

1. antennal nerve
2. anterior midgut
3. anterior pronotal muscles
4. blood meal #1
5. blood meal #2
6. body scale
7. brain
8. calyx
9. caudal ganglion
10. chorion (exo-, pale blue; endo-, red)
11. cibarial pump muscles
12. cibarium
13. common oviduct
14. compound eye
15. dorsal diverticulum
16. eggs
17. ejaculatory duct
18. esophagous
19. fat body
20. female accessory gland
21. follicular epithelium
22. ganglionic cell bodies
23. genital chamber
24. germarium
25. --------
26. ileum
27. intussuscepted foregut
28. Johnston's organ
29. lateral distal region of salivary gland
30. lateral oviduct
31. lateral proximal region of salivary gland
32. leg muscles
33. longitudinal flight muscles
34. male accessory gland
35. Malpighian tubules
36. median proximal region of salivary gland
37. neuropile
38. nurse cells/oocyte
39. ovarian sheath
40. ovariolar sheath
41. ovariole
42. ovary
43. penultimate oocyte
44. pharynx
45. pharyngeal pump muscle
46. pleuro-trochanteral muscles
47. hindgut
48. posterior midgut
49. proventriculus (cardia)
50. rectal epithelium
51. rectal papilla
52. rectum
53. salivary gland

54. seminal vesicle
55. spermatheca
56. spermatozoa
57. ---------
58. tergosternal flight muscles
59. testicle
60. thoracic ganglion
61. ultimate oocyte
62. vas efferens
63. ventral diaphragm
64. ventral diverticulum
65. ventral nerve cord
66. wing base
67. yolk granules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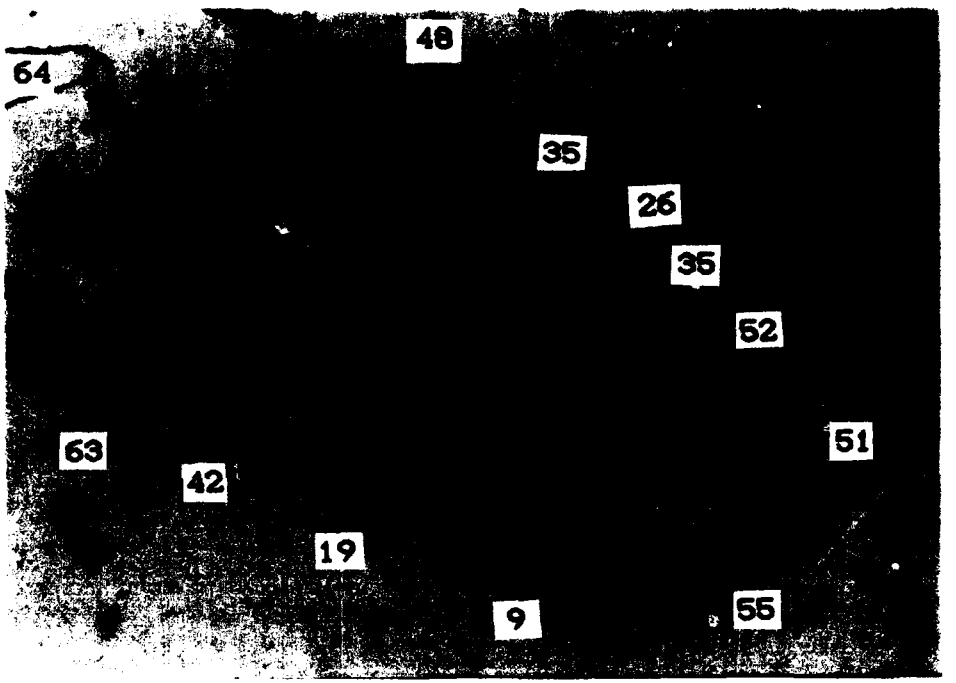
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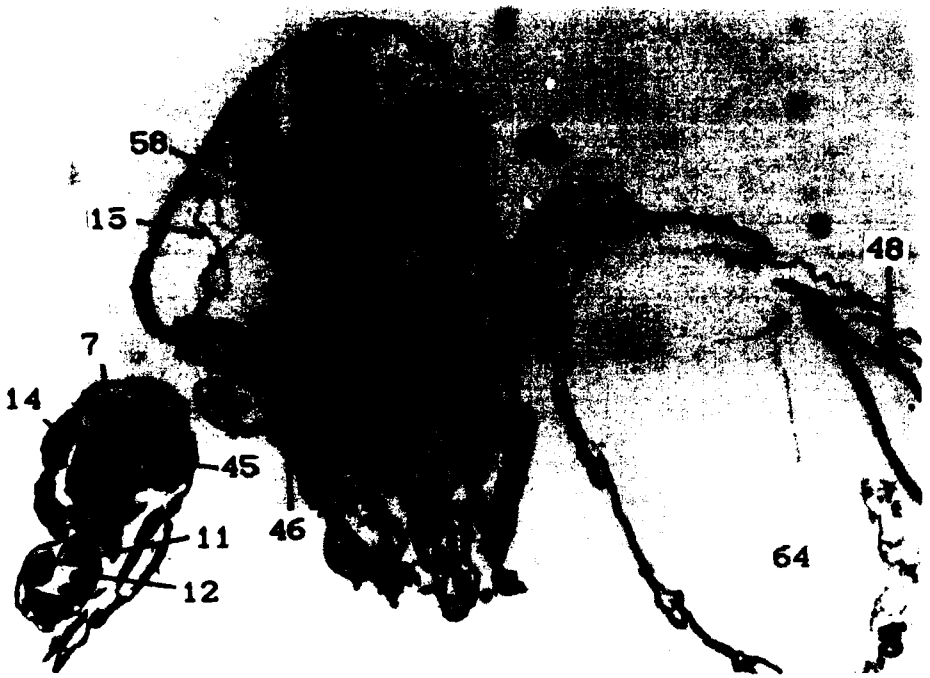
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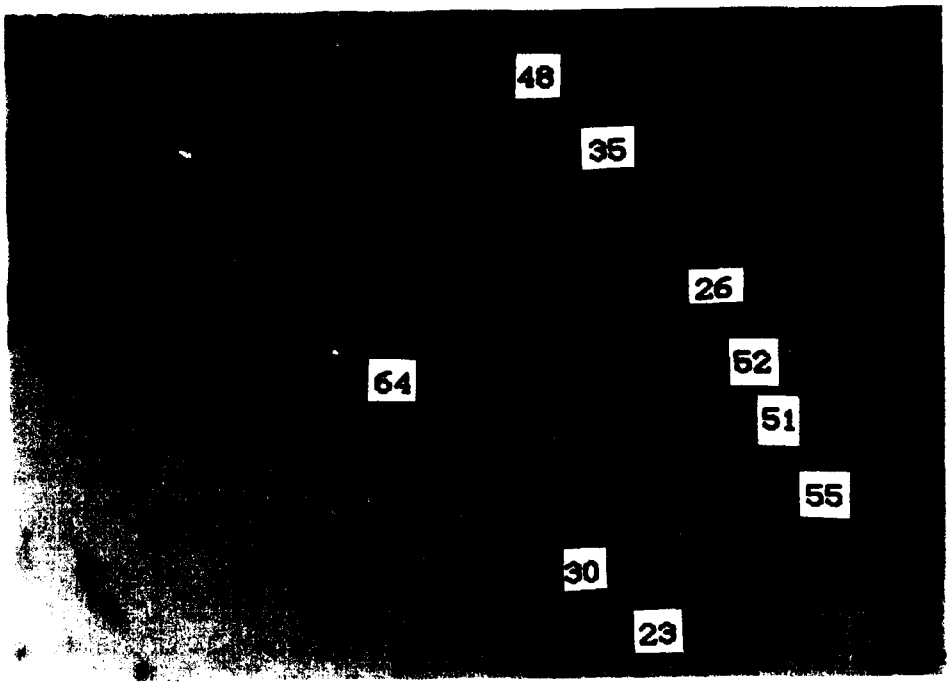
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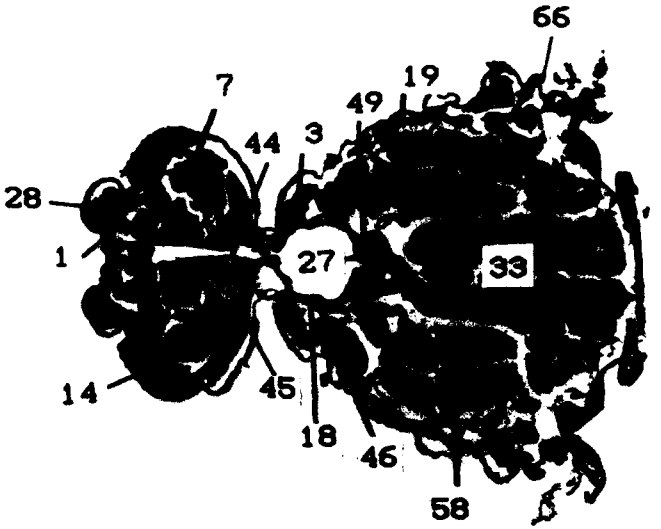
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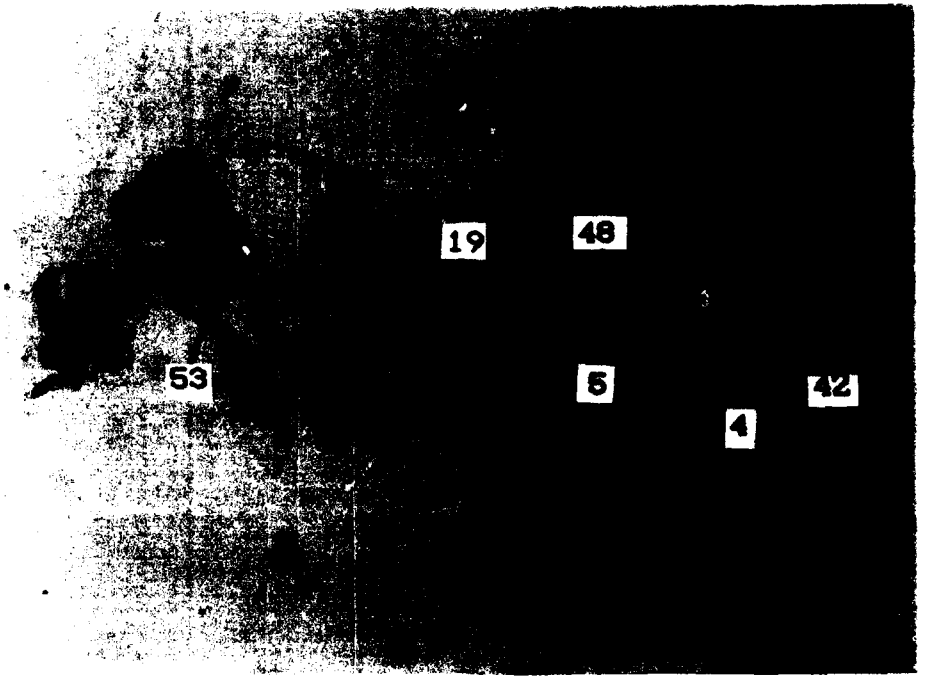
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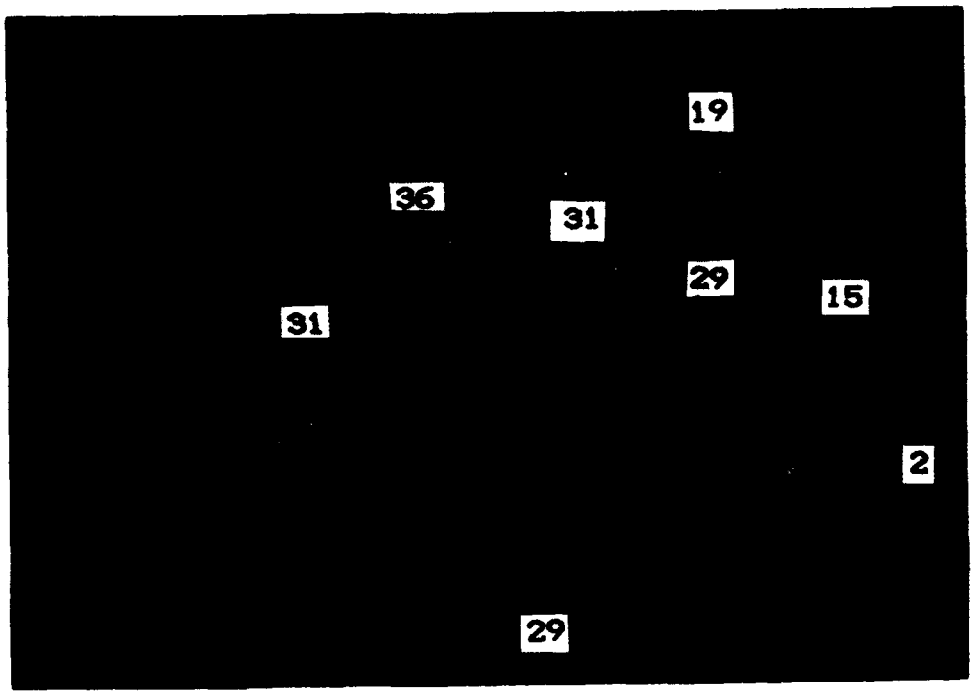
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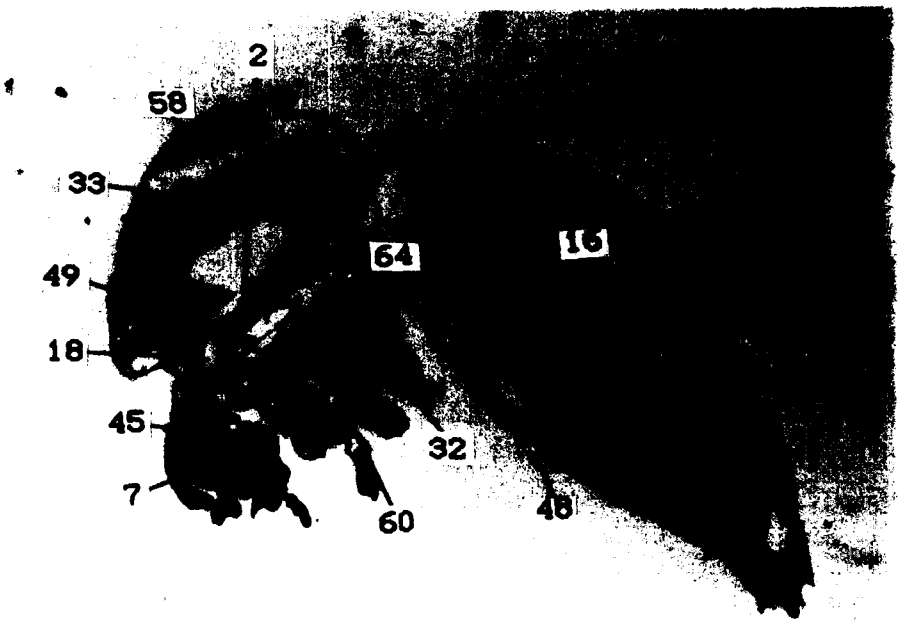
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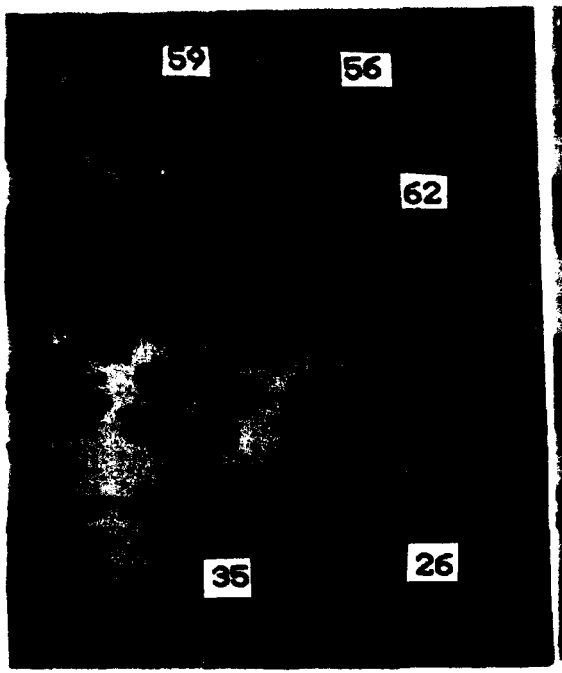
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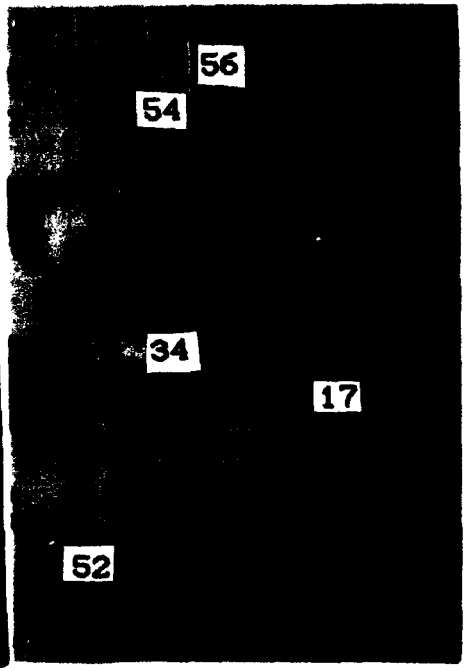
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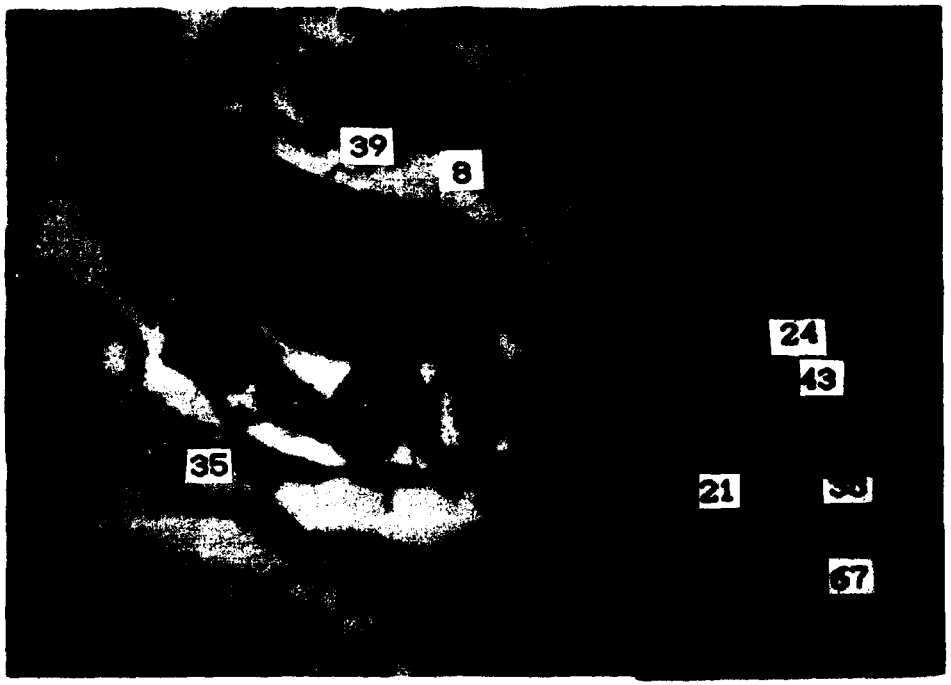
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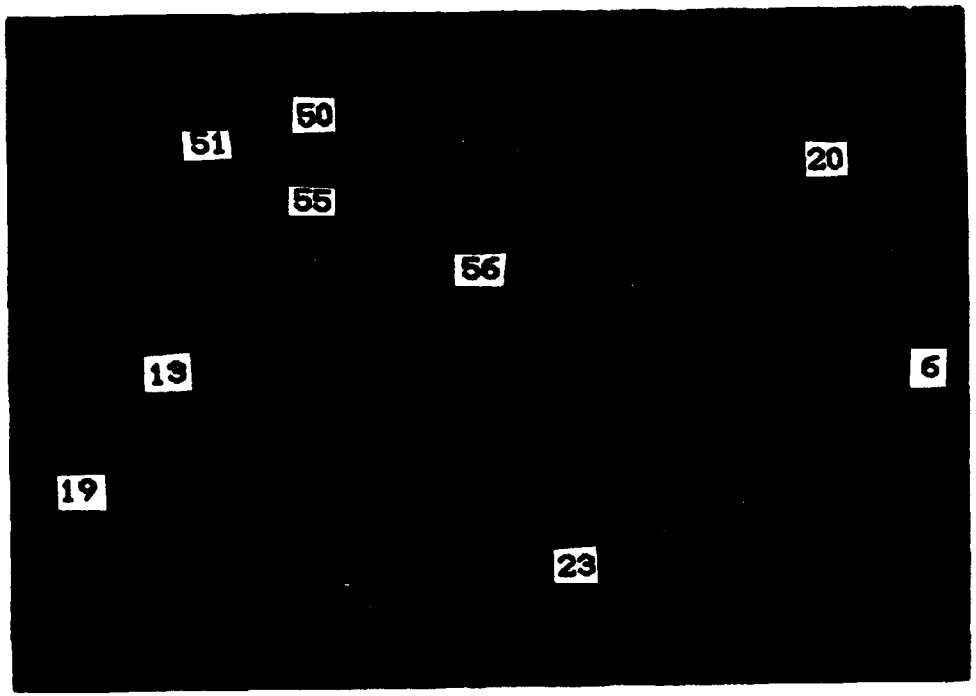
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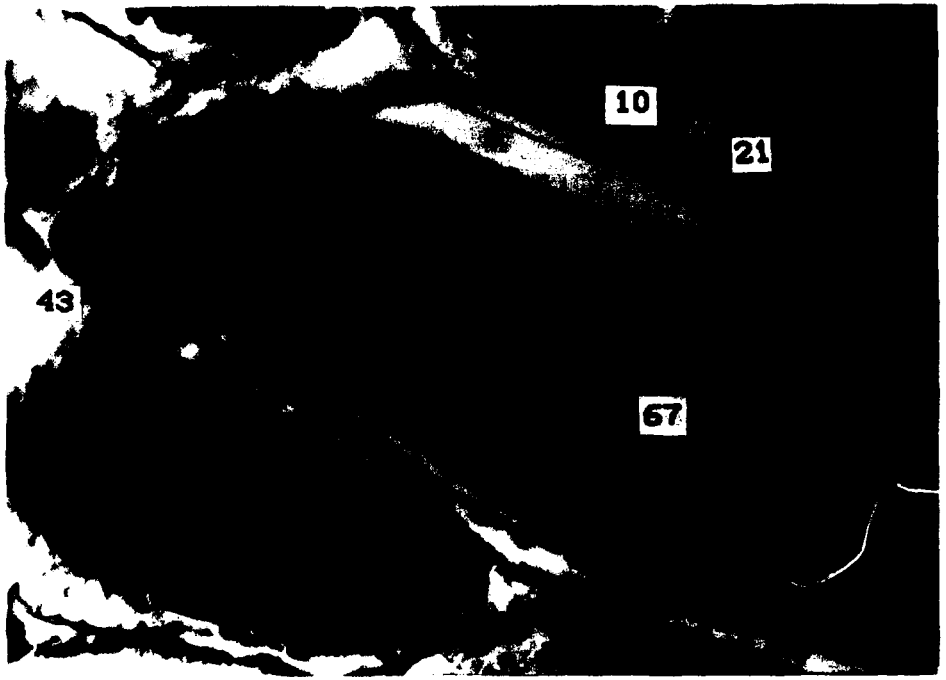
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CAPTIONS FOR ELECTRON PHOTOMICROGRAPHS

Em. 1. Section of integument. Note the lamellate nature of the endocuticle.

Em. 2. Section of integument with fat body cell beneath.

Em. 3. Section of the foregut epithelium with associated visceral muscle fiber and fat body cell.

Em. 4. Section of the foregut epithelium. Note the cross-sectional and longitudinal profiles of the muscle fibers.

Em. 5. Section of the epithelium of the ventral diverticulum.

Em. 6. Saggital section of the anterior part of the proventriculus (cardia) and associated foregut epithelium.

Em. 7. Saggital section of the proventriculus (cardia).

Em. 8. Section of the posterior end of the intussuscepted foregut as it faces into the midgut lumen.

Em. 9. Cross section of the proventriculus (cardia).

Em. 10. Sagittal section of a portion of the proventriculus (cardia) showing the anterior cardial epithelium.

Em. 11. Section within the proventriculus (cardia) in the region of the foregut-midgut junction.

Em. 12. Section of the cardial epithelium.

Em. 13. Section of the anterior midgut with ventral diverticulum beneath.

Em. 14. Section of the anterior midgut with epithelium of the ventral diverticulum beneath.

Em. 15. Section of the posterior hindgut epithelium.

Em. 16. Section of the posterior hindgut epithelium showing peritrophic membrane.

Em. 17. Section of Malpighian tubule.

Em. 18. Section of Malpighian tubule.

Em. 19. Section in region of midgut-hindgut junction.

Em. 20. Section of hindgut epithelium.

Em. 21. Saggital section of hindgut.

Em. 22. Section of hindgut showing bacteria in lumen.

Em. 23. Cross section of salivary gland.

Em. 24. Fat body cell.

Em. 25. Section of a trachea.

Em. 26. Section of a trachea.

Em. 27. Longitudinal section of skeletal muscle fibers.

Em. 28. Sections of flight muscle fibers.

Em. 29. Sections of skeletal muscle fibers showing myocuticular insertions.

Em. 30. Section of thoracic ganglion.

Em. 31. Section within brain.

Em. 32. Section of ventral nerve cord.

Em. 33. Neurosecretory granules within neurosecretory axon.

Em. 34. Section of ovarian follicle.

Em. 35. Section of ovarian follicle.

Em. 36. Section of ovarian follicle.

Em. 37. Spermatozoa within testicular follicle.

Em. 38. Spermatozoa within ejaculatory duct of male.

EM-ABBREVIATIONS

ACE	Anterior-cardial epithelium
AMG	Anterior midgut
AX	Axon
B	bacteria
BC	Brain cell
BL	Basal lamina
BLB	Basal labyrinth
BM	Basement membrane
C	Cuticle
CB	Cell bodies
CE	Cardial epithelium
CuH	Cuticular hair
CuL	Cuticular lining
D	Desmosomes
E	Epidermis
ED	Ejaculatory duct
EL	External lamina
EnC	Endocuticle
EpC	Epicuticle
ExC	Exocuticle
FB	Fat body
FC	Follicular epithelium
FE	Foregut epithelium
FI	Foregut intima
FL	Foregut lumen
GC	Gut content
GG	Glycogen granule
GH	Ganglion sheath
GlC	Glial cells
H	Hemocoel
HG	Hindgut epithelium
HI	Hindgut intima
HL	Hindgut lumen
I	Ileum
IF	Intussuscepted foregut
LD	Lipid droplet
M	Mitochondria
MeC	Mesocuticle
ME	Midgut epithelium
ML	Midgut lumen

MpT	Malpighian tubule
Mt	Microtubules
Mu	Muscle
Mv	Microvilli
N	Nucleus
No	Nucleolus
Nr	Neurites (Axon & Dendrites)
NC	Nurse cell
NF	Nerve fiber
NG	Neurosecretory granule
O	Ovary
OS	Ovariole sheath (wall)
PA	Pyloric ampulla
PG	Protein granule
PM	Peritrophic membrane
PMG	Posterior midgut
RER	Rough endoplasmic reticulum
RC	Regenerative cell
RW	Reflected wall of IF
SBL	Spongy basal lamina
SC	Sphero-crystal
SG	Salivary gland
SGD	Salivary duct
SPZ	Spermatozoa
T	Trachea
TD	Taenidium
TG	Thoracic ganglion
TRC	Tracheolar cell
TT	Testes
Y	Yolk
VD	Ventral diverticulum
ZC	Zonula continua
ZP	Zonula pellucida (space between the oocyte & follicular cell)

Em. 1. Section of integument.
Note the lamellate nature of the endocuticle.

Em. 2. Section of integument with fat body cell beneath.

Em. 3. Section of the foregut epithelium with associated visceral muscle fiber and fat body cell.

Em. 4. Section of the foregut epithelium.
Note the cross-sectional and longitudinal profiles of the muscle fibers.

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Em. 13. Section of the anterior midgut with ventral diverticulum beneath.

Em. 14. Section of the anterior midgut with epithelium of the ventral diverticulum beneath.

Em. 15. Section of the posterior hindgut epithelium.

Em. 16. Section of the posterior hindgut epithelium showing peritrophic membrane.

Em. 17. Section of Malpighian tubule.

Em. 18. Section of Malpighian tubule.

Em. 19. Section in region of midgut-hindgut junction.

Em. 20. Section of hindgut epithelium.

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Em. 38. Spermatozoa within ejaculatory duct of male.

Fig. 1. Section of integument.
Note the lamellate nature of the endocuticle.

Fig. 2. Section of integument with fat body cell beneath.

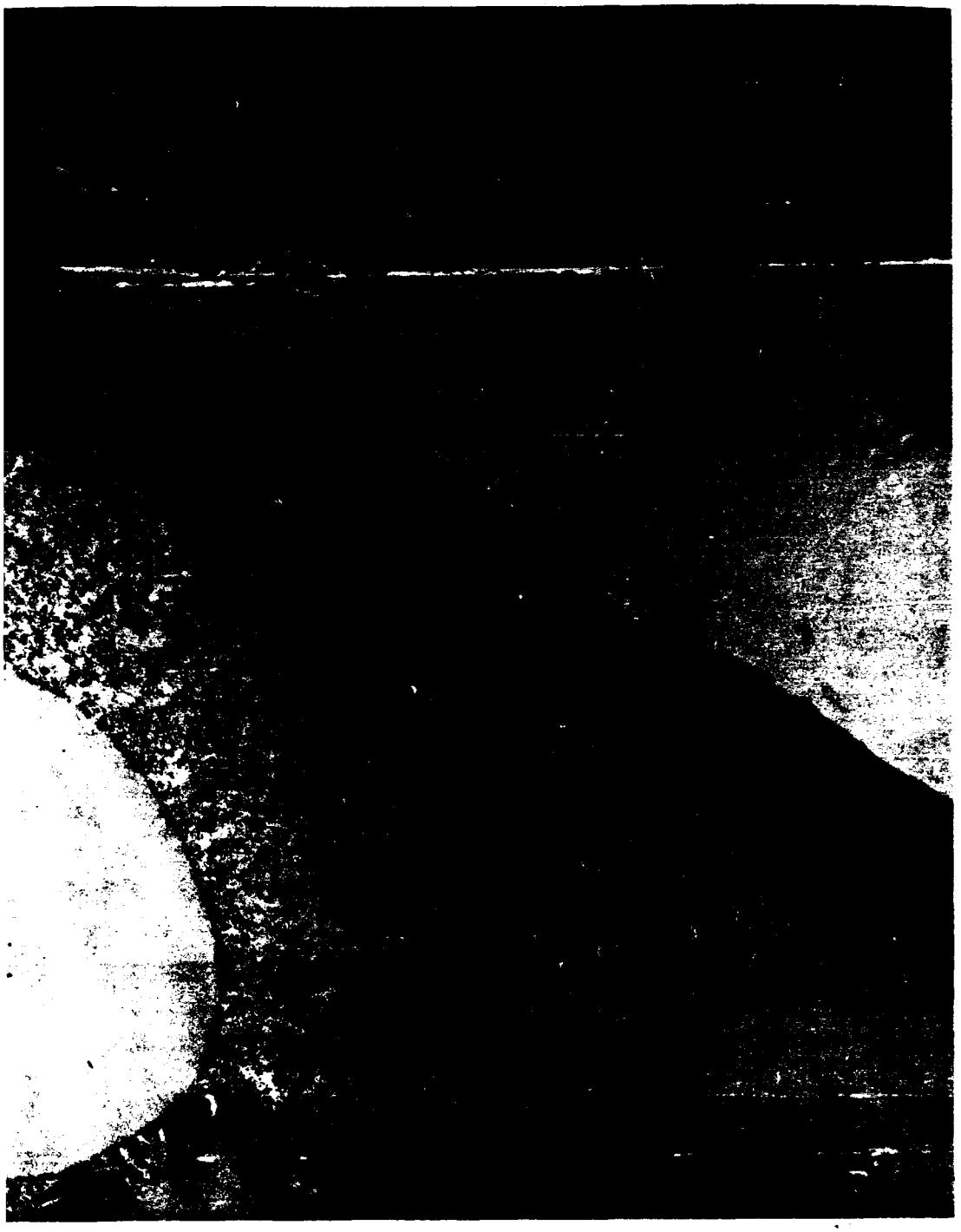
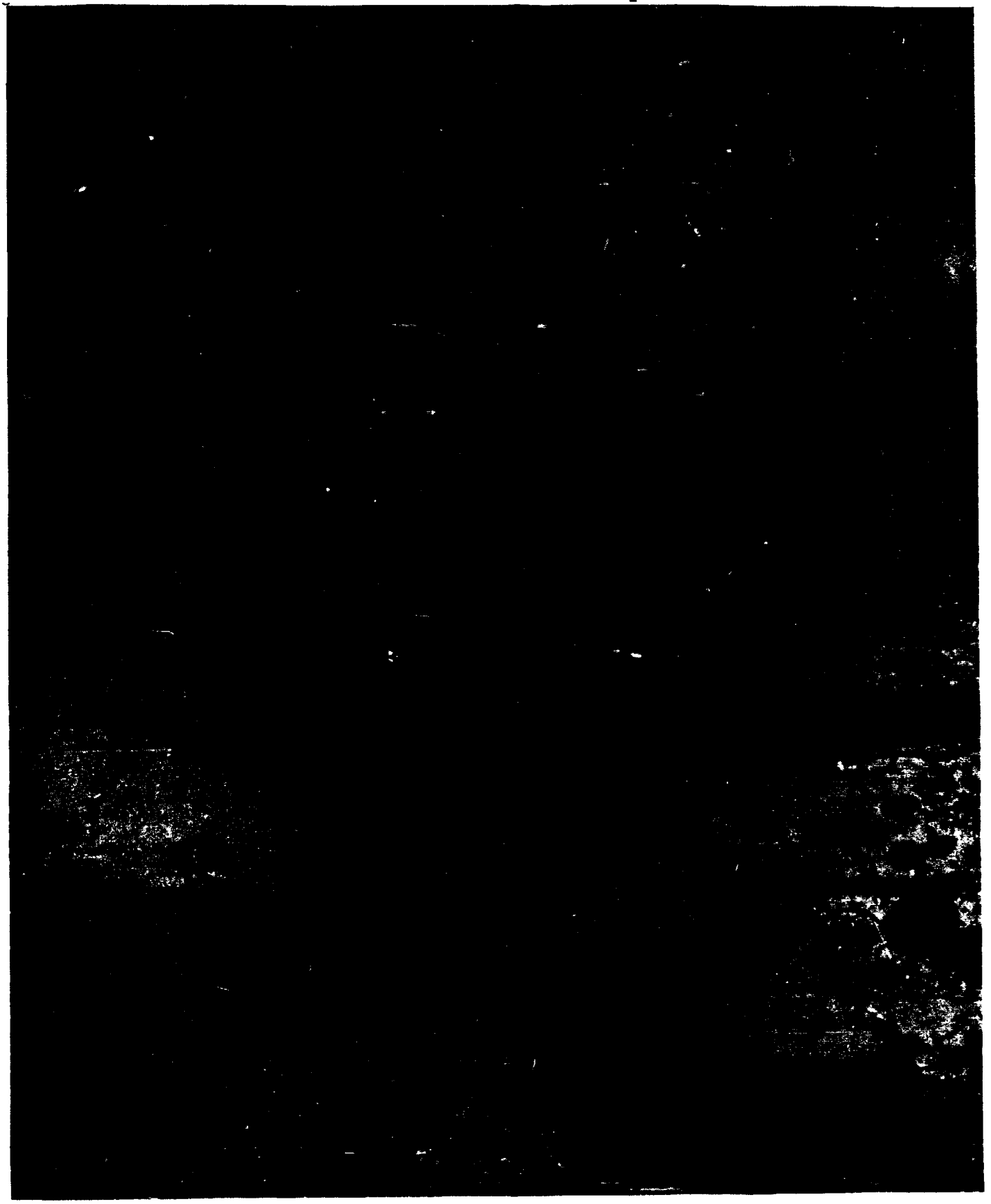


Fig. 3. Section of the foregut epithelium with associated visceral muscle fiber and fat body cell.



Em. 4. Section of the foregut epithelium.
Note the cross-sectional and longitudinal profiles of
the muscle fibers.

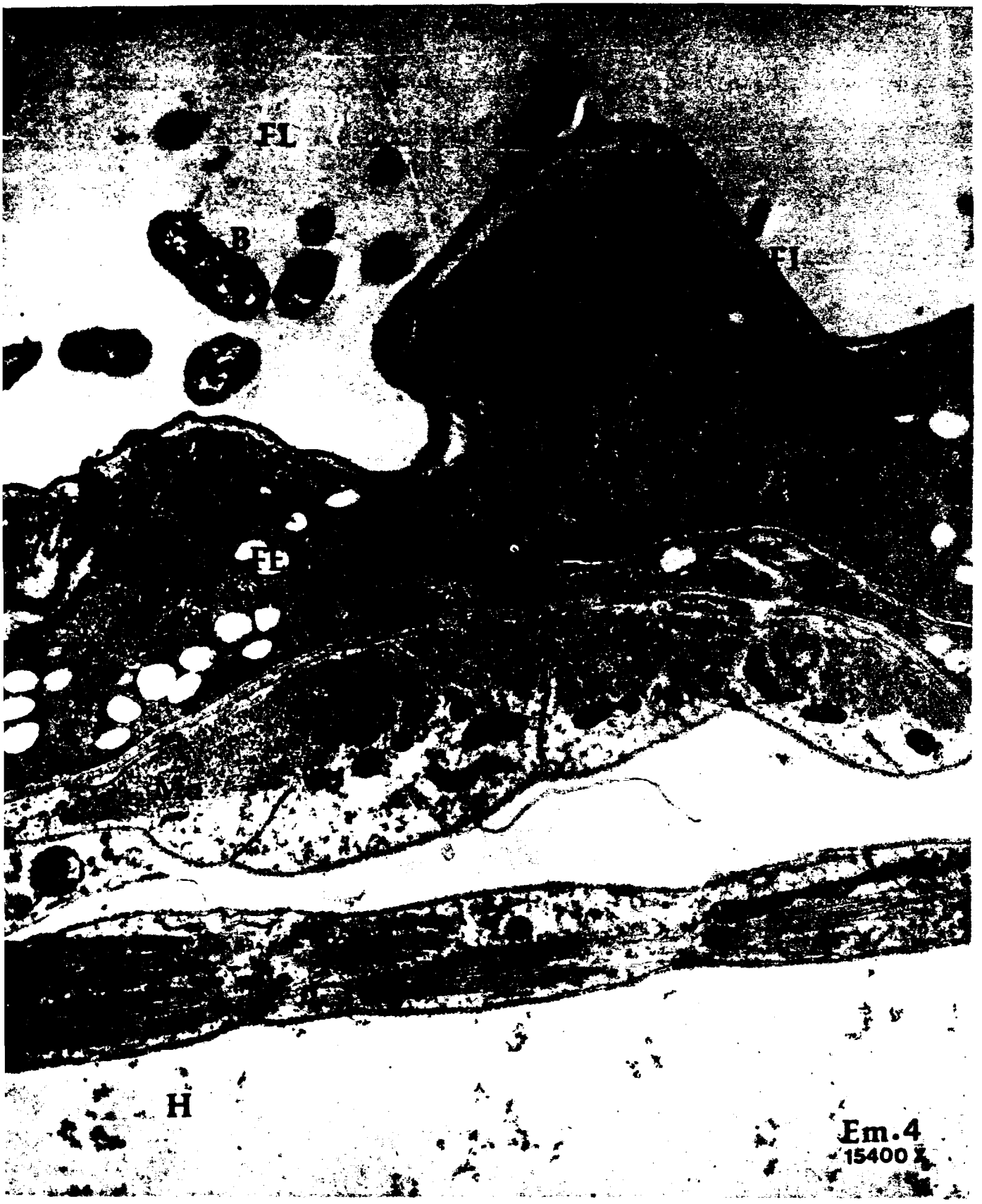
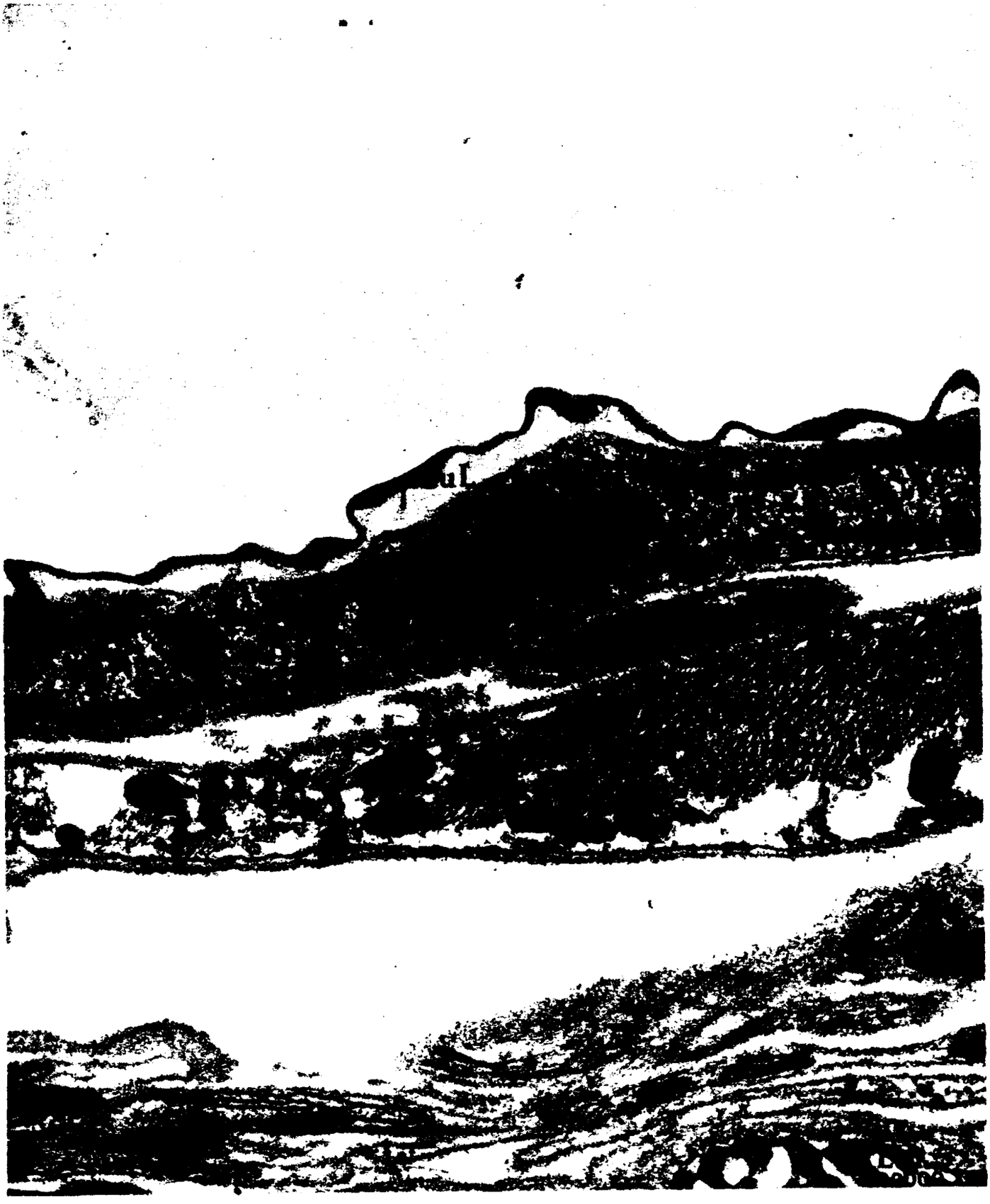
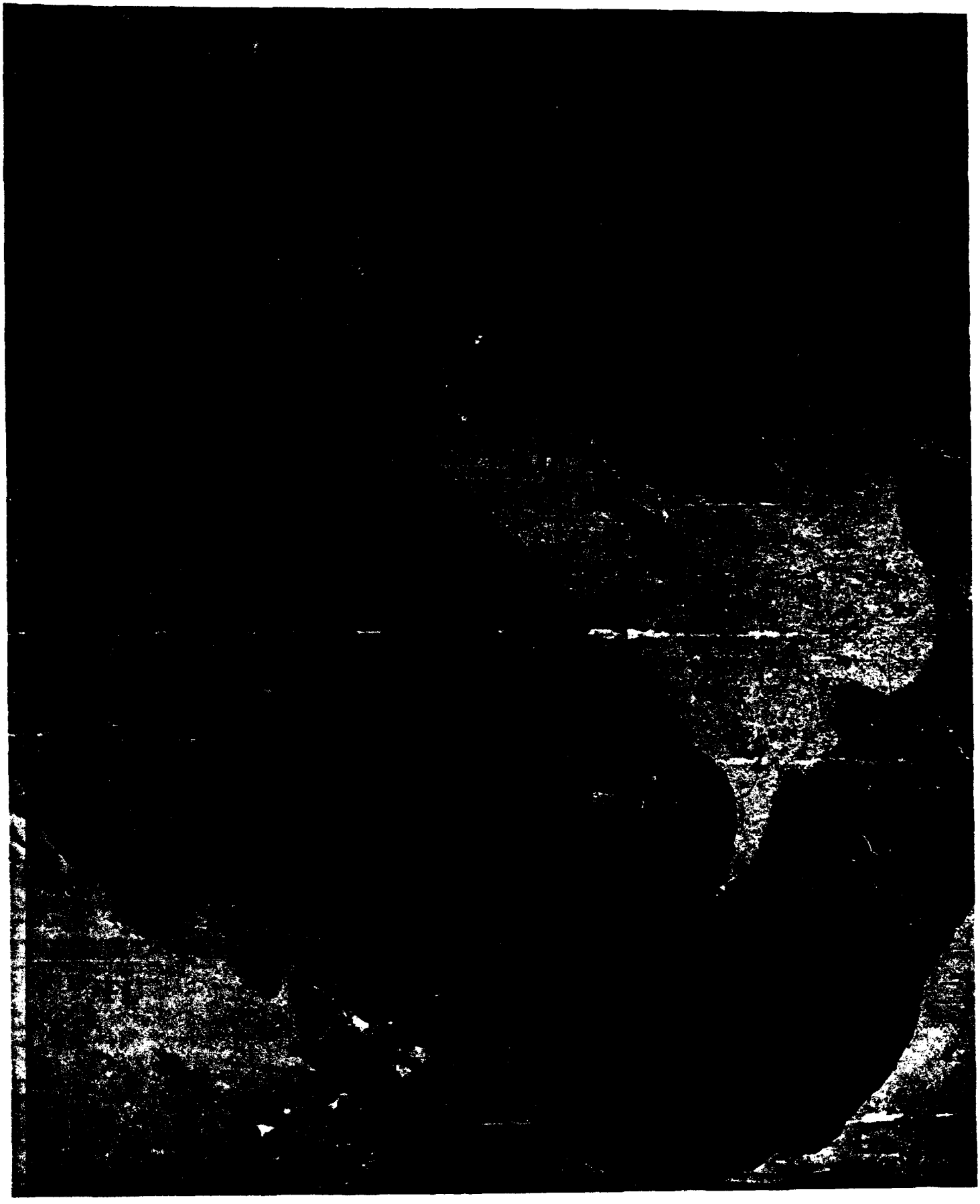


Fig. 5. Section of the epithelium of the ventral diverticulum.

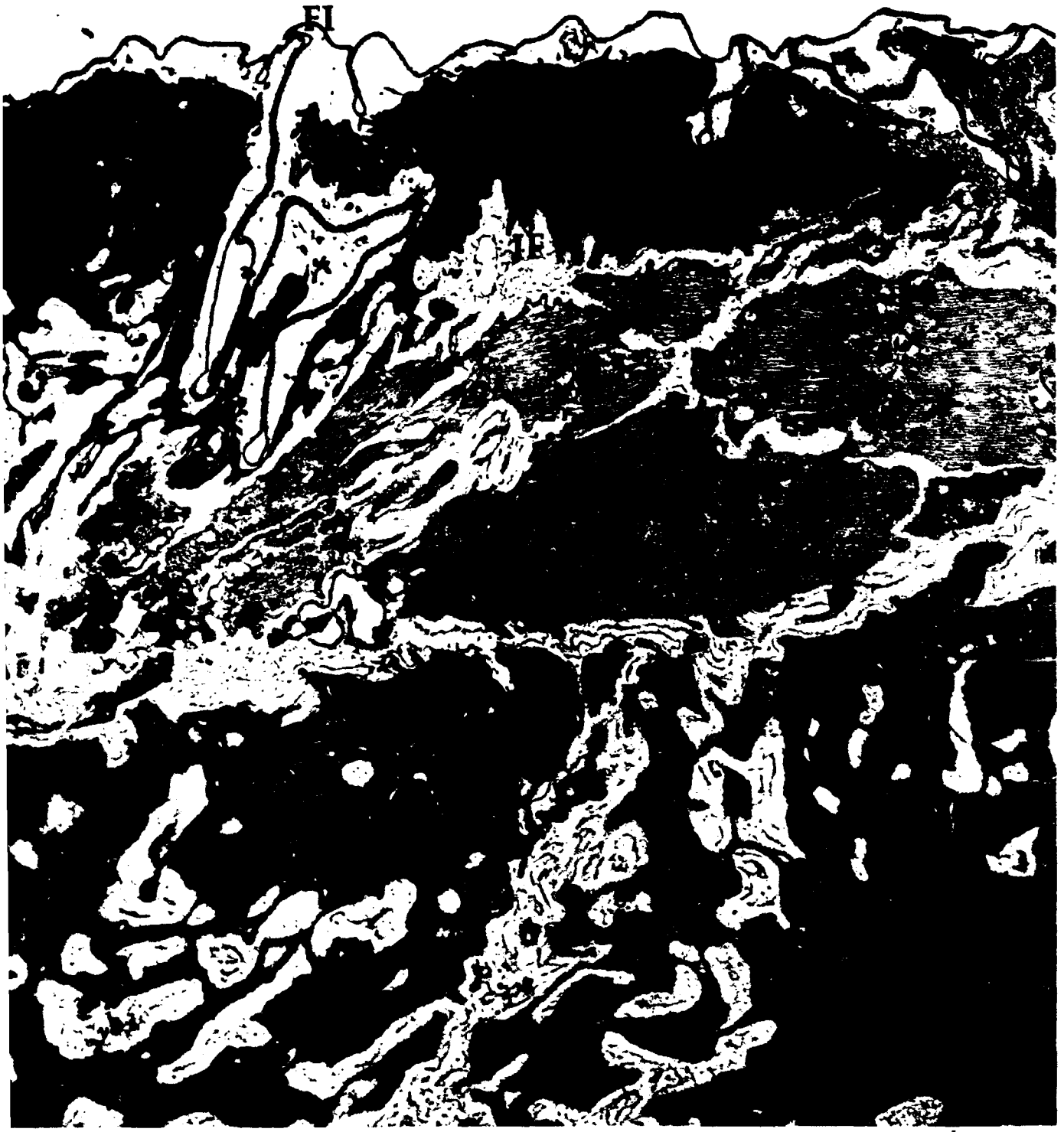


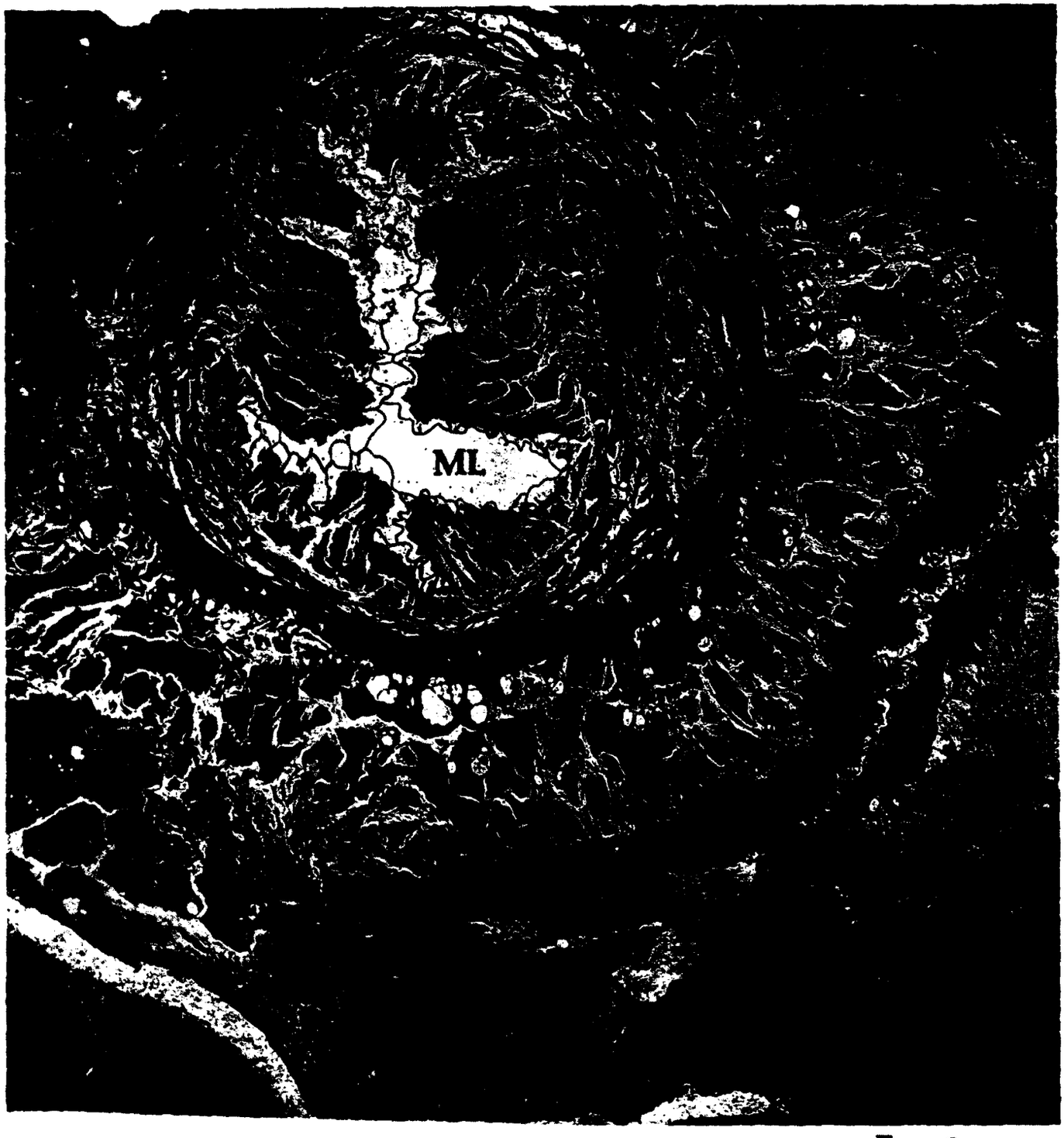
Em. 6. Saggital section of the anterior part of the proventriculus (cardia) and associated foregut epithelium.

Fig. 7. Saggital section of the proventriculus (cardia).



Em. 8. Section of the posterior end of the intussuscepted foregut as it faces into the midgut lumen.





Em. 9. Cross section of the proventriculus (cardia).

Em. 10. Sagittal section of a portion of the proventriculus (cardia) showing the anterior cardial epithelium.

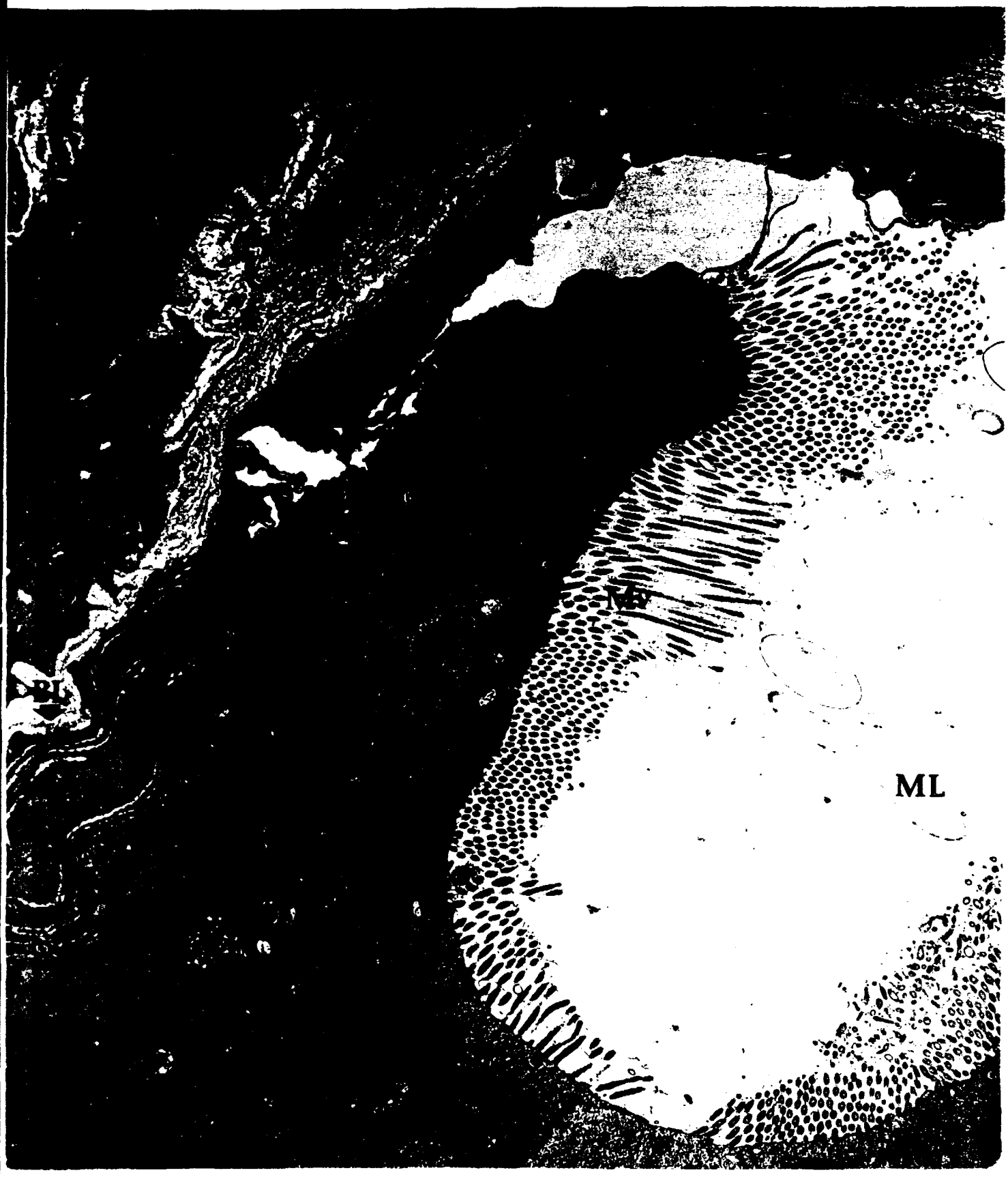
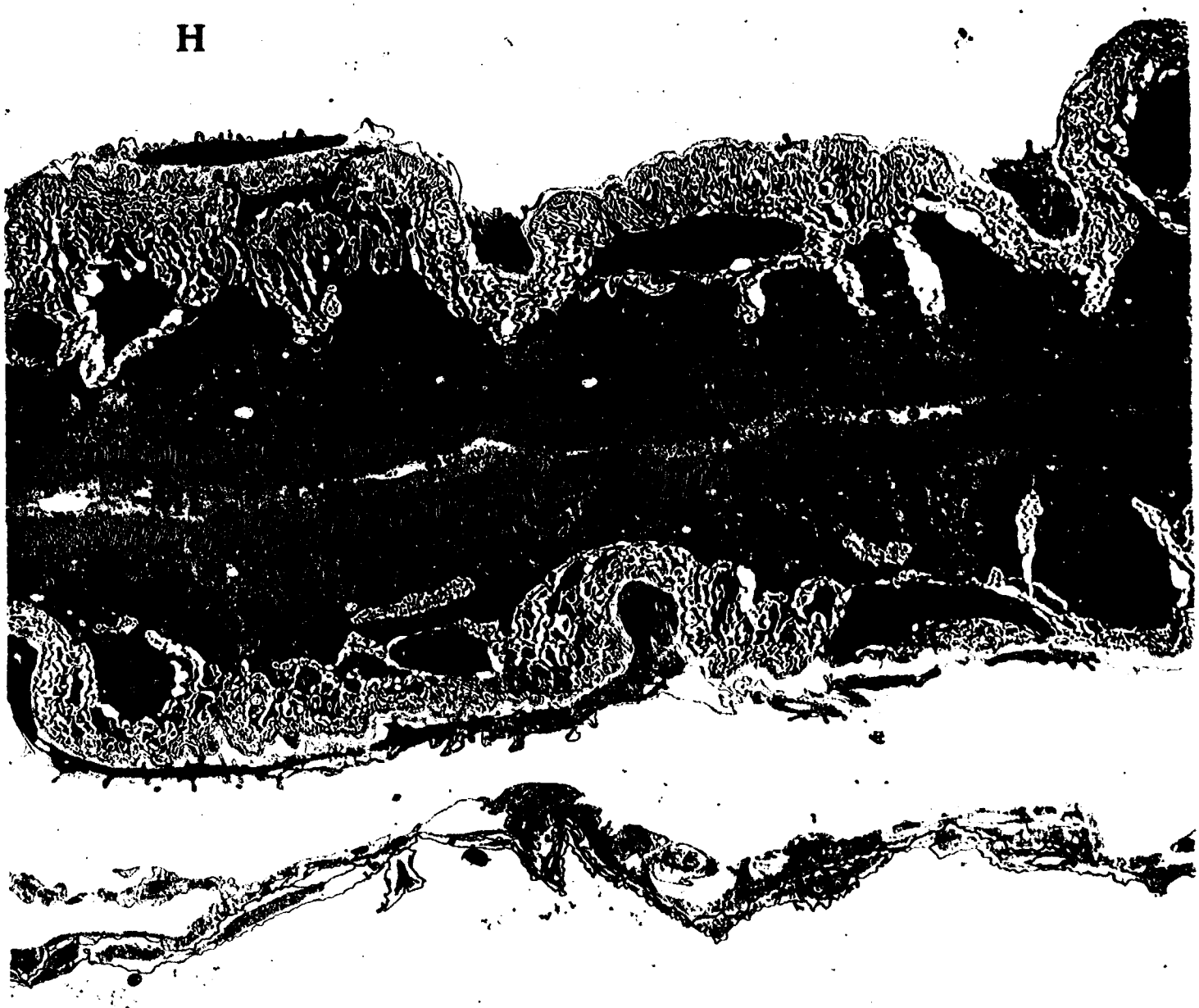


Fig. 11. Section within the proventriculus (cardia) in the region of the foregut-midgut junction.

Em. 12. Section of the cardial epithelium.

Em. 13. Section of the anterior midgut with ventral diverticulum beneath.



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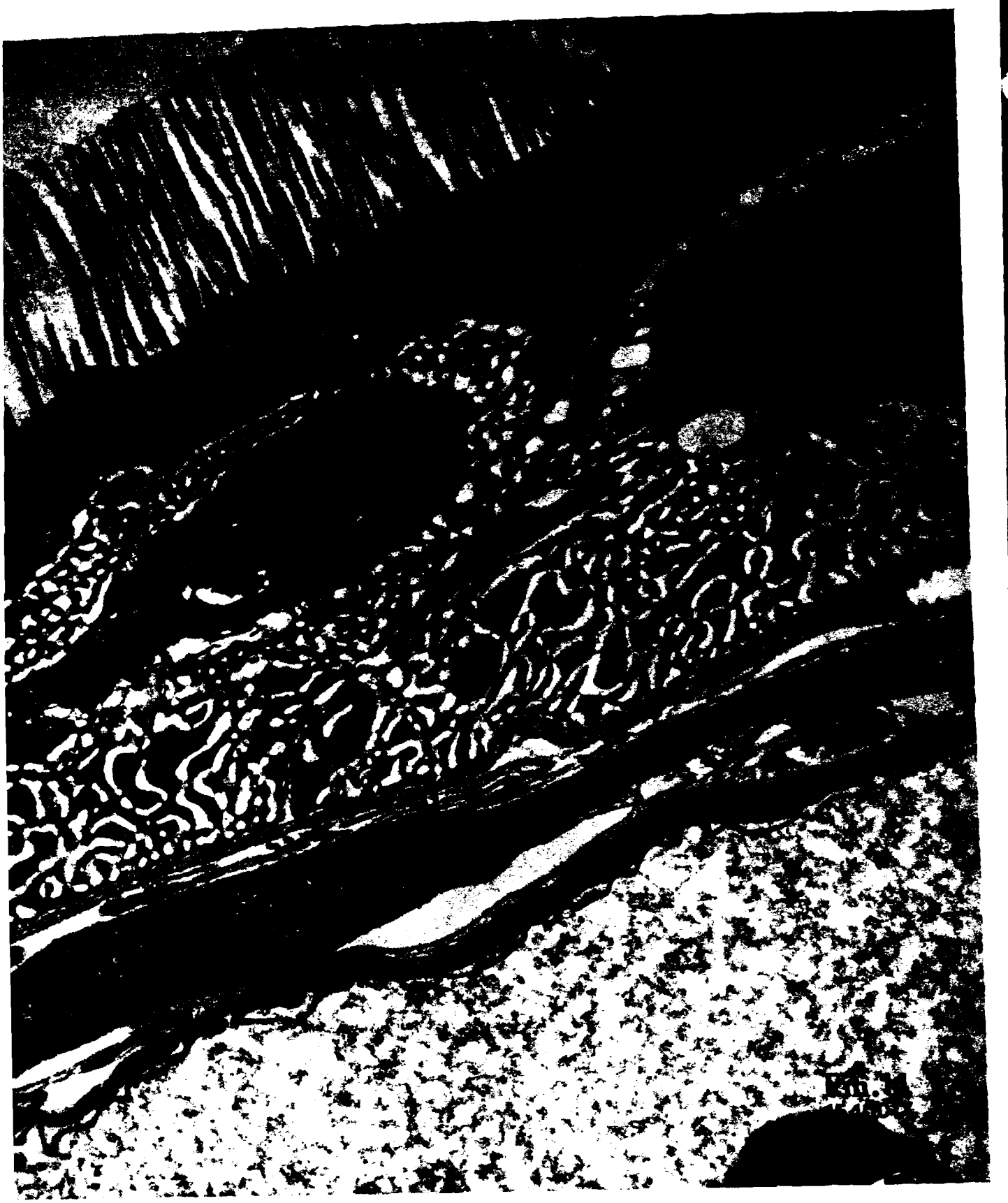
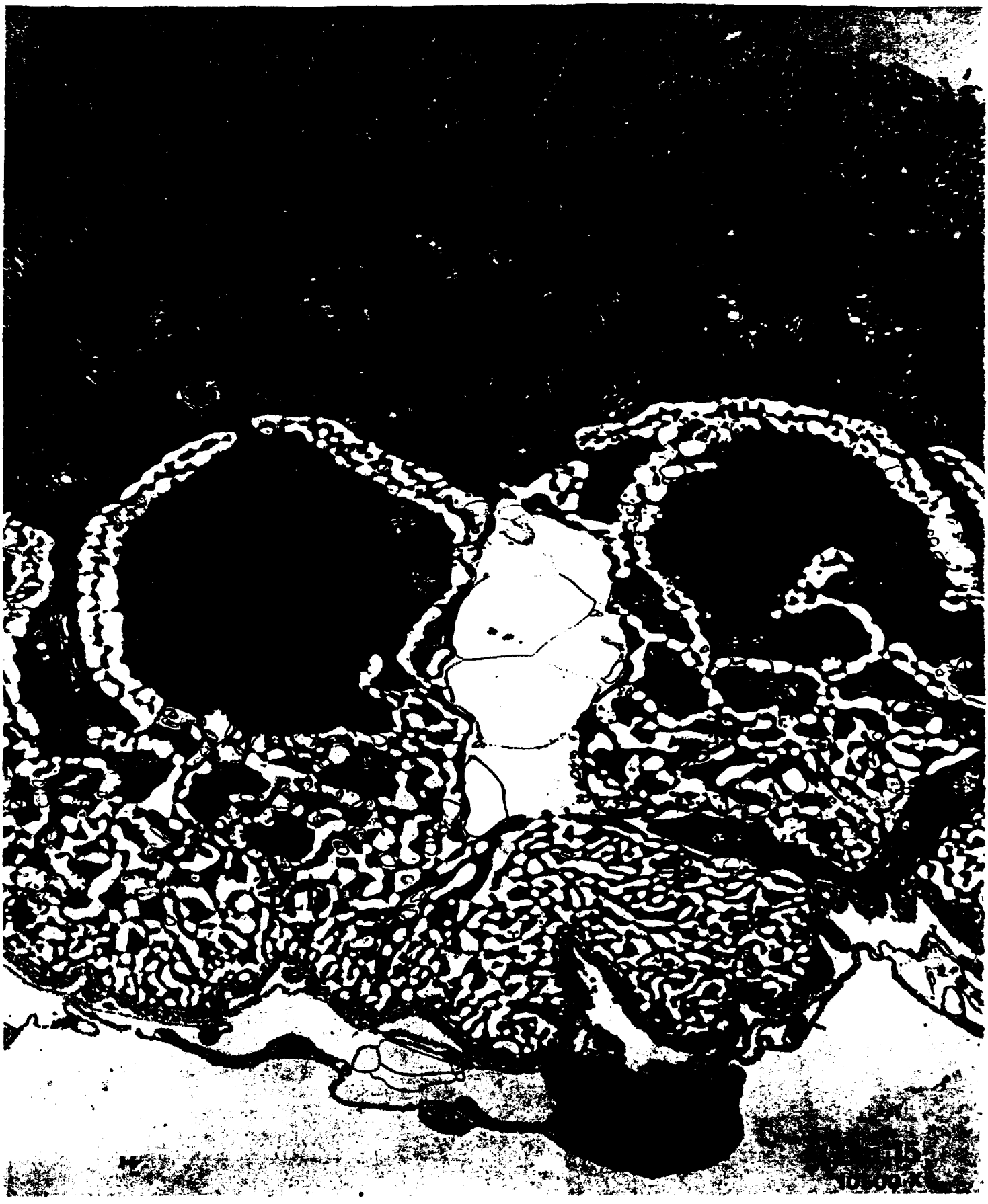


Em. 14. Section of the anterior midgut with epithelium of the ventral diverticulum beneath.

Em. 15. Section of the posterior hindgut epithelium.



Em. 16. Section of the posterior hindgut epithelium showing peritrophic membrane.

Em. 17. Section of Malpighian tubule.

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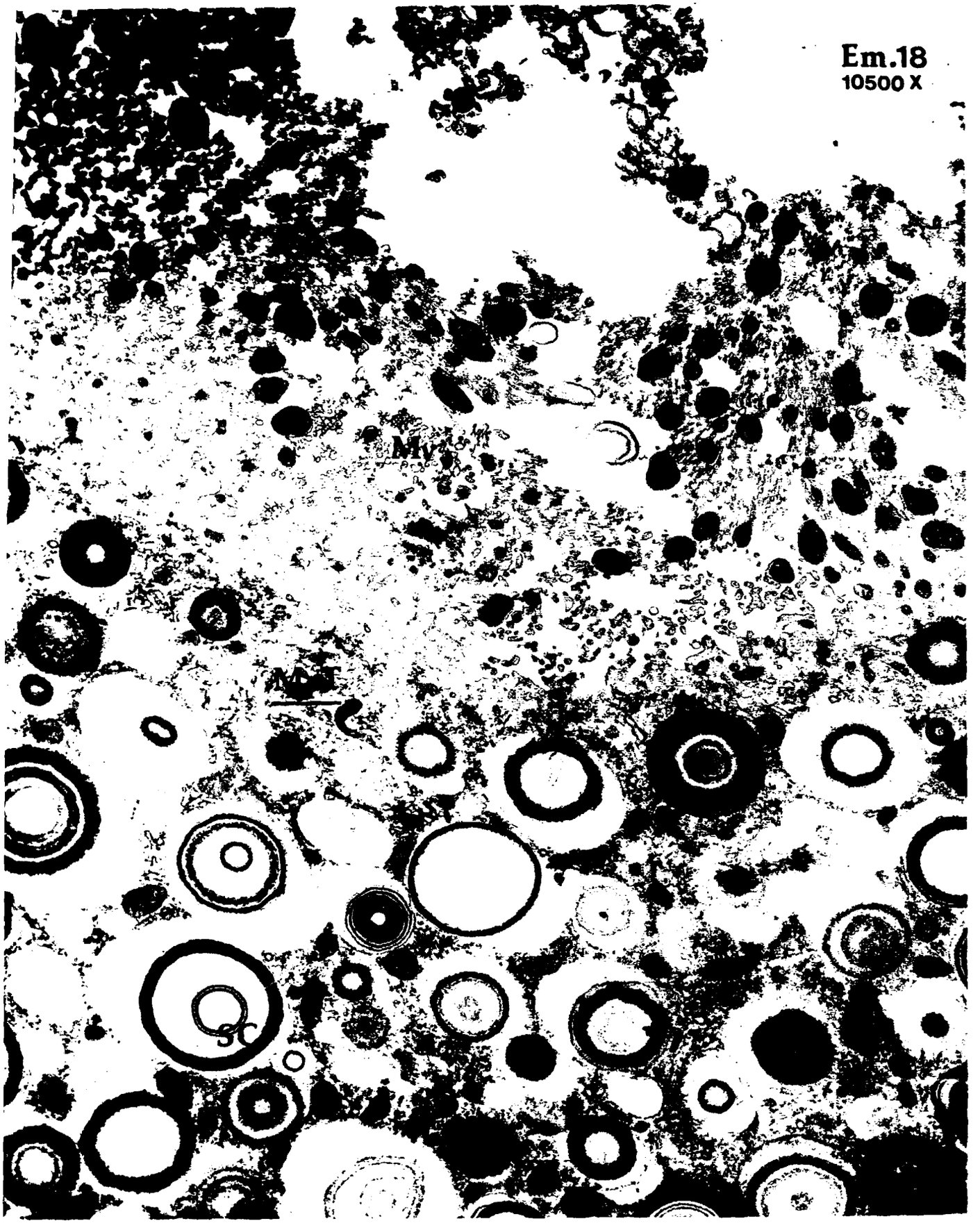
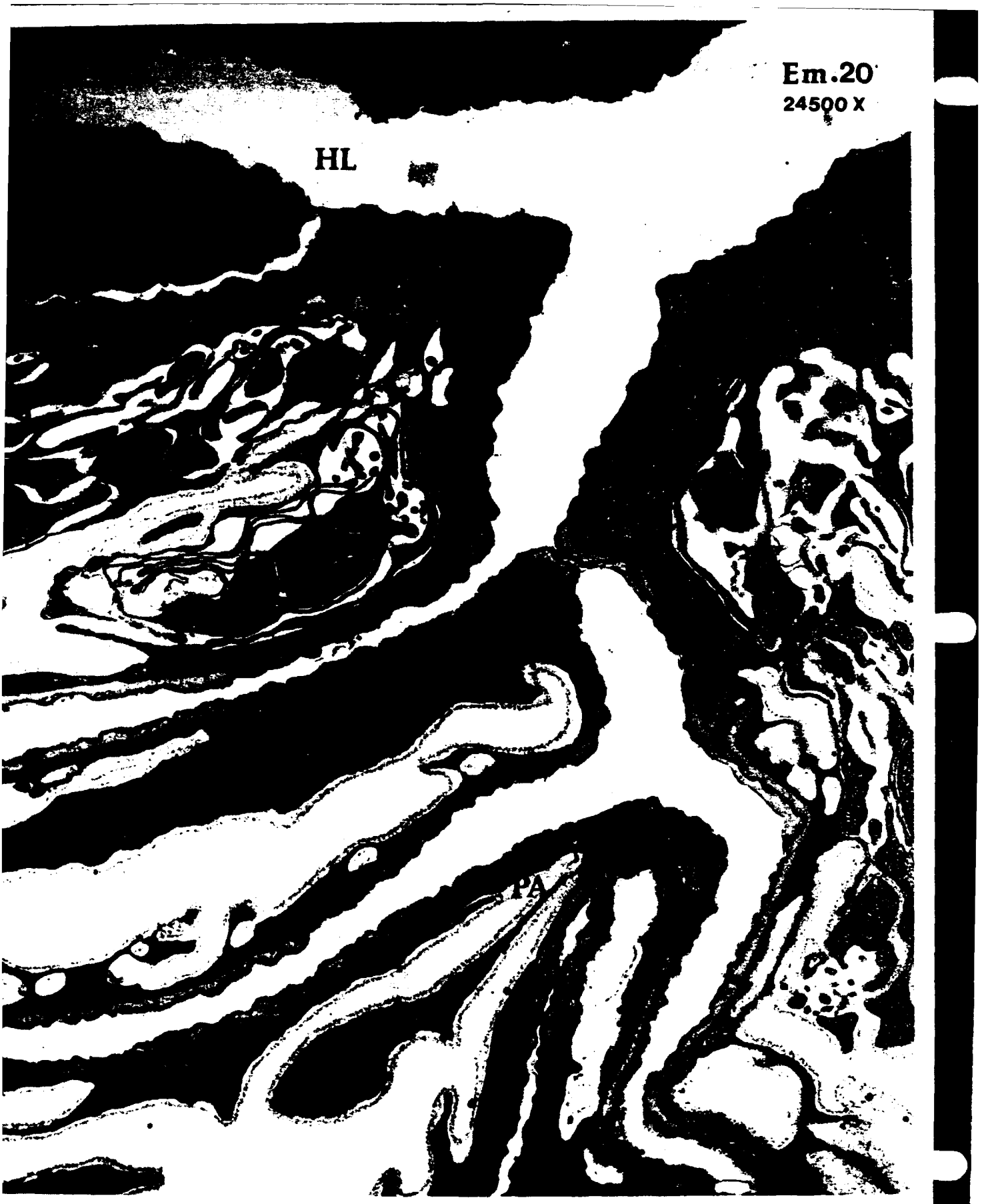


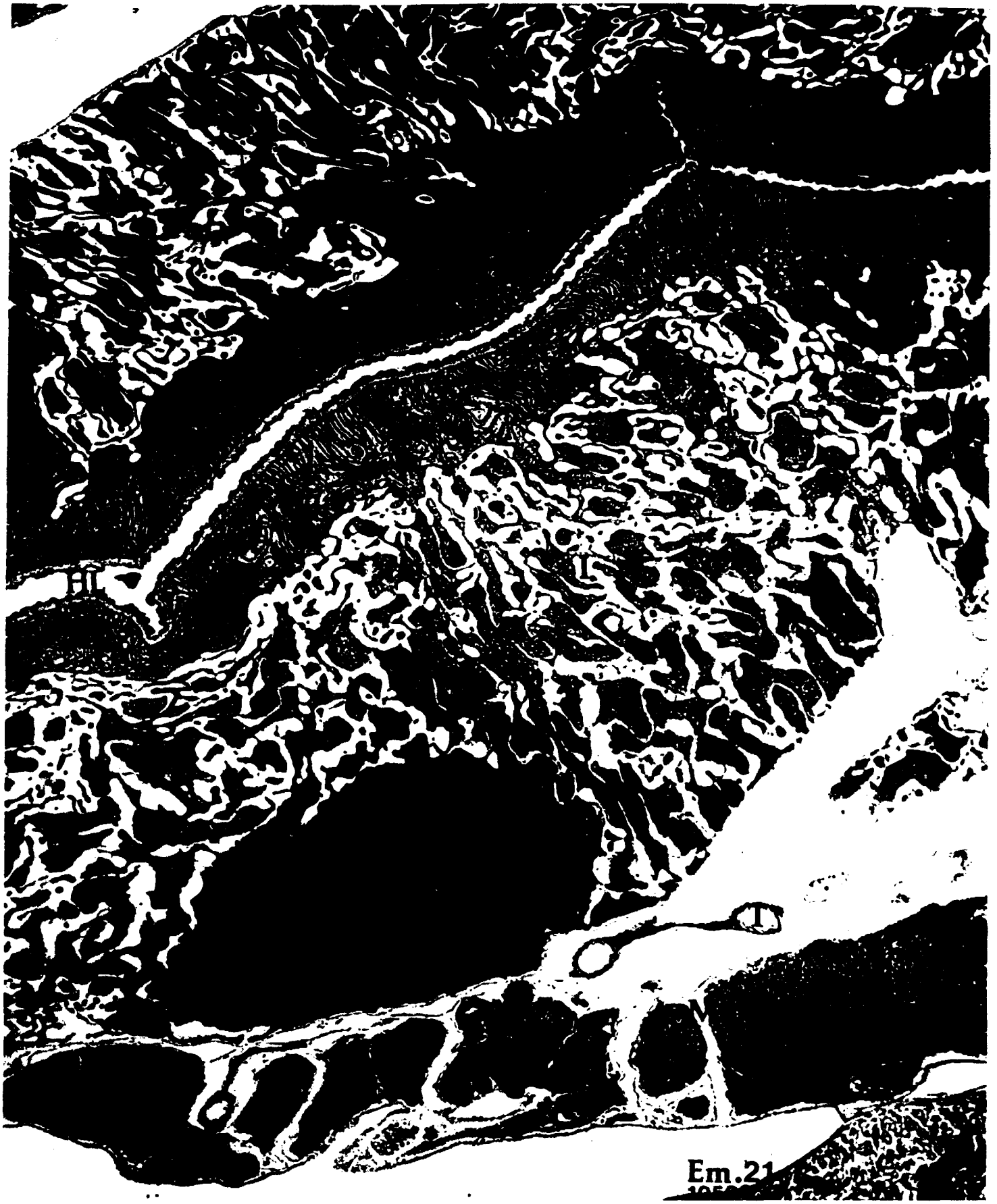
Em. 18. Section of Malpighian tubule.

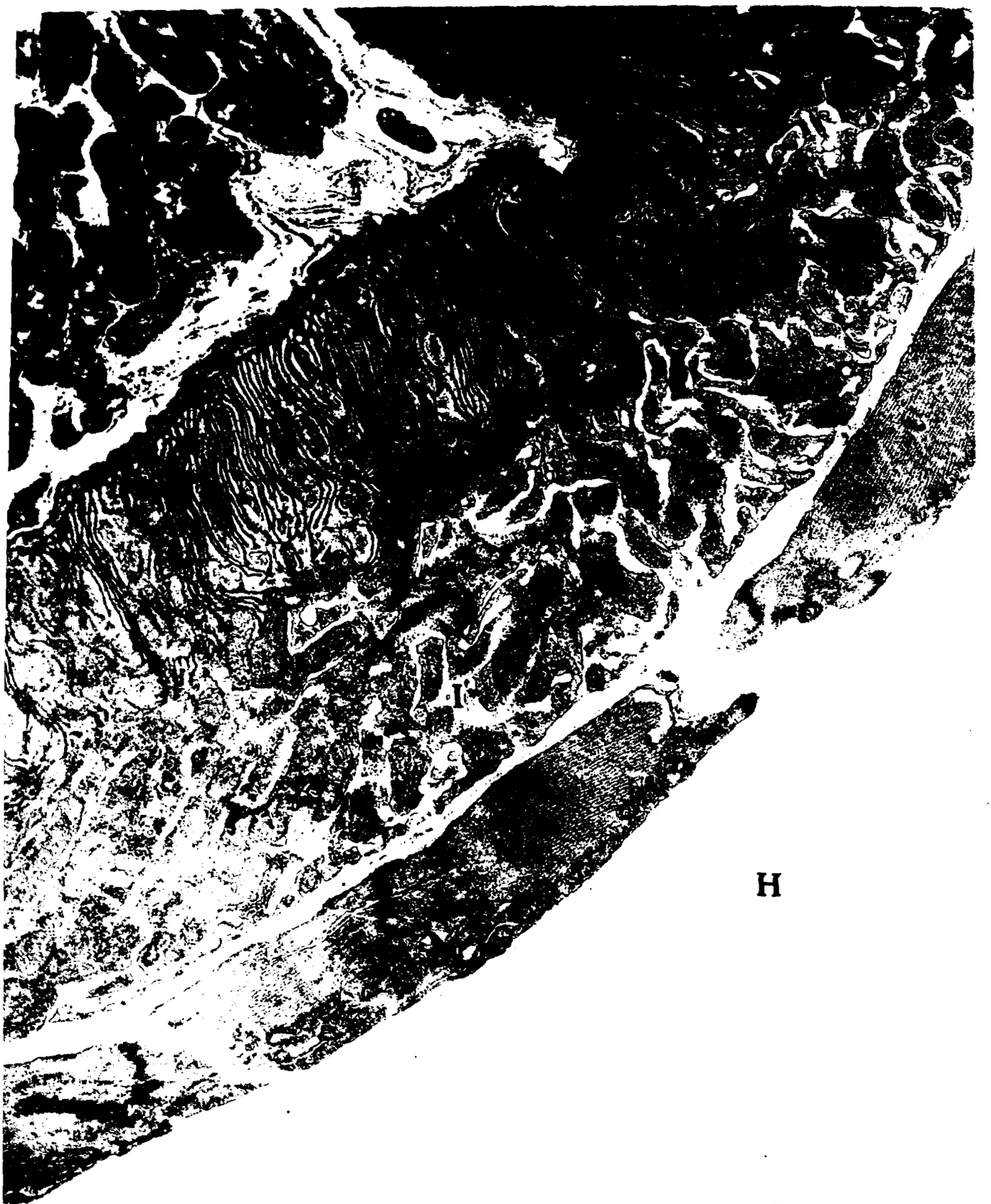
Em. 19. Section in region of midgut-hindgut junction.



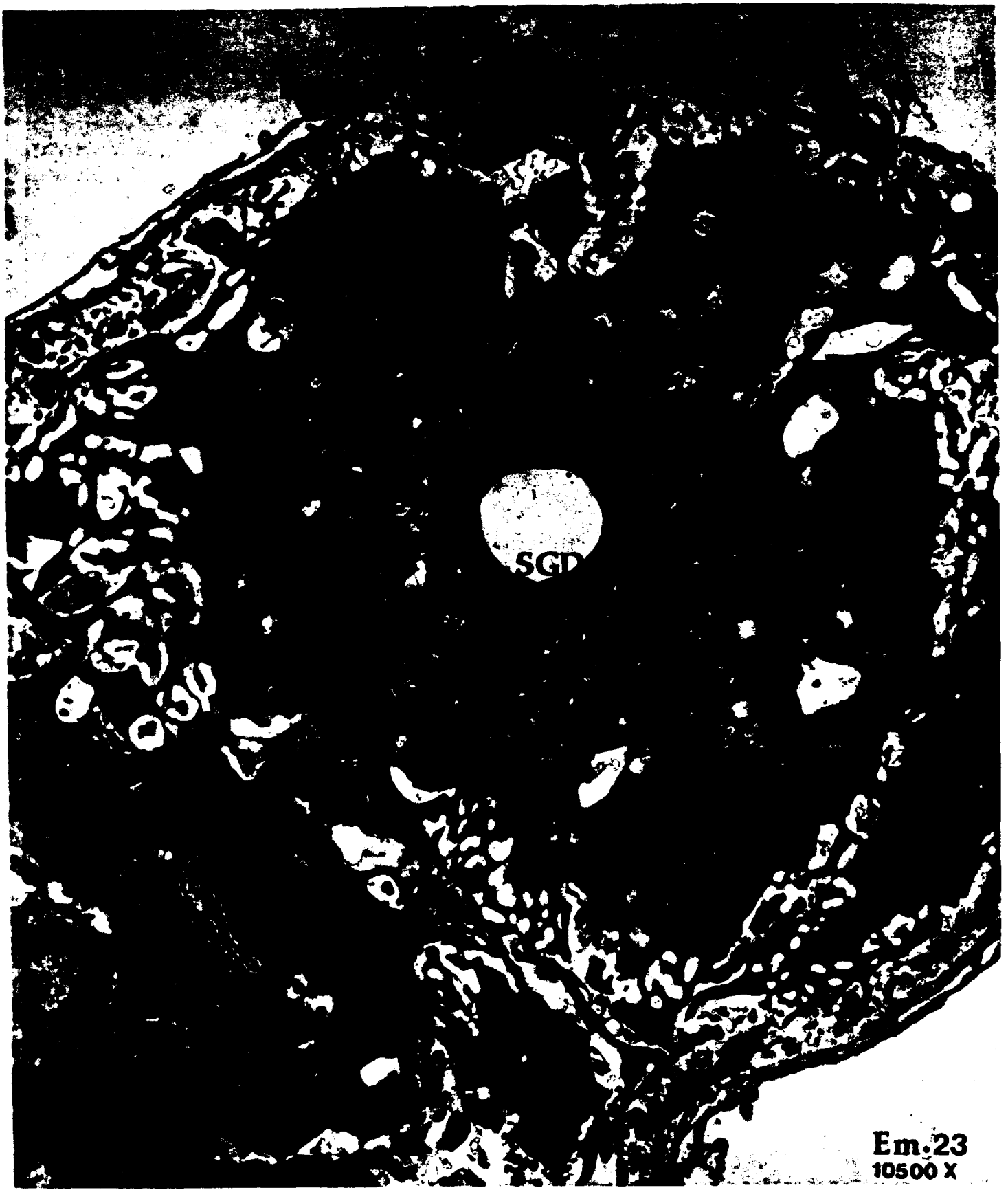
Em. 20. Section of hindgut epithelium.

Em. 21. Saggital section of hindgut.



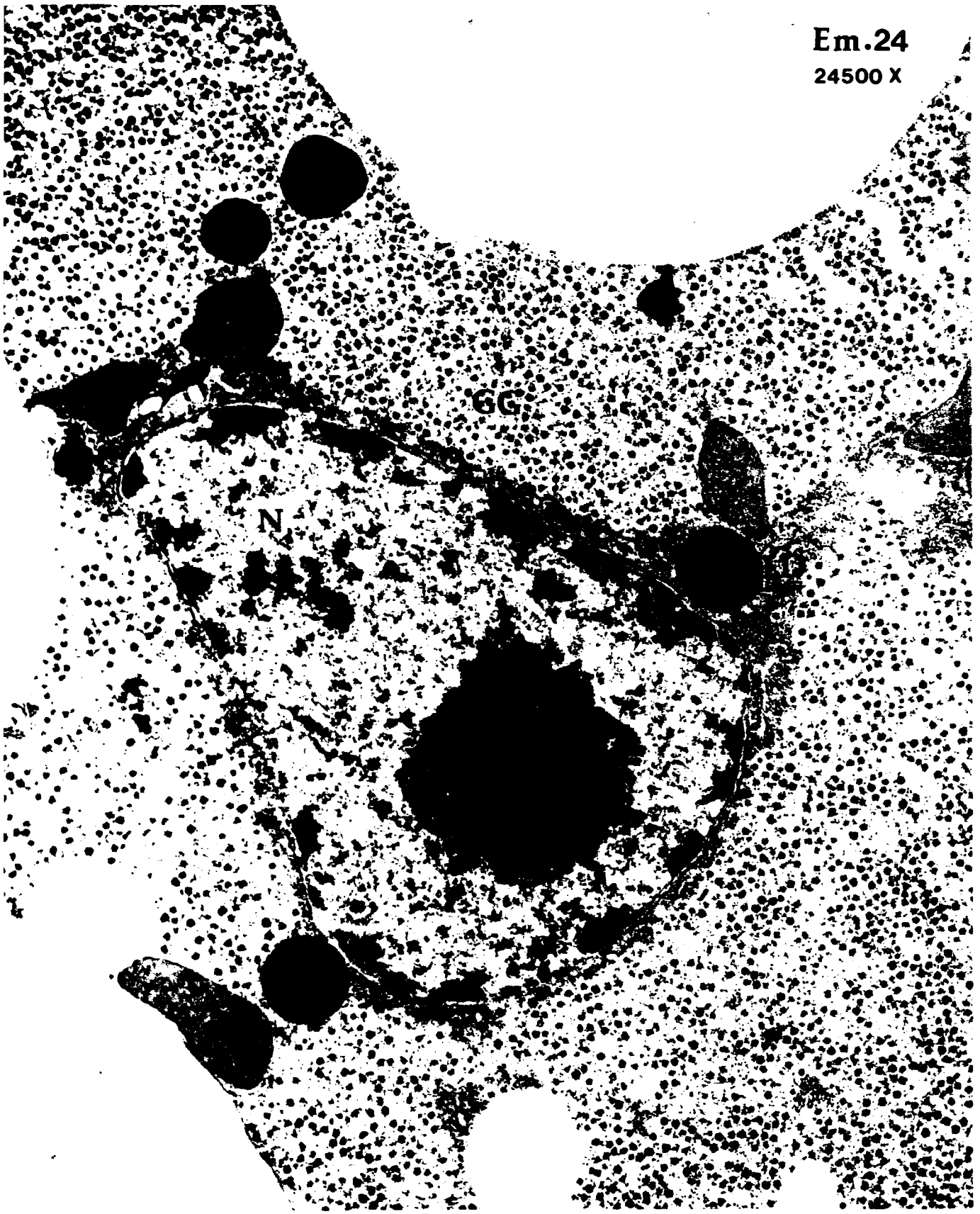


Em. 22. Section of hindgut showing bacteria in lumen.



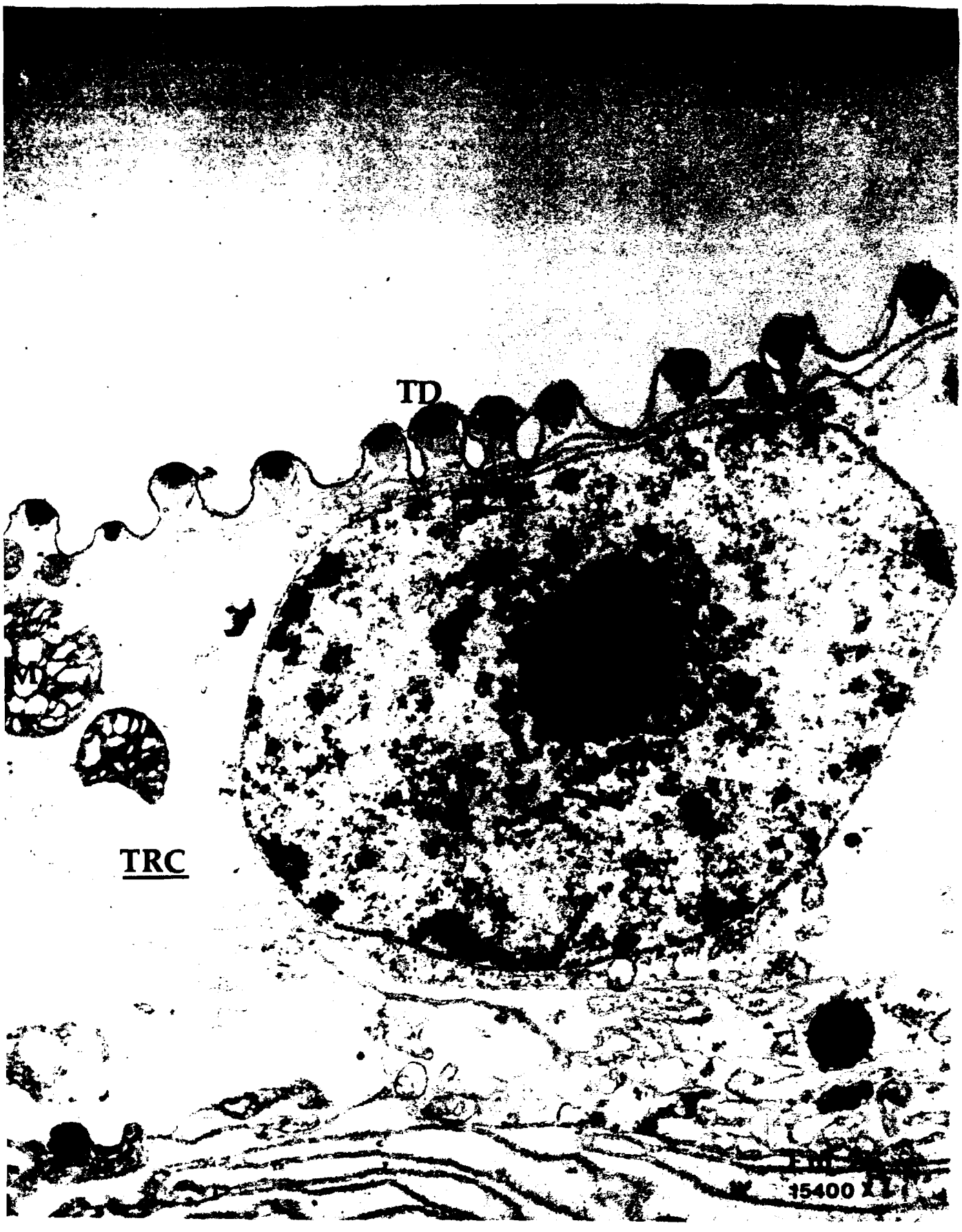
Em. 23. Cross section of salivary gland.

Em. 24. Fat body cell.

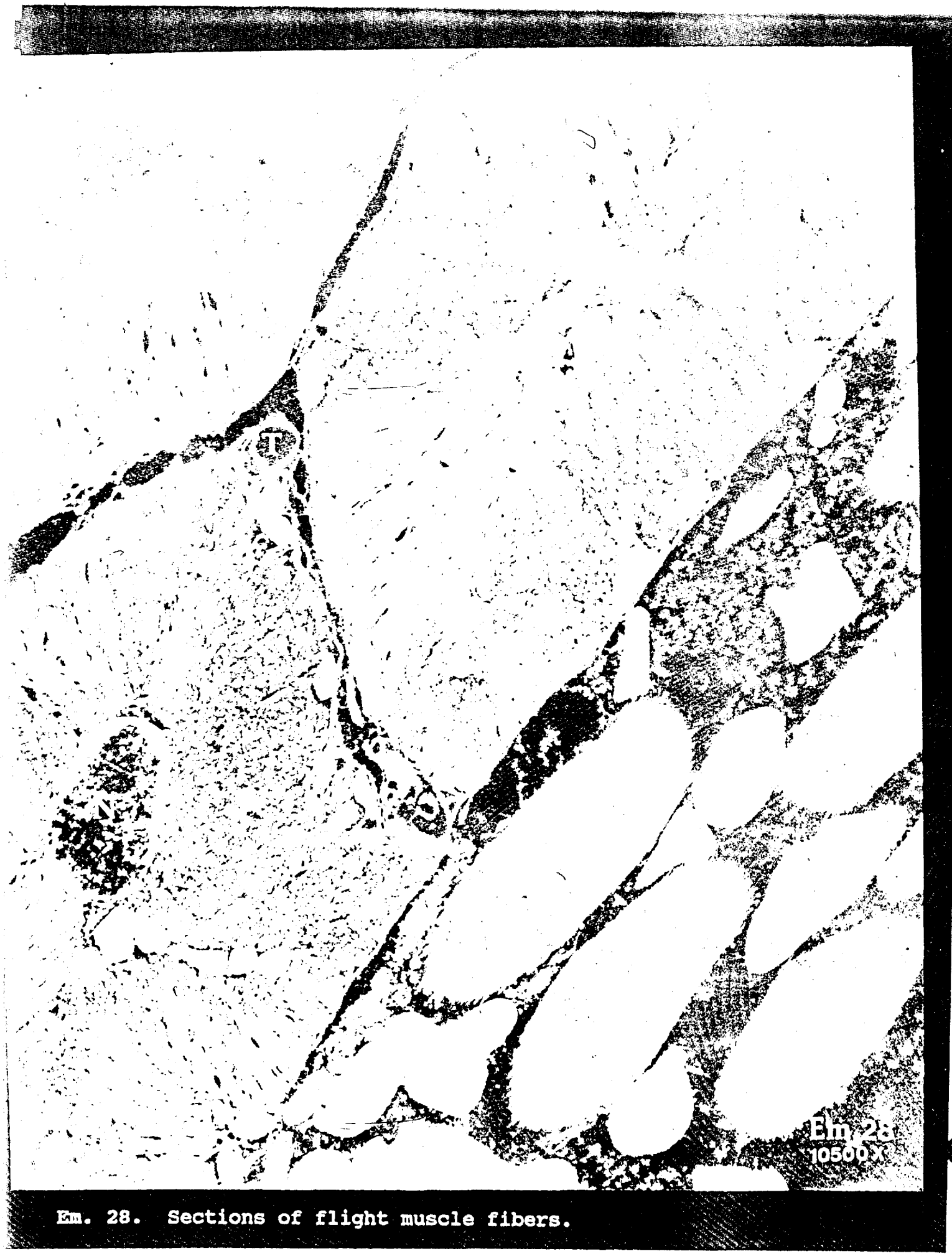


Em. 25. Section of a trachea.

Em. 26. Section of a trachea.



Em. 27. Longitudinal section of skeletal muscle fibers.



Em. 28. Sections of flight muscle fibers.

Em. 29. Sections of skeletal muscle fibers showing myocuticular insertions.

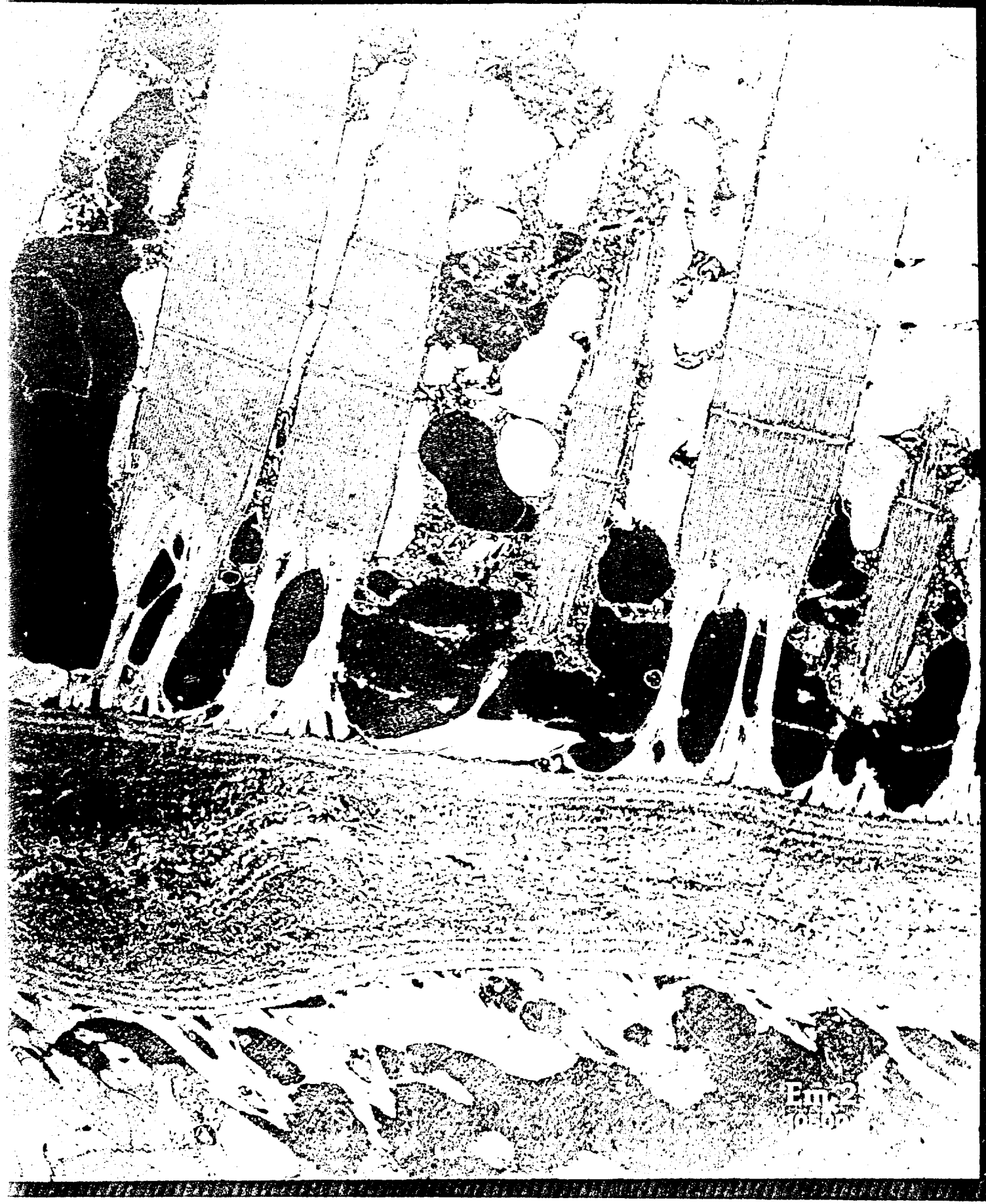
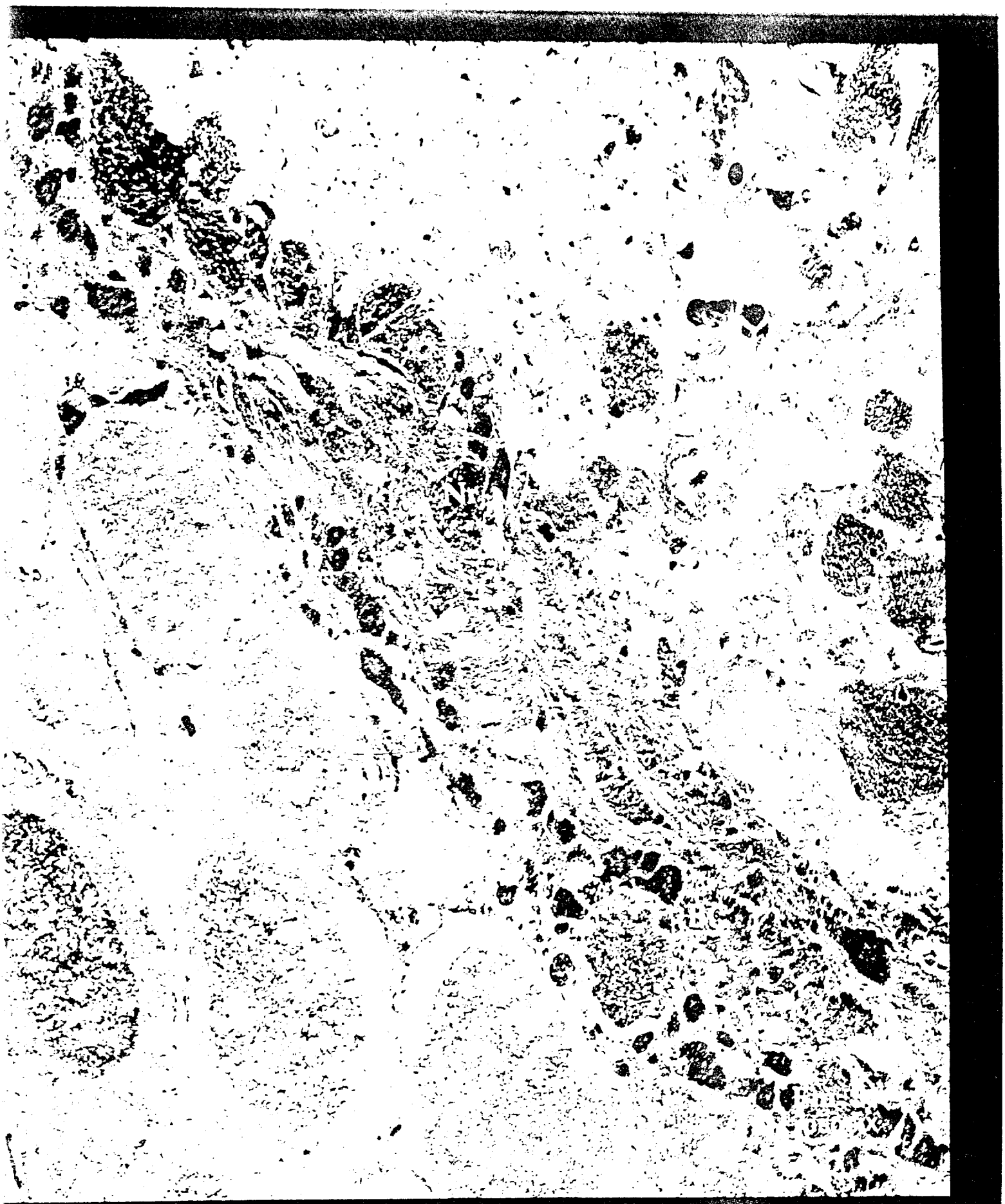
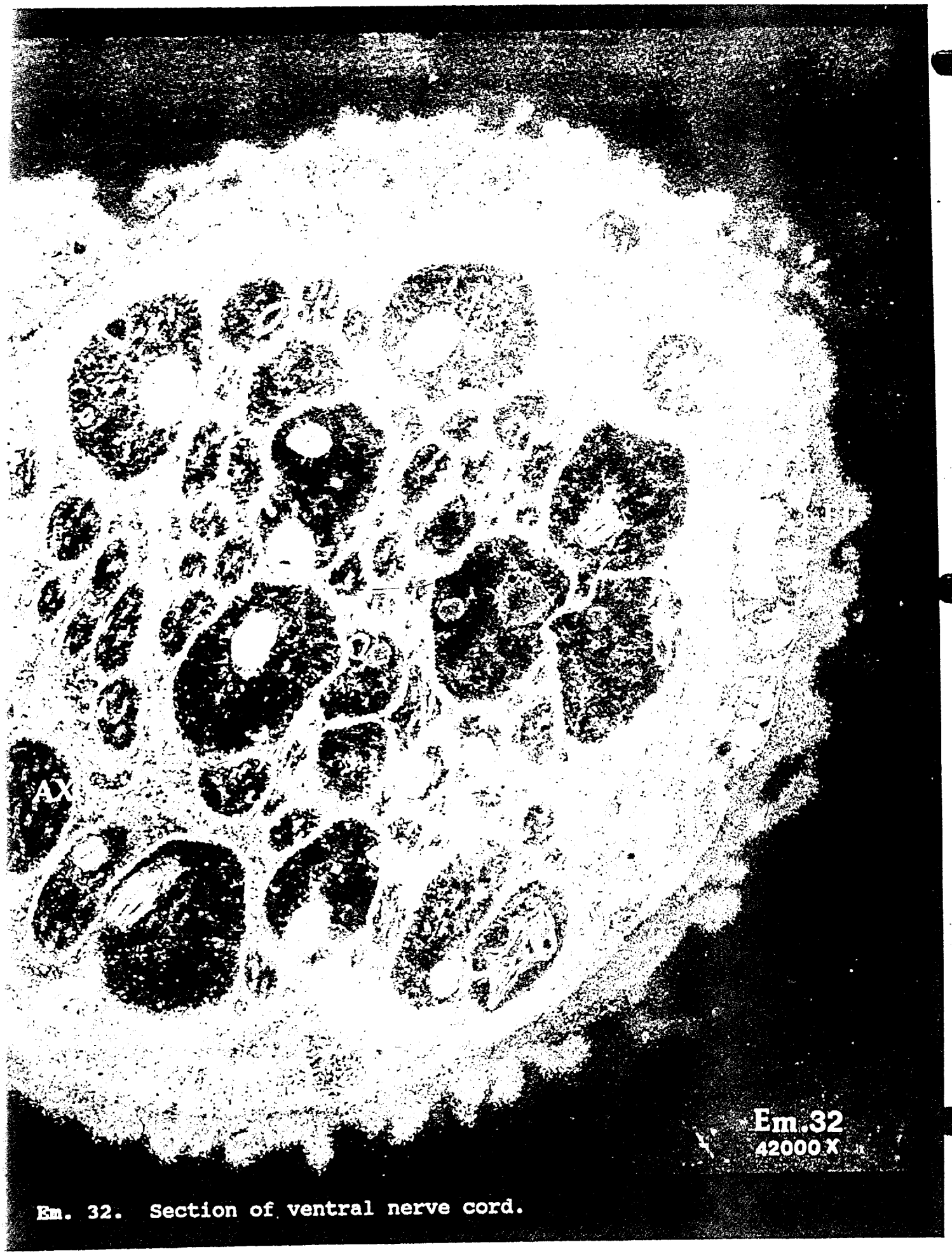


Fig. 30. Section of thoracic ganglion.

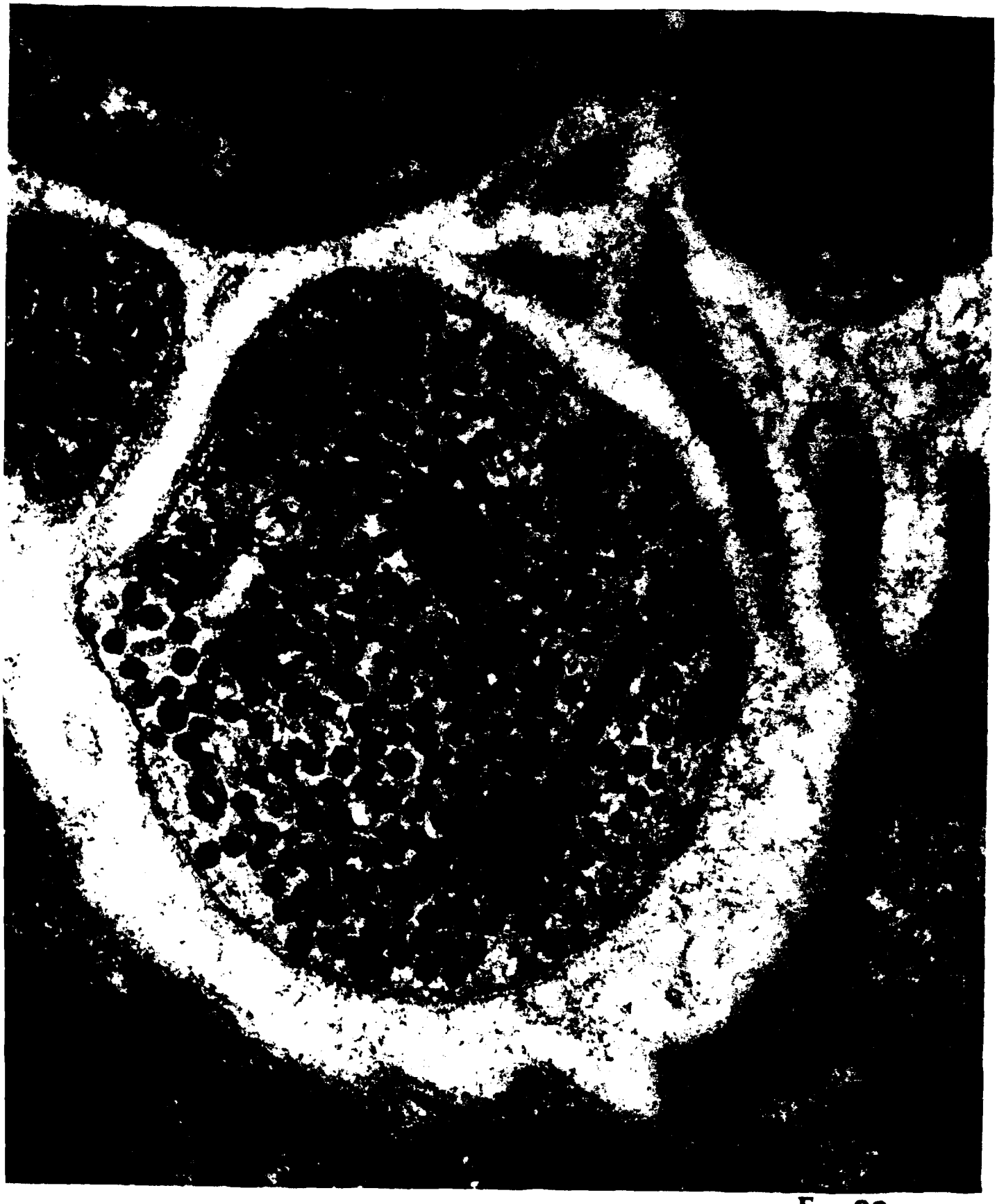


Em. 31. Section within brain.



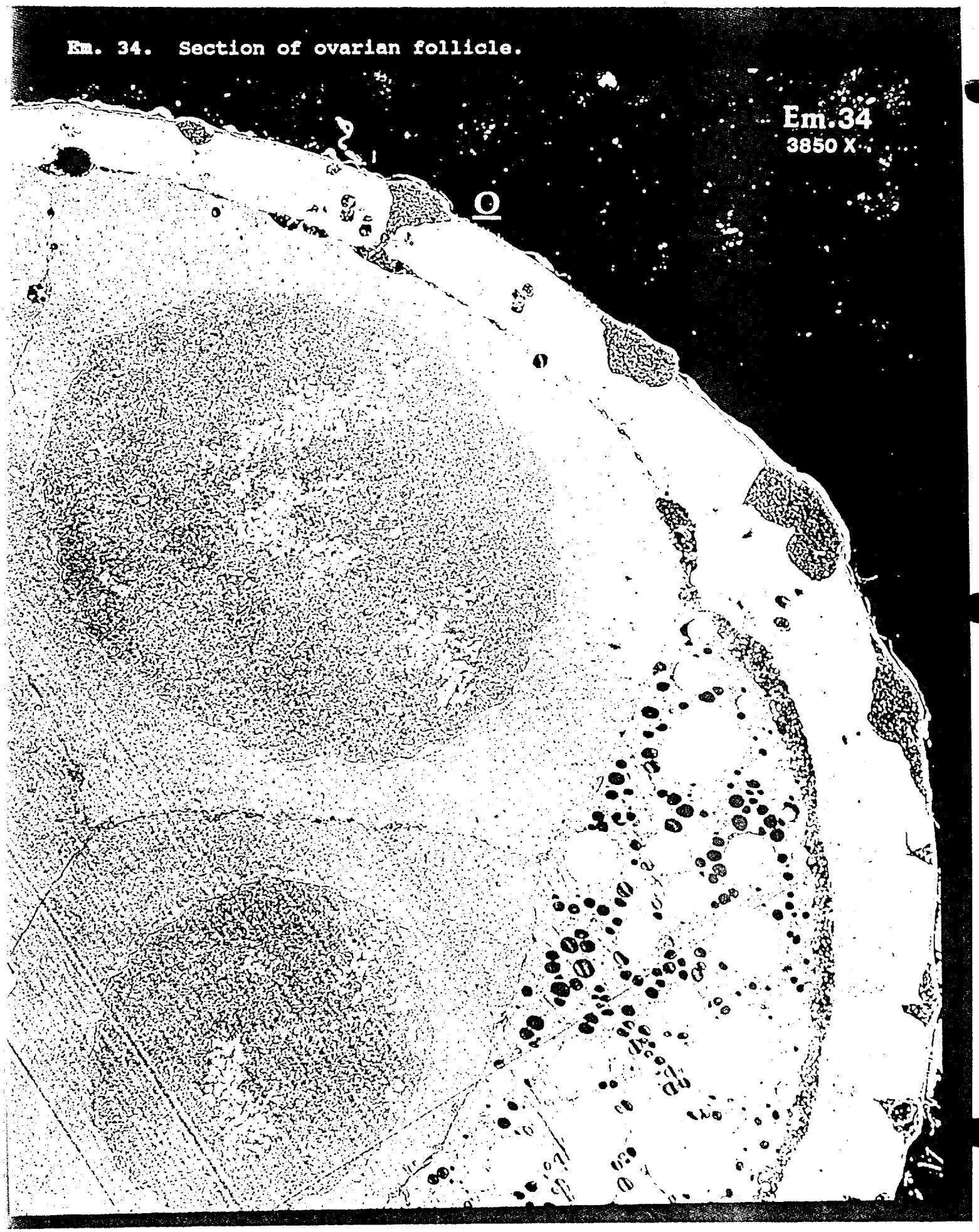
Em. 32. Section of ventral nerve cord.

Em. 33. Neurosecretory granules within neurosecretory axon.



Em.33
70000 X

Em. 34. Section of ovarian follicle.



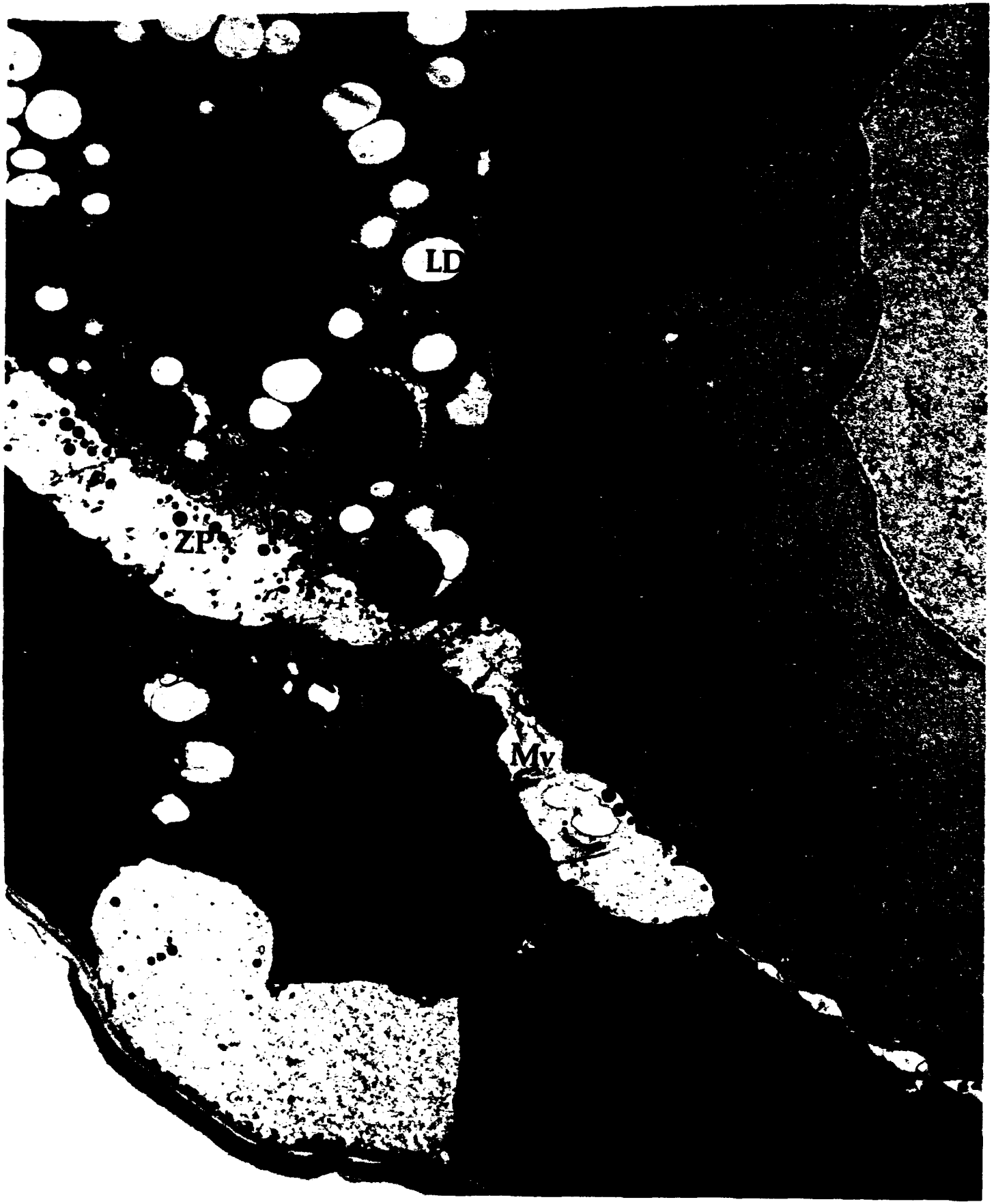
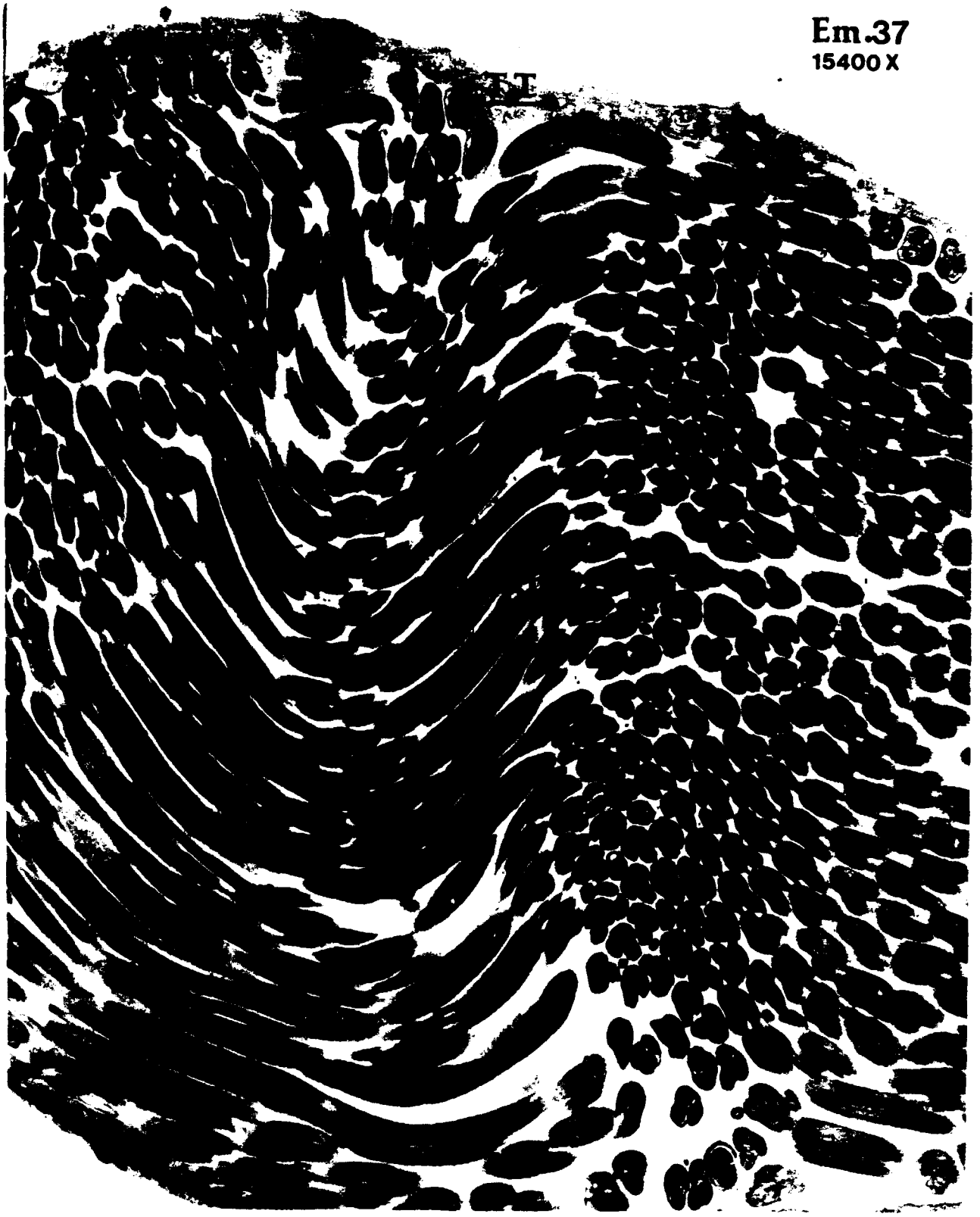
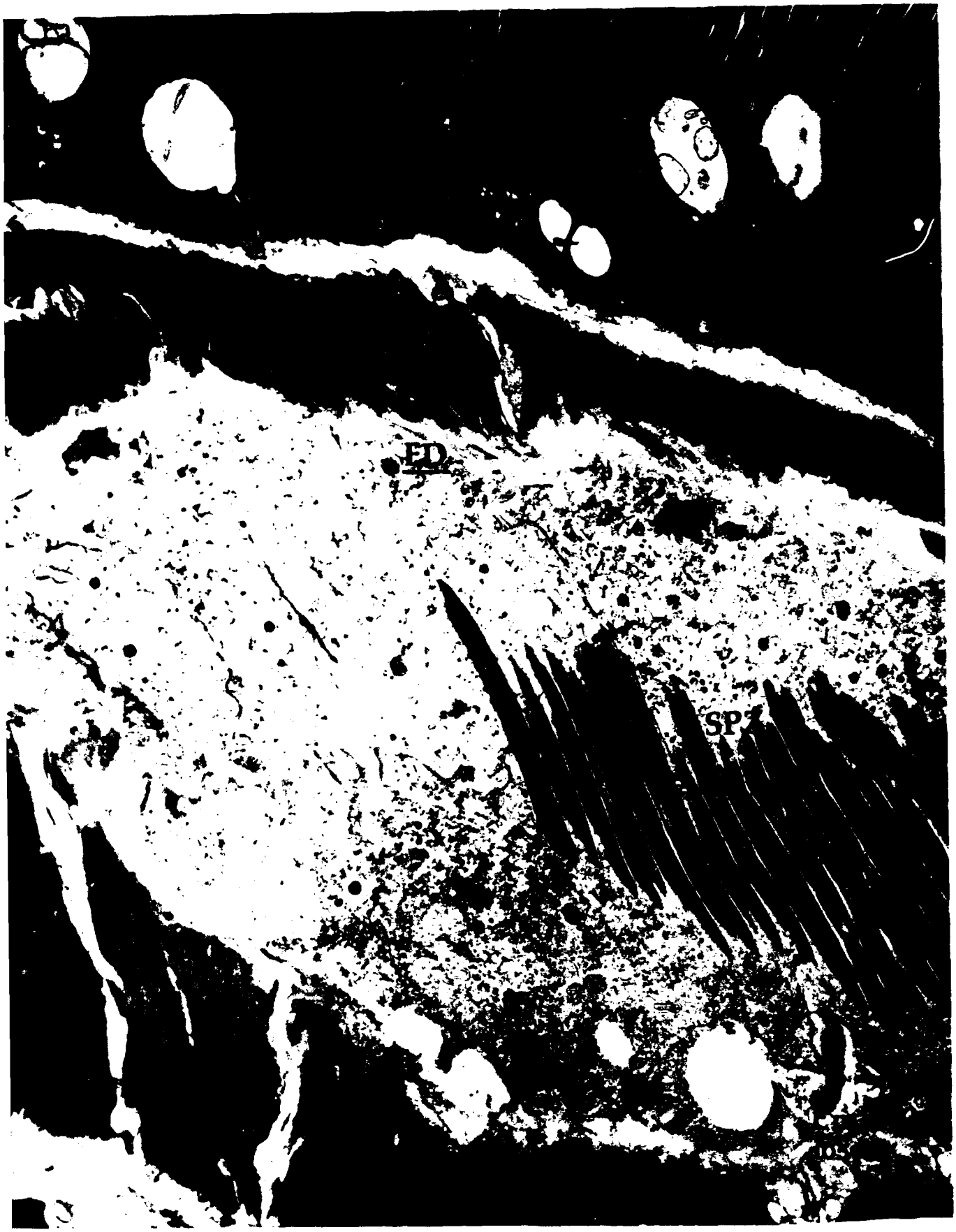


Fig. 35. Section of ovarian follicle.

Em. 36. Section of ovarian follicle.

Em. 37. Spermatozoa within testicular follicle.





Em. 38. Spermatozoa within ejaculatory duct of male.

PREPARATION OF MOSQUITO TISSUES FOR: HISTOLOGY, IMMUNOCYTOCHEMISTRY, & ELECTRON MICROSCOPY

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Department of Biological Sciences
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Smith's Modified Bouin's Fixative

Picric acid (Saturated aqueous soluiton)	45 ml
95% Ethanol	45 ml
Formalin, reagent grade	5 ml
Glacial Acetic acid	5 ml

:-Mix in the order listed
:-Prepare fixative just before using and preheat in the oven (60-65 C) for approximately 10 min. before plunging the specimens into fixative
:-Return hot Bouin's containing the specimens to the oven for 10 min., remove and allow the solution to cool to room temperature
:-Fix specimens for 24 hours, then wash in 70% ethanol 3-4 times until the alcohol no longer turns yellow
:-Store specimens in 70% ethanol until ready to infiltrate

Paraffin Embedding of Mosquitoes

Dehydration	70%	60 min.
	95%	60 min.
	100% - I	60 min.
	100% - II	60 min.
	100% - III	60 min.
Clearing	Hemo-De - I	30 min.
	Hemo-De) II	30 min.
Infiltration	Hemo-De:Paraplast (1:1)	30 min.
(in the oven 55-60 C)	Paraplast	60 min.
	In Vacuum (380-500 torr)	30 min.
	Fresh Paraplast	60 min.
Embedding	Proboscis Right/Head Up	

Simplified Azan Staining Technique

(Hubschman,J.H. 1962: A simplified azan process well suited for crustacean tissues. Stain Technol. 37:39-40, 1962)

Azan solution:

Solution I:- Dissolve 0.25 gm of Azocarmine in 250 ml dist.water, boil the solution for 5 min., allow to cool and add 5.0 ml glacial acetic acid. Filter before using.

Solution II:- add the following stain in order:
0.30 gm Aniline Blue
1.10 gm Orange-G
2.50 gm Phosphotungstic acid

:- Mix well in 250 ml dist. water

Staining Procedure

Hemo-De I	5 min.
Hemo-De II	2 min.
100% Ethanol	2 min.
95% Ethanol	2 min.
70% Ethanol	2 min.
dist. Water	2 min.
Azan I	15 min.
dist. Water	Rinse slides by dipping 2-3 times
1% Aniline in 95% Ethanol	30 sec.
dist. Water	Rinse slides by dipping 5-6 times
Azan II	15 min.
100% Ethanol	1 min.
100% Ethanol	1 min.
100% Ethanol	10 min.
Hemo-De I	2 min.
Hemo-De II	5 min.

Mount in Permount solution & place on slide warmer.

Eosin Y staining solution for H & E staining

Prepare 0.1-0.5% Eosin Y solution by:
adding 0.5 gm Eosin Y in 100 ml 95% ethyl alcohol,
then adjust pH to 5.4-5.6 with 0.1N HCl.

Stain for 2-5 min.

Hematoxylin & Eosin

Solutions:

1. Gill's III (no.3) hematoxylin (Polysciences)

Hematoxylin	4.00 gm
Sodium iodate	0.40 gm
Aluminum sulfate (or Potassium alum)	35.20 gm
Ethylene glycol	250.00 ml
Glacial acetic acid	40.00 ml
Distilled water	710.00 ml

2. Counterstain - Buffered Eosin Y/Phloxine B

Solution 1:	Glacial acetic acid	5.75 ml
	Distilled water	1000.00 ml
Solution 2:	Sodium acetate	8.2 gm
	Distilled water	1000.00 ml

:- Mix 295.00 ml of solution 1 with 705.00 ml of solution 2,
:- Add 5.00 gm Eosin Y & 0.50 gm Phloxine B,
:- Add two crystals of Thymol to prevent mold.
This gives a consistently reliable quality with a shelf life of several months.
The concentration of the counterstain is 0.50% Eosin Y and 0.05% Phloxine B with a final pH of 4.98.

3. Scott's tap water substitute:(GMA does not like Ammonium water)

Tap water	1000.00 ml
Magnesium sulfate (anhy.)	10.00 gm
or Magnesium sulfate x 7 H2O	20.00 gm
Sodium bicarbonate	2.00 gm

Staining Procedure

1. Place in Gill's III hematoxylin for 10 minutes (Don't put in water before staining),
2. Rinse in distilled water (pH 7.0) - 2 changes - 10 dips each,
3. Place in Scott's tap water substitute - 2 minutes,
4. Rinse in distilled water - 2 changes - 10 dips each,
5. Counterstain in buffered eosin Y/phloxine B - 6 minutes,
6. Rinse in distilled water - 10 dips,
7. Dehydrate in absolute ethyl alcohol - 5 dips,
8. Transfer to mixture of ethyl alcohol:xylene (1:1) - 5 dips,
9. Clear in xylene - 2 changes - 5 dips each,
10. Mount from xylene with permount.

Reference: Castro, M.D. 1985 A hematoxylin-eosin phloxine stain for tissues embedded in glycol methacrylate. J. Histotechnol. 8:23-24.

Avidin-Biotin-Complex (ABC) Staining Procedure

The avidin-biotin-complex (ABC) system, developed by Hsu et al (1981a), is used to localize monoclonal antibody. This method is more sensitve than the peroxidase-antiperoxidase method (Hsu et al 1981b).

In this procedure, the primary antibody in low concentration is used and nonspecific tissues background staining is absent or minimized. This methodology employs primary antibody, a biotinylated secondary antibody and a preformed avidin-biotinylated horseradish peroxidase complex.

One advantage of the ABC system is that it utilize the same peroxidase complex fro all primary antisera irrespective of its origin in different animal species.

ABC reagents use in the study were obtained from Vector Laboratories, Inc. (Burlington, CA.)

Phosphate Buffer Solution (PBS)

Stock solution (4X):

KCl	3.2gm
K2HPO4	3.2gm
Na2HPO4	18.4gm
NaCl	128.0gm

In 4000ml of dist. water
Add 32ml 5% Thimerosol Solution (5gm in 100ml)

Working solution: Dilute 1:3 with dist. water

Adjust pH to 7.4

Tris solution: (0.05 M - $\frac{6.057gm \times 1000ml}{MW\ 121.4}$)

Add 6.057gm Tris in 1000ml dist water

Adjust pH to 7.6 (with Strong HCl)

ABC-Paraffin Sections

		Minutes
Deparaffinize:	Hemo-De I	3
	Hemo-De II	2
Hydrate:	100% Ethanol	2
	95% Ethanol	2
	70% Ethanol	2
	dist. water	2
Wash:	PBS I	5
	PBS II	5
	PBS III	5

Destroy Endogen Peroxidase activity:

(0.456gm/200ml dist.water) 0.01 M Periodic Acid	10
(2ml of 1% + 198ml dist.water)0.01% Na Borohydride	10

Wash:	PBS I	5
	PBS II	5
	PBS III	5

Block Non-specific sites:

(mouse anti-rabbit; 4drops/10mlPBS) Mouse-Horse Serum	30

Primary Antibody:

(50 microL. in 25ml PBS) Mouse anti-Rift	60

Wash:	PBS I	5
	PBS II	5
	PBS III	5

Biotinylated Secondary Antibody:

(1 drop secondary antibody in 10mlPBS) Horse anti-mouse	30

Wash:	PBS I	5
	PBS II	5
	PBS III	5
ABC Reagents:		
	Avidin-Biotinylated HRP complex (2drops of Reagents A & B in 10ml PBS)	60
Wash:	PBS I	5
	PBS II	5
	PBS III	5
	Tris I	5
	Tris I	5
	Tris III	5
Stain:		
	Diaminobenzidine (DAB) {20ml of 0.03% H2O2 in Tris (0.1ml of 30% H2O2 in 100 Tris)}	6-8
Wash:	Tris I	5
	Tris II	5
Counterstain:	Mayer's Hematoxylin	1
Rinse:	Dist. Water	A few dips
Wash:	Dist. Water	5
Dehydrate:	70% Ethanol	2
	95% Ethanol	2
	95% Ethanol	2
	100% Ethanol	2
Clear:	Hemo-De I	5
	Hemo-De II	2

ELECTRON MICROSCOPY --- TECHNIQUES

EM - Fixation & Embedding Procedure

1. Remove tissues and place in fixative for 15-30 min. in refrigerator, then dice tissues with razor blade if needed, fix tissues for about 2 hr.

2. Remove fixative and place in buffered sucrose rinse according to what type of fixative used, for 15 min. or overnight

3. Post-fix in 1% osmium tetroxide solution at 4 C for 1 hr

4. Dehydrate tissues in the following ethanol:

70% ethanol	5 min.
95% ethanol	5 min.
100% ethanol	10 min.
100% ethanol	10 min.

(Note: if tissues are very delicate or easily damage, thus it needs a more gradual series of ethanol, then adding 30%, 50% and 80% steps)

5. Transfer tissues to propylene oxide for 20 min.

6. Remove propylene oxide, then add 1 ml fesh propylene oxide & 1 ml of freshly prepared resin, for 1 hr.

7. Add 1 ml of feshly prepared resin, swirling vials for good mixing for 3-24 hr.

8. Embed tissues in oven at 60 C for 48 hr.

Fixative for conventional/regular EM specimens

2% formaldehyde, 2% glutaraldehyde in 0.1M sucrose & 0.1M sodium cacodylate buffer pH 7.2

:- Add 2.50gm paraformaldehyde to make 2% formaldehyde solution $\left(\frac{2.50gm}{125ml} \times 100\%\right)$ to 100ml dist. water

Heat & stir to 60 C, and add 0.1N NaOH to clear solution, and let it cool down

:- Add 3.555ml of 70% glutaraldehyde to make 2% glutaraldehyde $\left(\frac{3.555ml}{125ml} \times 70\%\right)$

:-Add 4.2928gm sucrose to make 0.1M $\left(\frac{4.2928gm \times 1000ml}{125ml \times MW\ 342.3}\right)$

2.684gm sodium cacodylate $\left(\frac{2.684gm \times 1000ml}{125ml \times MW\ 214.03}\right)$

to make 0.1M buffer

:- Bring solution up to 125ml & adjust pH to 7.2

0.1M sodium cacodylate buffered-sucrose rinse

:- Add 17.17gm sucrose

10.736gm sodium cacodylate

1.45gm NaCl

to 400ml dist. water

:- Bring solution up to 500ml & adjust pH to 7.2

5% Paraformaldehyde in 0.1M sodium cacodylate buffer
(Fixative for normal mosquito-injection)

:- Add 50gm paraformaldehyde, to make 5% solution, to 750ml dist. water

Heat & stir to 60 C and add 0.1N NaOH to clear solution, and let it cool down

:- Add 21.403gm sodium cacodylate, to make 0.1M buffer

:- Bring solution up to 1000ml & adjust pH to 7.2

Osmium tetroxide solution for EM Post-fixation

Stock solution of 2% Osmium tetroxide

:- Add 1gm of Osmium tetroxide to 50ml dist. water

0.2M sodium cacodylate & 0.2M sucrose buffer

:- Add 4.28gm sodium cacodylate

:- Add 6.846gm sucrose
to 75ml dist. water

:- Bring solution up to 100ml & adjust pH to 7.2

0.2M Phosphate buffer in 0.2M Sucrose Solution

:- Add 0.773gm sodium dibasuc phosphate (Na_2HPO_4)

2.044gm sodium monobasic phosphate (NaH_2PO_4)

6.846gm sucrose

to 75ml dist. water

:- Bring solution to 100ml & adjust pH to 7.2

Working solution = 1% Osmium tetroxide in 0.1M buffer

:- Mix equal volume of 0.2M sodium cacodylate (or 0.2M phosphate buffer solution) in 0.2M sucrose buffer and osmium tetroxide stock solution

For Osmium tetroxide

:-Use a diamond pencil to scratch around the neck of the ampule
:-Do not touch ampule with bare hands, but with gloves or kimwipes
:-Have 50 ml dist. water in a wide-mounted jar that closes securely with a lid
:-To facilitate dissolving of osmium, run hot water onto the ampule until the osmium crystals have melted. Crack the ampule & immediately drop it into dist. water, shake the solution for several hours (6-8 hrs.)
:-Keep the container of osmium within another container, e.g. peanut butter jar that have a good seal
:-The vapor of osmium solution is dangerous, when disposing this solution, mix with twice amount polysaturated oil (vegetable oil will do)

Phosphate Buffer Solution = PBS

0.1M Phosphate buffered in 0.1M sucrose solution

:- Add 3.864gm Sodium dibasic phosphate (NaH_2PO_4)

10.224gm Sodiummonobasic phosophate (Na_2HPO_4)

34.230gm Sucrose

2.923gm NaCl

to 1000ml dist. water

Resin recipe

	Full cup	Half cup	1/4 cup	2/3 cup (in gm)
Epon 812	13.1	6.6	3.3	8.8
Araldite 502	10.0	5.0	2.5	6.6
DDSA	27.7	13.9	7.0	18.4
DMP 30	30 drops	15 drops	8 drops	20 drops

:-Adequate stirring, at least 5 min. with a tongue depressor, to accelerate the mixture, then let it stands for at least 20 min.

:-When finish with resin, leave it in the oven for overnight then discard later.

(Note: Araldite 502 can be substituted with NMA, but 502 give the resin softness and easy to work with, while NMA tends to be more crystalize and easy to break)

EM Post-staining

Uranyl acetate

1. 2% of uranyl acetate in dist. water

   :- Stain sections for 45 min to 1 hr at Rm Temp.

2. 5% of uranyl acetate in 70% EtOH

   :- Stain sections for 15-20 min at Rm Temp.
   (Providing more contrast on micrograph)

Lead citrate

:- Add 1.33gm $Pb(NO3)2$ - Lead nitrate

1.76gm $Na3(C6H5O7)2.H2O$ - Sodium citrate

in 30 ml dist. water in 50ml volumetric flask

:- Shake vigorously for 1 min, then allow to stand with intermittent shaking in order to insure complete conversion of lead nitrate to lead citrate

:- After 30 min, 8.0ml 1N NaOH is added

:- The suspension diluted to 50ml by adding 12ml dist. water and mixed by inversion several times

:- Adjust pH to about 12 $\pm$ 0.1

:- Note: the solution is stable for about 6 months when stored in glass or polyethylene bottle.

Immuno-gold Staining Technique

Various types of resin have been used, with various success for immunomarking of ultrathin sections of resin-embedded tissues. The hydrophilic resins such as the Lowicryls, LRWhite and glycol methacrylate give the best immunoreactivities.

:-Lowicryl K4M can be combined with low temperature dehydration and polymerization at -35 C.

:-LR White embedding has been used with good results in combination with glutaraldehyde/ paraformaldehyde fixation. Some special care must be taken to make sure that tissues undergoing embedding are not exposed to temperatures in excess of 55 C if antigenicity is not to be impaired. Therefore, polymerization should be done by incubation the specimens in an accurate oven or incubator set at 50-55 C for 24-48 hr, or at room temperature for 5 days. The "cold-cure" procedure, using the cold cure accelerator should no t be used, because the exothermic reaction may exceed 60 C.

The fixation method and embedding material used will depond on the kind of antigen. It is recommended to try a range of fixatives for every antigen. (See Fixatives for Immuno-gold technique).

Usually, pretreatment with OsO4 (Osmium tetroxide) before embedding is omitted.

For incubations with immune reagents, the grids are floated, sections down on top of drops of immune reagents displayed on a sheet of parafilm and are kepts covered in moist chamber (petri dishes with moistened filter paper on the bottom).

There are several ways of washing "on grid" marking. They may be washed on larger drops of buffer, or with a procedrue called "jet washing", by rinsing individual grids held by a foreceps with a stream of buffer from a syringe or squeezed-bottle.
Another way to rinse and wash grids is by using the Microtest plates with small wells, 48-well assay plates. The grids are floated on the meniscus of the washing fluid, dispensed into a well of the 48-well plate. The fluid is kept in constant motion by putting the 48-well on the rocking table. The grids move around and this yields a smooth but very efficient type of washing. The grids are transferred from each wash by the fine foreceps.

The Actual Procedure:

1. Tissues embedding in hydrophobic resins, i.e. epon-resin, and tissues fixed with aldehyde and osmium tetroxide need etching before incubation with immune reagents.

Etching procedure:- there are various etching procedures for thin sections on grids:

a. for tissues embedded in epon-resin and fixed with aldehyde, incubation in a saturated aqueous solution of sodium meta-periodate for 30-60 minutes,

b. for LR White sections, incubation for 10 minutes in saturated sodium meta-periodate, followed by a rinse in water and 15-30 minutes 10-1 N HCl giving a good result.

2. After the etching, the grids are washed in distilled water and filtered 0.05M TBS pH 7.6 (Tris buffer solution--- Tris HCl buffer (MW 157.64) 7.882gm in 1000ml to make 0.005M) 2 x 5 minutes each on the shaking/rocking table;

3. Blocking non-specific sites---the washed grids are incubated in Normal Goat Serum (1:10) in TBS for 30-45 minutes;

4. Primary antibody---the grids are transferred to drops of specific primary antibody for 60 minutes. (Antibody concentration and incubation time may be optimized by trying an antibody dilution series with other incubation times.) For example, Rift Valley Fever Virus - anti-nucleocapsid, 1:100 in TBS at room temperature for 90 minutes,

For washing, the grids are transferred to 48-well plate filled with TBS for 4 x 2 minutes on the shaking/rocking table;

5. Secondary antibody conjugated with colloidal gold reagent--- the grids are transferred to drops of the appropriate secondary antibody conjugate with colloidal gold solution (1:10) diluted in TBS for 60-120 minutes;

Auro Probe EM Goat anti-mouse IgG 15nm, (Janssen Cat.# 14759)

Auro Probe EM Goat anti-mouse IgM 30nm, (Janssen Cat.# 14747)

For washing, the grids are transferred to 48-well plate filled with TBS and distilled water for 4 x 2 minutes each on the shaking/rocking table;

6. Post-fixation---(Option) for tissues embedded in epon-resin and or fixed in paraformaldehyde alone, without a pretreatment of osmium tetroxide, grids shoul be post-fixed :

a. by placing the grids on 2% glutaraldehyde in PBS for 10 minutes, then rinse with filtered PBS 4 x 2 minutes, and

b. by transferring them into drops of 1% osmium tetroxide in PBS for 10 minutes, follwed by rinsing with filtered PBS 4 x 2 minutes, and distilled water 3 x 2 minutes.

6. Post-staining---the grids are stained/contrasted using conventional techniques

:- Uranyl acetate in 70% ethanol for 15 minutes, then wahed with distilled water,

:- aqueous Lead citrate for 45 seconds, then washed with distilled water.

Fixatives & Buffer for EM-Immuno-Gold Experiments

1) .5% Paraformaldehyde in 0.05M Sod. Cacodylate buffer pH 7.2

:- Add 6.25gm paraformaldehyde ($\frac{6.25gm}{125ml} \times 100\%$) to make 5% formaldehyde

to 100ml dist. water

Heat & stir to 60 C and add 0.1N NaOH to clear solution, and let it cool down

:- Add 1.342gm Sodium cacodylate ($\frac{1.342gm}{125ml \times MW\ 214.03} \times 1000ml$) to make 0.05 M buffer

:- Bring solution up to 125 ml & adjust pH to 7.2

0.05M sodium cacodylate buffer

:- Add 5.368gm sodium cacodylate to 400ml dist. water to make 0.05M buffer

:- Add 0.725gm NaCl

:- Bring solution up to 500ml & adjust pH to 7.2

2). 2% Paraformaldehyde, 0.25% Glutaraldehyde in 0.05M Sucrose & 0.05M Sodium cacodylate buffer pH 7.2

:- Add 2.50gm paraformaldehyde ($\frac{2.5gm}{125ml}$ X 100%) to make 2% formaldehyde

to 100ml dist. water

Heat & stir to 60 C, and add 0.1N NaOH to clear solution, and let it cool down

:- Add 0.455ml of 70% glutaraldehyde ($\frac{0.455ml}{125ml}$ X 70%) to make 0.25% glutaraldehyde

:- Add 2.1464gm sucrose ($\frac{2.1464gm}{125ml \text{ X MW } 342.3}$ X 100ml) to make 0.05M sucrose

:- Add 1.342gm sodium cacodylate ($\frac{1.342gm}{125ml \text{ X MW } 214.03}$ X 1000ml) to make 0.05M buffer

:- Bring solution up to 125ml, & adjust pH to 7.2

0.05M sodium cacodylate buffer-sucrose rinse

:- Add 8.5856gm sucrose to make 0.05M

5.368gm sodium cacodylate to make 0.05M

0.725gm NaCl to 400ml dist. water

:- bring solution up to 500ml & adjust pH to 7.2

LR White -- Infiltration & Embedding

1. Fix specimens in fixative for 2-4 hrs in refrigerator

2. Rinse specimens 2-3 times for 15 min with buffer (according to what kind of fixative used; specimens can be stored in the buffer overnight)

3. Dehydrate specimens by transferring them to

70% EtOH I - 30 min
70% EtOH II - 30 min
70% EtOH III- 30 min

4. Infiltrate specimens by soaking them up in a mixture of 2:1 LR White:70% EtOH for 1 hr (swirling vials or putting them on a shaker to insure good mixing), then transfer specimens to

LR White I - 30-45 min
LR White II - 30-45 min
LR White III - overnight
LR White IV - fresh LR White before embedding

(Note: Specimens can be stored in this unpolymerized LR White at 4 C in refrigerator)

5. Embed specimens in gelatin capsules, fully filled & tightly capped, and incubated in the oven at 50-55 C for at least 48 hrs.

IMMUNOCYTOCHEMISTRY DATA SHEET

Slide Code _____

Epidermis: Head _____
Thorax _____
Abdomen _____

Fat Body: Head _____
Thorax
peripheral _____
visceral _____
Abdomen _____

Alimentary Canal:	Epithelium	Muscles
Esophagous	_____	_____
Ventral diverticulum	_____	_____
Dorsal diverticula	_____	_____
Intussuscepted Foregut	_____	_____
Proventriculus (cardia)	_____	_____
Anterior midgut	_____	_____
Posterior midgut	_____	_____
Malpighian tubules	_____	_____
Pyloric ampulla	_____	_____
Ileum	_____	_____
Rectal epithelium	_____	_____
Rectal papillae	_____	_____

Salivary glands:

lateral distal _____
lateral intermediate_____
lateral proximal _____
median distal _____
median proximal _____

Dorsal vessel:

heart _____
aorta _____
pericardial cells _____

Tracheal system:

tracheae _____
end cells _____

Skeletal muscle:

Head _____
Indirect flight _____
Leg _____
Abdomen _____

Ganglia:

	Cell bodies	Neuropile
Brain	_____	_____
Subesophageal	_____	_____
Thoracic	_____	_____
Abdominal	_____	_____
Connectives & nerves	_____	_____

Reproductive system:

Female

Degree of egg development _____
Inseminated ? _____
Oocytes/nurse cells _____
Follicular epithelia _____
Ovarian sheath _____
Ovariolar sheath _____
Calyx _____
Lateral oviduct _____
Common oviduct _____
Genital chamber _____
Accessory gland _____

Male

Follicular epithelium _____
Testes _____
Vas efferent _____
Vas deferens _____
Seminal vesicle _____
Accessory gland _____
Ejaculatory duct _____

Miscellaneous:

COMMENTS

Ommatidia _____
Oenocytes _____
Corpora allata _____
Corpus cardiacum _____
Frontal ganglion _____
Johnston's organ _____
Nephrocytes _____
Hemocytes _____
Ventricular ganglion _____

Immunocytochemistry: Brief Protocols

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The Direct Method

The one-step (direct method) methods utilize primary antibodies labeled with a variety of markers that allow the location of the antigen to be visualized. Antibodies can be directly labeled with fluorescent compounds such as fluorescein or rhodamine, with enzymes such as horseradish peroxidase (HRP) or glucose oxidase (GO) or with colloidal gold particles. The use of directly labeled antibodies insures that staining is dependent solely upon the specificity and affinity of the primary antibody. When trying to locate two antigens in the same section, there is a distinct advantage to direct labeling if both antibodies were raised in the same host. In this case, simultaneous labeling for both antigens is possible only with directly labeled primaries antibodies. The disadvantages of the direct method outweigh these advantages in most situations: (1) the process of labeling the primary antibody may reduce the ability of the antibody to bind with the antigen in the tissue due to conformational changes in the antibody binding site; (2) labeling every primary antibody is time consuming; (3) a directly labeled antibody offers no flexibility in choice of label from one experimental situation to the next; (4) direct methods are far less sensitive than indirect and three-step methods. The lack of sensitivity is the major reason that directly labeled antibodies are not widely used today.

Indirect Method:

Labeled Secondary Antibody Techniques

Indirect labeling methods are among the most popular immunocytochemical methods used today. These techniques utilize a labeled secondary antibody, directed against immunoglobulin of the host animal in which the primary antibody was raised. This labeled secondary antibody binds to the primary antibody. Example: goat-anti-mouse secondary antibodies conjugated to a fluorophore or enzyme can be used to detect primary antisera generated in mouse. Fluorescence-markers are most widely used for indirect labeling. Indirect methods provide significant increases -in sensitivity when compared with direct methods where the primary antibodies themselves are conjugated to a marker. Signal amplification is achieved by adding one or two layers of antibody molecules during the labeling sequence. Several secondary antibodies can attach to each primary antibody, and several molecules of label are attached to each primary antibody.

The use of labeled secondary antibodies also has the advantage of offering flexibility in the choice of a visualization marker. With the direct technique, the primary antibody itself is labeled, and that marker must always be used. With indirect methods, the particular experimental application can guide the choice of label. For instance, if rapid screening of tissue is favored fluorescence markers are often chosen. However, for permanent preparations or for electron microscopy, enzyme and electron dense markers are preferable. Therefore, in order to increase sensitivity and experimental flexibility over direct methods, labeled secondary antibody methods are commonly chosen.

Peroxidase Anti Peroxidase

Advances are continually being made to increase the sensitivity of immunocytochemical methods. By using three-step methods that take advantage of "sandwiching" of antibodies, and using multiple copies of the label on the third layer, new levels in sensitivity have been achieved. Peroxidase-anti-peroxidase (PAP), double bridge, and the avidin biotin complex (ABC) techniques are the most sensitive methods available today for most biological applications. It is estimated that PAP and ABC methods are 100 to 1000 times more sensitive than labeled secondary antibody methods.

The PAP technique utilizes an unlabeled primary antibody. The secondary antibody is bound to the primary as with the labeled secondary antibody technique, but in this case the secondary antibody is also unlabeled. The tertiary antibody is a peroxidase anti-peroxidase (PAP) conjugate; the anti-peroxidase and primary antibodies must have the same host. The major disadvantage of this technique, in some applications, is that the PAP molecule is very large compared with labeled secondary antibodies. When processing whole mounts or thick sections of tissue, achieving adequate penetration of the PAP molecule can be difficult and can result in sensitivity loss. Generally, however, by adjusting incubation times, temperatures, and detergent concentrations it is possible to overcome this obstacle.

The double bridge method theoretically affords even more sensitivity than the PAP technique, upon which it is based. This variation again capitalizes upon the idea that by sandwiching antisera, extra binding sites are created, and hence, more label is attached. By this technique, after completing the series of antibody incubations prescribed by the PAP method, the tissue is re-incubated in the secondary (bridge) antibody. This is followed by another incubation in PAP. Recycling of reagents is repeated one or more times. For some applications, the sensitivity of the double bridge method is equal to or greater than that of the three-step ABC technique.

Avidin Biotin Complex

Since the staining intensity of the immunoperoxidase reaction is a function of peroxidase activity, heightened sensitivity would be achieved if one could further increase the numbers of peroxidase molecules bound to the tissue. This has been accomplished with the ABC (avidin-biotin complex). Biotin is a small vitamin molecule, and avidin is a 68,000 MW glycoprotein found in egg white and some bacteria. Avidin has an extraordinary affinity (the highest noncovalent affinity known) for biotin and provides four active binding sites for biotin. The binding of these molecules can therefore be used as the basis for an immunocytochemical detection system. In this technique, tissue is incubated in unlabeled primary antibody, followed by incubation in a biotin labeled secondary antibody (directed against the antibody of the host in which the primary antibody was raised). Multiple biotin molecules are bound to this antibody. Finally, the preparation is incubated in the avidin-biotin complex. Although the exact molecular structure of the ABC complex has not been defined, many peroxidase molecules are believed to be attached. The major limitation of this method, in some applications, is the large size of the avidin-biotin complex, which can hinder penetration. However, this is not a serious problem as discussed later. Biotin is also available conjugated to alkaline phosphatase, glucose oxidase, beta galactosidase, colloidal gold, and a variety of fluorophores. This method provides the sensitivity of a three-step method with the labeling flexibility of a two-step method. For this reason, avidin-biotin methods are very popular today.

CHOICE OF METHOD: SUMMARY OF PROS AND CONS

Issues to consider when choosing the type of method and label.

1. sensitivity
2. reliability
3. permanence
4. penetrance (size of molecules)
s. processing time
6. expense
7. photography
8. counterstaining
9. electron microscopy

The choice of label will also depend on the amount of time that will be needed to study the staining pattern. Fluorophores fade (in spite of techniques available that retard fading,) with time. Therefore, if many hours might be needed to examine the staining pattern, or if many photographs will be required, a permanent enzyme marker will be required. Fluorescein is extremely light sensitive, rhodamine fades less rapidly, and Texas Red has the reputation of being relatively bright and long lasting. Fading problems necessitates that photography of fluorescent preps be done with high speed film while enzyme labels can be photographed in normal fashion. Both fluorescent and enzyme labeled cell preparations can be counterstained. Fluorophores are generally recommended for double labeling experiments. One should maximize the quality of each immunoreactivity before the two are combined in double staining experiments.

To summarize, sensitivity, while very important, is not the only issue to be considered in choosing an immunocytochemical method. There are many situations where two step methods may be more sensible to use than three step techniques. When choosing the label, the primary considerations are the permanence of the results, the photographic requirements, whether counterstaining is needed, and whether double labeling or electron microscopy is the ultimate goal. No matter which method or label is decided upon, the method of fixation will be a critical factor in obtaining high quality results.

Specificity

The issue of specificity can be divided in the groups listed below. Problems are often encountered in these procedures. The following may be of some assistance.

Method Specificity

1. Autofluorescence of tissue, when using fluorescein labeling techniques.
   Solution: use rhodamine or HRP as alternative.

2. Endogenous peroxidase activity (as in RBCs) when using HRP as a label.
   Solution: treat with (a) methanol followed by hydrogen peroxide, (b) methanol nitroferricyanide, or (c) phenylhydrazine.

3. Binding of antibody to free reactive groups still present after fixation.
   Solution: preincubate tissue with (a) serum not recognized by the immunoreagents or (b) sodium borohydride or (c) use buffers containing small molecules (lysine,glycine, or tris) or gelatin, serum albumin.

4. Ionic or hydrophobic interaction of antibodies with the tissue.
   Solution: use buffer with enhanced ionic strength (i.e., add NaCl).

Antibody Specificity

To be more confident in the specificity of a particular reaction the following should be performed:

1. Preabsorption of antisera with antigen. Note: Preabsorption studies should always be done when using a new antiserum, new animal, new bleed, or new tissue.

2. Preabsorption of the antisera with other compounds, to test for the presence of other antibodies or cross reactivities.

3. Use several different types of antisera against the same antigen (a) from different animals; (b) produced using antigens that were chemically linked to carrier molecules via different functional groups).

4. Convergence of immunocytochemical results with biochemical characterization and quantification of the antigen (RIA, HPLC, molecular sizing, chromatography).

If problems still persist they may be occurring for one of the following reasons. Some solutions are given.

1. Natural antibodies present in serum as a result of prior, unrecognized antigen stimulation occasionally caused by adjuvant.

   Solutions: affinity purification of antibodies; use of more dilute antisera; preabsorption with"other" antigens.

2. Contaminating antibodies caused by impure immunogen.

   Solutions: difficult to solve but possibly by affinity purification w/purer form of antigen than the one used as the immunogen; higher dilution of serum might help, if titer of contaminating, antibodies is low relative to the titer of antigen specific antibodies.

3. Antibodies directed against carrier molecules used with haptens.
   Solution: preabsorption w/carrier molecule (i.e., BSA, thyroglobulin, KLH)

4. Antibody recognizes antigen, but also cross-reacts w/ related compounds.
   Solution: preabsorption w/the cross-reactive compounds; characterize the cross-reactivity & accept it.

Post-Fixation Treatment For Improving Penetration Of Antibodies

In addition to physically sectioning the tissue there are a number of methods that can be used to improve antibody penetration. Yet, these are likely to hinder the preservation of morphology. All of the methods described below are used after fixation and before the 1° antibody incubation.

Agents that improve antibody penetration

1. Detergents
2. Freeze-thaw
3. Solvents
4. Proteolytic enzymes
5. Sodium borohydrate

The most popular of the treatments listed above is the use of detergents in buffers and antibody diluents . The majority of protocols (with the exception of those for electron microscopy) include either Triton-X 100 or saponin (0.3% - 1.0%) in the buffers as a solubilizing agent. Triton-X 100 is the most popular of the two detergents. The inclusion of one of these agents in the protocol is a standard remedy for the penetration problems caused

by fixation. If penetration of immunoreagents is still difficult there are a number of second order treatments that can when used to increase antibody penetration. The first of these methods is to freeze and thaw the tissue. This causes the formation of ice crystals in the tissue, resulting in some tearing of membranes and thereby allowing antibody penetration. Solvent treatment of the tissue extracts lipids, thereby leaving microscopic holes in membranes. The most common solvent treatment is to dehydrate the samples with ethanol and passing them through zylene followed by rehydration. Another solvent treatment that has been used with success in insect tissues is methanol dehydration followed by treatment with a chloroform/methanol/acid solution (200 parts chloroform/100 parts MeOH/1 part HCl. The tissue is then rehydrated and antibody incubations proceed as the normal.

Proteolytic enzyme (e.g. pronase) and sodium borohydride treatments can also be used to enhance antibody penetration. Dilute pronase solutions have successfully been used to unmask antigenic activity of protein antigens and sodium borohydride treatment is used to unmask glutaraldehyde fixed antigens. These methods should be used with caution since both pronase and sodium borohydride can damage the antigenicity and tissue morphology. The correct concentration and time for a particular tissue must be determined empirically.

Immunofluorescent Staining of Sections

After the tissue has been fixed in an appropriate fixative (some fixative protocols are given in the appendix), sectioned and washed the following protocol can be used as a starting point for the development of an appropriate immunocytochemical procedure for your particular system.

Presoak Solution:
0.1M phosphate buffer containing
3% normal serum (from host animal of your secondary antibody)
0.4% Triton X-100 (aids penetration of antibodies)

1) Presoak sections for 30 min. at room temperature in solution above.

2) The presoak is aspirated off the sections and the primary antisera is applied. Do not let the sections dry out at any point in the procedure. Incubate for 2 hr to 12hrs at 4°C. The timing of this and all other incubations must be empirically determined.

Note: The optimal antisera dilution is determined through experimentation. It is important to use the most dilute concentration of primary antibody that will give good staining. Often the manufacturer will be able to give you suggestions for good starting dilutions. In the absence of such directions, start with a 1:1,1:10 and 1:100 dilution for monoclonal antibodies, and 1:10, 1:100, 1:1000, and 1:10,000 for polyclonal antisera. The primary antibody is more concentrated for immunofluorescence with labeled secondary antibodies than those labeled with biotin. Use the presoak solution as the diluent.

3) Rinse sections after the primary antibody with at least 3 changes of O.lM phosphate buffer over 15-30 mins.

4) Incubate the sections in secondary antibodies diluted with O.lM phosphate buffer with or without normal serum from the host animal of the secondary antibody for 1 hr at room temperature.

Labeled Secondary Antibody Procedure: secondary antibodies are labeled with FITC, TRITC, or Texas Red and are generally used at 1:50 - 1:100. Keep the slides away from light.

OR

Avidin Biotin Procedure: secondary antibodies labeled with biotin are usually used at 1:200 (each manufacturer will differ).

5. Rinse the slides in O.lM phosphate buffer 3 times over 30 min.

6. Labeled Secondary Antibody Procedure: Rinse the sections in 4mM sodium carbonate for 10 min. Mount sections onto slides with glycerol containing para-phenylenediamine (PPD) (recipe is given in appendix). PPD slows down the fading of the fluorescence. Surround the coverslip with a thin rim of red nail polish. objectives).

OR

Avidin Biotin Procedure: incubate sections in avidin labeled fluorochrome of choice which is diluted to 1:100 - 1:1000 (according to the manufacture directions) in O.1M PBS. Incubate for 1 hr at room temperature. Keep the slides out of the light.

7. Avidin Biotin Procedure: wash the slides in O.1M PBS 5 times over 30 min.

8. Avidin Biotin Procedure: Rinse the slides in 4mM sodium carbonate for 10 min. Follow mounting procedure outlined in step #6 for "Labeled Secondary Antibody Procedure"

Immunofluorescence on Whole Mounts

1. After fixation each tissue chosen for whole mount fluorescence immunocytochemistry is placed in a beaker or tissue culture plate and rinsed at least six times over 6-12 hours with 0.1M phosphate buffer with 0.4% (v/v) Triton-X 100.

2. Presoak tissues in presoak buffer for 2-10 hours at room temperature (for short incubations) or in the refrigerator (for incubations longer than 5 hours) .

3. Presoak solution is exchanged for primary antiserum diluted with PBS/Triton. Dilutions can range from 1:50 - 1:1000. Tissues are incubated in primary antibody for 2-48 hours depending upon size. Azide O.1% w/v can be added to prevent bacterial growth for longer incubations.

4. Rinse with at least 6 changes of PBS/Triton over 4-24 hours.

5. Labeled Secondary Antibody Procedure: Tissues are incubated in secondary antibodies diluted in PBS/Triton (with or without normal serum from the host animal for the secondary antibody) for 4-24 hours. Dilutions of 1:50-1:100 are common for fluorescently-labeled affinity-purified secondary antibodies. Incubations longer than 5 hours should be at 4C.

OR

Avidin Biotin Procedure: Secondary antibodies are labeled with biotin and are generally used at 1:200 (check manufacturers instructions).

6. Tissues are rinsed in 6 changes of 0.1 M PBS/Triton in 2-12 hours.

7. Labeled Secondary Antibody Procedure: A final rinse is done with 4mM sodium carbonate (pH 10) for 15-30 minutes. The tissues can then be mounted in glycerol containing para phenylenediamine (PPD) (see appendix for recipe).

OR

Avidin Biotin Procedure: Tissues are incubated in avidin labeled with fluorochrome generally diluted to 1:100-1:1000 in O.1M phosphate buffer. Incubate for 4-24 hours. Keep the samples dark.

8. Avidin Biotin Procedure: Rinse samples in O.1M phosphate buffer at room temperature, six changes in 6 hours.

9. Tissues are mounted in 50% glycerol in 20 mM sodium carbonate or mounted in PPD-glycerol mounting medium.

Immunoperoxidase Labeling of Sections

The most popular permanent label for immunocytochemical methods is horseradish peroxidase (HRP). The three step ABC-peroxidase method is the most sensitive of the peroxidase techniques, but it can occasionally be subject to background staining if the tissue contains biotin-like molecule (this problem can be solved by pretreating the tissue with free avidin followed with free biotin). The three step, PAP method is more sensitive than HRP-labeled secondary antibodies (two step method), but for electron microscopy, labeled secondary antibodies are occasionally preferred since the molecules are smaller and able to penetrate the tissue better.

Horseradish peroxidase can be visualized by enzymatic reaction with hydrogen peroxide together with a variety of different chromagens (3,3-diaminobenzidine tetrahydrochloride (DAB), p-phenylene diamine with pyrocatechol (Hanker-Yates reagent), 4-chloro-1-naphthal, 3-amino-9-ethylcarbozole, or o-dianisidine. While the reaction products formed from some of these chromagens will fade with time, the product produced with DAB is sensitive and permanent. In addition, DAB can be used for electron microscopy since the reaction product can be made electron dense if it is followed by osmium tetroxide treatment which will also amplify the signal. To do this, the sections are first reacted with DAB, rinsed, and treated with O.O5% osmium tetroxide in 0.1 M phosphate buffer for 30-60 seconds. These factors make DAB the chromagen used most often for immunoperoxidase techniques.

Immunoperoxidase Protocol

1) Follow the same protocol outlined for fluorescent labeling of sections except for the reaction below.

2) React sections with 0.02 - O.05%. DAB (3 ,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 0.1M phosphate buffer with hydrogen peroxide (0.001%-0.003%). Be sure that the 30% stock solution of hydrogen peroxide is fresh (less than 6 months old). Use the DAB solution within 1 hr. Add the hydrogen peroxide immediately before incubating the sections. Keep both the DAB and the DAB-hydrogen peroxide out of the light. React sections for 5 to 15 min. Stop reaction with 2 changes of phosphate buffer. Some investigators use O.lM Tris buffer for DAB reactions. This may improve your staining. Neither buffer, however. should contain sodium azide.

CAUTION: DAB is a carcinogen. Chlorox can be used to inactivate DAB.

3) Rinse sections in 0.1M phosphate buffer for 20 - 30 min.

4). Coverslip the sections. The sections may be dried overnight and then dehydrated and coverslipped or dehydrated in ethanol, cleared in three changes of xylene, and coverslipped with Permount right away.

Counterstains for Immunocytochemistry

Fluorescence

Sections with fluorescent-labeled antibodies or reagents usually cannot be counterstained with conventional dyes since many dyes will fluoresce themselves. Thus, for immunofluorescent labels it is best to counterstain with another fluorescent marker that is not visible under the viewing conditions for your label. An excellent counterstain for fluorescein and rhodamine is the DNA stain - Hoechst (Aldrich). This compound is used at a 10% dilution in phosphate buffer and appears pale blue when viewed with a filter set designed for blue fluorescence. The advantage of this counterstain is that it allows you to stain with fluorescein or rhodamine or both. It is best to use filters that eliminate bleeding from one channel of fluorescence into the others.

Before the sections are coverslipped, they are dipped into dilution of Hoechst for 1-5 minutes. The sections are then rinsed in buffer and coverslipped with glycerin mounting media containing PPD, aqueous mounting medium or dehydrated, cleared, and coverslipped with a non-fluorescent mounting medium.

Peroxidase:

Sections stained with a peroxidase marker can be counter stained with cresyl violet. Cresyl violet stains Nissl substance thus cell bodies blue and can be used on paraffin and cryostat sections or mounted Vibratome and sliding microtome sections.

1. The lipid should be removed from the section before staining. (This step is not necessary for paraffin sections). This is done by dehydrating the sections through ethanol and clearing in xylene; then the sections are run through the alcohols to distilled water.

2. The hydrated sections are stained in cresyl violet acetate (0.5%.) for 1 to 6 minutes

3. Stained sections are rinsed in distilled water for 1 minute and in 70% ethanol for 2 minutes.

4. The background staining with cresyl violet is removed by soaking the sections in 95% ethanol containing 1% acetic acid.

5. After 95% ethanol the slides are transferred through 2 changes of 100% ethanol for 2 minutes each, cleared in 3 changes of xylene for 3 minutes each and are mounted.

Interpretation of Results

Interpretation of immunocytochemical data is difficult. These data are essentially qualitative and should not be quantified without careful analyses. The advent of image analysis and scanning confocal microscopy make it possible to legitimately quantify *in situ* labeling. A proper interpretation of results requires careful comparisons of experimental and control staining patterns that have been performed simultaneously. In order to validate results, several controls are necessary: (1) replace primary antiserum with nonimmune serum; (2) positive tissue control; (3) negative tissue control; (4) preabsorption control.

In addition, to corroborate immunocytochemical labeling it is valuable to have information about the particular antigen from other biochemical and anatomical methods. The most difficult aspect to evaluate is whether or not the staining constitutes specific labeling. There will always be different concentrations of antigens in different cells, regardless of tissue type. This variation in antigen concentration will be reflected in the staining intensity. By comparing control and experimental tissues, the level of staining required to be considered positive can be ascertained. It is also important, in addition, to running the standard control experiments to try variations in the fixation protocol as part of the data evaluation. If tissue has been underfixed antigen may be lost. Underfixed tissue may also be difficult to interpret because the morphology is poorly preserved and because unfixed portions of tissue may stain non-specifically. Excessive fixing, on the other hand may mask antigens and therefore can also result in a staining pattern that is not representative of the true antigen localization. The use of proteolysis in order to unmask antigens carries the destroying antigenicity. Therefore, in order to validate the staining pattern following proteolysis of the tissue, control tissue should be treated in same way.

Other problems to be aware of include (l) tissue that had dried during the staining procedure and improperly rinsed tissue (2) dead tissue (3) wrinkles in sections, edges of tissue, and knife marks in sections can differentially stain (3) poor quality or old immunoreagents can leave fine precipitates in the tissue. If variable staining persist with a particular antibody preparation it is advisable to process the tissue using other antibodies directed against the antigen of interest. It is always valuable to demonstrate the same staining pattern with more than one antibody preparation.

Fixative Solutions

A.) 2% paraformaldehyde & 0.15% picric acid

To make 500 ml

distilled water at 60°C	160 ml
paraformaldehyde	10 grams

stir 20 minutes and clear solution with 1N NaOH]

1% picric acid solution	75 ml
distilled water	bring volume to 250 ml
0.2M PBS	bring volume to 500 ml

Adjust pH to 7.3-7.4 and filter.

B.) 1% paraformaldehyde and l.25% glutaraldehyde

for 500 ml

distilled water at 6O°C	75 ml
paraformaldehyde	5 grams

stir 20 minutes and clear solution with lN NaOH

0.2 M PBS	250 ml
50%	glutaraldehyde 12.5 ml
distilled water	bring volume to 500 ml

Adjust pH 7.3-7.4 and filter.

C.) 4% Paraformaldehyde and 0.2% glutaraldehyde

(fixative for some small molecules and for some EM immunocytochemistry)

To make 500 mL

distilled water at 6OC	220 ml
paraformaldehyde	20 grams

Stir 20 minutes and clear solution with NaOH
Allow to cool to room temperature and add the following:

distilled water	bring volume to 248 ml
50% glutaraldehyde	2 ml
0.2M PBS	bring volume to 500 ml

Adjust pH 7.3-7.4 and filter.

D.) Periodate-lysine-paraformaldehyde

This fixative was initially developed for immunoelectron microscopy but it is very good for many immunocytochemical techniques because it effectively stabilizes proteins without effecting their antigenicity.

Solution A: Lysine-phosphate buffer

To 0.2M lysine hydrochloride add 0.1M dibasic sodium phosphate until the pH is 7.4. Then dilute the lysine HCl to 0.1 m with 0.1 M PBS (pH 7.4).

Solution B: Paraformaldehyde

Dissolve 40g paraformaldehyde in 100 ml distilled water heated to 60C & clear with 1N NaOH
Add 5.4 grams glucose

When ready to use mix 3 parts of solution A to 1 part solution B and add solid sodium m-periodate to a concentration of 0.01M.

Fluorescent Mounting Media

0.1% para-phenylenediamine (PPD)
10% 0.OlM PBS in O.15M NaCl
90% glycerol

To make lOOml

PBS	10ml
PPD	100mg (dissolve buffer)
glycerol	90ml

Adjust pH to 9.0 with sodium carbonate buffer (O.5M)

Store in dark and keep refrigerated. when not in use With proper storage it should be good for a few weeks or until medium gets turns darker.

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In Situ Hybridization

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In situ hybridization (ISH) is a procedure whereby labeled nucleic acid probes (DNA or RNA) are hybridized to complementary (target) nucleic acid sequences in tissue sections, dispersed cells and chromosomal preparations. The advantage of using ISH is that it yields both molecular and morphological information about the distribution of target nucleic acid within a tissue. ISH has been most widely used by surgical pathologist to detect viral infections in cells and to study viral pathogenesis and by developmental biologist to study gene expression in plant and animal cells and tissues. ISH holds great potential for use in the detection of vector-borne infectious agents and in studies elucidating the developmental cycles and kinetics of infectious organisms in both vertebrate and invertebrate hosts.

In a nutshell, the ISH technique starts with cells or tissues that are fixed and adhered to slides. The slides are treated to render the cell membranes permeable to the labeled probe and to prevent nonspecific binding during the hybridization steps. Labeled probe either DNA or RNA is applied to the slide and if the probe finds complementary target the probe will hybridize. Hybridization is a reaction whereby two single-stranded nucleic acid molecules recognize one another and bind by means of hydrogen bonding of complementary base pairs. The slides are then washed under conditions of buffer and temperature that permit maximum specific hybridization and minimun nonspecific binding of the probe. Probe bound to the cells or tissues is then detected by autoradiography for probes labeled with radioisotopes or by an enzymatic reaction for probes labeled without radioisotopes. Slides are then examined by light microscopy for specific "staining" in the cells or tissues.

The specificity of ISH is conferred by the base matching of the probe to the target nucleic acid and the stringent requirement for complementarity in order for stable hybrids to form. The sensitivity of ISH depends on the number of copies of the target nucleic acid expressed in the tissue or cell which is typically in the picogram range.

There are three types of nucleic acid hybrids: DNA-DNA, DNA-RNA, and RNA-RNA. Each successful hybridization is dependent on several critical parameters: construction and sequence of the nucleic acid probe; preparation of the tissue sections containing target nucleic acids; optimal hybridization and washing conditions; and a means of identifying the probe.

1) Nucleic acid probe - Probe selection is a critical decision. Probes can be obtained by cloning fragments of genomic DNA (DNA of the target organism) or complementary DNA (cDNA) (DNA that is derived from its messenger RNA using reverse transcriptase, an enzyme purified from RNA tumor viruses), by using DNA synthesizers to prepare synthetic oligonucleotide probes, by the synthesis of RNA probes from cloned cDNA, and by polymerase chain reaction.

2) Preparation of the tissue sections containing the target nucleic acid.

A) Fixation - the most sensitive parameter. Length of fixation time and type of fixative should be emperically determined for the tissue of interest. To optomize diffusion of the fixative the tissues should be cut into small pieces or perfused with fixative.

Fixation must --- preserve cellular morphology,
preserve cellular DNA and RNA, and
allow probe access to the tissue.

***fresh 4% paraformaldehyde**

B) Embedding - use standard histologic procedures

Paraffin (Paraplast)
Plastic (glycol methacrylate)
Cryostat (OTC compound)

C) Sectioning and Adhesion to Slides -

1) Clean microscope slides are required for ISH
   Chromerge acid wash
   Detergent wash
   DEPC treatment (diethylpyrocarbonate)
2) Section thickness may affect ISH efficiency
   Plastics 2-4 um
   Paraffin 5-6 um
3) Adhesives
   Elmer's glue
   Poly-L-lysine
   Silane
   Fisher brand "Probe-On Plus" slides

   * Do not use gelatin in the water bath.

3) Pretreatment of the tissue sections -

A) Deparaffinization - remove all paraffin wax to allow penetration of the probe, e.g. Hemo-de.

B) Permeabililization - to increase accessibility of the probe, e.g. Triton-X-100.

C) Deproteinization - proteolytic enzymes are used to enhance penetration of the probe, e.g. Proteinase K, dilute HCl.

D) Acetylation - acetic anhydride is used to reduce background and nonspecific binding of nucleic acid probes by blocking basic protein groups.

E) SSC washes - removes ethanol from the tissues, sodium chloride and sodium citrate.

F) Block - depends on the detection system used. Endogenous enzyme activity is often blocked with nonfat dry milk.

4) Prehybridization - The prehybridization buffer contains inhibitors to reduce background by competitively inhibiting nonspecific binding of the nucleic acid probe. The prehybridization solution should be the same as the hybridization solution without the labelled probe. Follow the protocol of the "kit" you are using.

These solutions typically contain -

A) Formamide - a denaturant that decreases the Tm (the temperature at which 50% of the hybrids "melt").
B) Dextran sulfate - a polymer that increases hybridizationefficiency by forming networks in the hybridization solution.
C) Carrier DNA - prevents nonspecific binding of labelled probe, e.g., salmon sperm DNA.
D) Denhardt's reagent - a protein solution that reduces background.

5) Denaturation of probe and/or target sequences - Probe and target nucleic acid sequences must be single stranded for hybridization to occur. Depending on the probe and target nucleic acid denaturation or melting into two single strands can be achieved by using an acid, alkali, organic solvent or heat. The optimum temperature of denaturation is determined by trial and error; the temperature that produces the strongest signal without affecting morphology is ideal.

For example, using the Genius kit to detect Rift Valley fever viral RNA , the digoxigenin labeled cDNA probe is boiled for 10 min to separate the double strands and then placed on ice to prevent reannealing before it is added to the hybridization solution. In this case, the target viral nucleic acid is ssRNA and therefore does not require denaturation.

To form DNA-DNA hybrids, DNA in the tissue section and the DNA probe must first be denatured. This can be done while the hybridization solution is on the slide by placing the slide in an incubator or on a heating block (90 C, 6 min) and then placing the slide into an ice water solution. These slides must be coverslipped and sealed with rubber cement before denaturation and hybridization.

6) Hybridization - During hybridization the probe enters the cell and if the nucleic acid of interest is expressed it hybridizes. There is no fixed protocol for hybridization. Follow the protocol of the "kit" you are using. Slides should be level during incubation in a humidified chamber and tissues should be covered with a removable coverslip such as siliconized coverslips or coverslips made of Parafilm to prevent the hybridization buffer from drying out.

A) Stringency - Hybridization specificity depends on the stringency of the conditions used during incubation and subsequent posthybridization washes. The set of conditions known as stringency describe the kinetics under which complementary probe and nucleic acid strands hybridize. In other words it describes the forces of hydrogen bonding. Physical variables that influence the stringency include: probe length and concentration, salt concentration (SSC), hybridization temperature, guanine-cytosine content of the sequences (low G+C content ,<45%, will tend to reduce thermal stability of the hybrid, high G+C

content, >65%, may lead to elevated background labeling of the tissues), and composition of the hybridization buffer, e.g. formamide concentration.

The stringency of hybridization can be adjusted to allow for some degree of mismatch of base pairs (less stringent conditions) or to minimize the hybridization of probes to related but nonidentical sequences (very stringent conditions).

B) Probe concentration - The required amount of probe is influenced by the type of fixation, the denaturation temperature, type and time of proteolytic digestion, hybridization time and temperature, and size of the probe. In order to compensate for all these variables and to minimize the hybridization time required to detect picogram quantities of target sequences the probe concentration must greatly exceed the target concentration.

7) Posthybridization - Posthybridization washes remove unbound probe and allow you to discrimminate between precisely and imprecisely matched hybrids. Stringency can be adjusted in the posthybridization washes through extensive washing in SSC buffer (sodium citrate, sodium chloride) of decreasing concentrations to remove unhybridized probe from the tissue sections.

8) Detection of the hybrids - Probes labeled with radioisotopes require lengthy autoradiographic procedures to detect hybridized probe. Probes labeled by nonradioactive procedures often utilize an enzymatic reaction and color producing substrates to detect hybrids. Slides are then examined by light microscopy for specific "staining" in the cells and tissues.

9) Controls - Controls insure the specificity of ISH.

Probe choice has some bearing on which control procedures you are able to perform.

Your controls should include:

A) Tissues known to be positive in a well defined morphologic distribution.

B) Tissues known to be negative.

C) Hybridization and immunodetection reagents with unlabeled probe and without the labeled probe on positive and negative tissues.

D) Hybridization with nonspecific vector sequences.

E) Immunohistochemical staining on parallel sections of tissues helps validate specificity of the probe.

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AVIDIN-BIOTIN IMMUNOCYTOCHEMISTRY FOR PLASTIC SECTIONS

AND

PEPTIDE IMMUNOCYTOCHEMISTRY FOR ELECTRON MICROSCOPY

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AVIDIN-BIOTIN IMMUNOCYTOCHEMISTRY FOR PLASTIC SECTIONS

FIXATION: Dissected tissues or whole mosquitoes (wings and legs removed; last abdominal segments opened and thorax punctured) are fixed in a phosphate buffered (0.01 M, pH 7.2) and modified (no acetic acid) Bouin's solution plus 0.2-1.0% glutaraldehyde and 200 mg trehalose/10 ml for 1-2 h at 4°C.

DEHYDRATION: Steps of 30, 50, 70, 80, 90% ethanol in water for 10-15 min each; 2 X 100% ethanol, same time; 100% propylene oxide for 5 min.

INFILTRATION/EMBEDDING:

Prepare plastic mixture in 30 ml graduated plastic syringe barrel.

Epon 812	6.3 ml
Araldite 6005	3.3 ml
DDSA	13.8 ml

Squirt into 30 ml polypropylene beaker, mixed and then add

DMP	25-30 drops

When well mixed, measure and mix to infiltrate specimen, under vacuum if necessary:

2 propylene oxide: 1 plastic mix---1-3 h
1 " " : 1 " " ---1-3 h
1 " " : 2 " " ---3 h-overnight

Take specimen out of last solution and put in molds with paper labels (pencil marked only) and fill with plastic mix for 8h-overnight, position specimen in mold, place molds in oven at 60°C for 24-36 h to polymerize.

SECTIONING: Three-four micron thick sections are cut from the embedded tissues or longitudinally-oriented whole mosquitoes. Sections are transferred to gelatin-coated glass slides in a water drop with a wire loop and h ated at 45°C for approx 15 min-1h. Glass knives prepared for ultramicrotomy with affixed large boats to hold water to float the sections work fine on a ultramicrotome. After sections are dried on slide, scratch a line below the section on the back of the slide with a diamond-tipped pen to aid observation.

Gelatin-coated slides: Mix 2.0 g gelatin into 500 ml heated, distilled water, cool, and mix in 0.25 g chrome aluminum, and filter. Dip clean slides (soaked in detergent overnight and washed exhaustively with hot water and distilled water), dry, and store.

IMMUNOCYTOCHEMISTRY PROCEDURE

ROOM TEMPERATURE, UNLESS NOTED, AND SECTIONS SHOULD NEVER DRY.

1. Strip plastic from sections, 2-3 min in NaOH in ethanol (35 g NaOH/250 ml 100% ethanol, must age for at least one week in dark, and good for approx. a year). Quick check to make sure plastic is gone.

2. 100 and 70% ethanol and distilled water rinses, 2 min/step.

3. Endogenous peroxidase quench: 5 ml, 30% hydrogen peroxide in 200 ml distilled water, 10 min; followed by a distilled water rinse, 2 min.

4. TBS-NS, 200 ml, plus 100 ul, 20% Tween 20 detergent, 10 min rinse. Save solution for step 6.

TBS-NS

0.5 M TRIS stock, ph 7.2	25 ml
NaCl	2.25 g
Bring to 250 ml with distilled water	

5. Block with with serum, 15 min-1 h. Slides racked in humidity chamber.

6. TBS-NS with Tween rinse, again, 10 min; save for step 8.

7. Primary treatment: apply antiserum or control serum diluted in TBS-NS with Tween 20 to slides racked in level humidity chamber (optional: cover slides with parafilm to keep solution distributed), overnight, 4^{o}C.

8. TBS-NS with Tween rinse, 10 min or more.

9. Secondary treatment: anti-animal IgG-biotin diluted in TBS-NS with Tween for 30-60 min to slides in humidity chamber.

10. TBS with Tween rinse, 10 min or more.

TBS

TRIS stock, 0.5 M, pH 7.2	100 ml
NaCl	9 g
Bring to one liter with distilled water	

11. Tertiary treatment: apply avidin-peroxidase diluted in TBS with Tween (no serum) to slides in humidity chamber, 30-60 min. Vector lab kits give better results.

12. Two TBS rinses, first with Tween and second without, 10 min or more.

13. Peroxidase reaction for stain

Prepare stain solution approx. 5 min before adding slides

Hankers-Yates reagent (Polysciences)	100 mg
30% hydrogen peroxide	50 µl
Nickel chloride	1-5 mg

to 200 ml TBS, keep stirred.

Immerse Slides in solution for 15-60 min, CHECK PROGRESS OF REACTION

14. Water rinse, 70% ethanol for 10 min or more to reduce staining, then 2 X 100% ethanol, 2 X Xylene, and mount coverslips with Permount.

PEPTIDE IMMUNOCYTOCHEMISTRY FOR ELECTRON MICROSCOPY

FIXATION: Midguts are removed from the mosquitoes and fixed in the same fixative as used in the light ICC procedure or a 1% glutaraldehyde fix (1.25 ml 8% glut., 8.75 ml 0.05 M phosphate buffer (pH 7.6) and 200 mg sucrose). Same time, temperature, and rinses. I have done postfix stains with OsO_4 (1% OsO_4 in same phos. buffer, 650 mg sucrose) and 5% Uranyl Acetate in dH_2O and had no loss of antigenicity.

DEHYDRATION, EMBEDDING and SECTIONING same as for Light ICC (except thin sections on nickel grids).

ICC PROCEDURE: Grids are mounted in a Hiraoka grid holder (Polysciences) for all wash steps and if I have many to do, for the Ab steps. The holder is placed on a small plastic weigh boat (grids down) filled with the necessary solution (5-9 ml is sufficient) and stirred with a small magnetic bar (especially with the DAB stain). All steps at room temperature, but I have ran at 37° for Ab steps. ALL SOLUTIONS (EVEN dH_2O) ARE MILLIPORE FILTERED.

1. Etch with 3% H_2O_2, 10 min.

2. dH_2O wash, 3 min, then TBS-1%NS 10 min.

3. TBS (pH 7.6) wash, 3 min.

4. 1° TRT: Ab or control in TBS, 30-60 min. (Make ABC if using Vector kit, same dilution as Light ICC).

5. TBS wash, 3 min.

6. 2° TRT: Ab against first Ab alone or conjugated to biotin in TBS (Vector kit or separate order same dilutions as Light ICC), 30 min.

7. TBS rinse, 2 min

8. 3° TRT: PAP or ABC or avidin-peroxidase (1:3000) in TBS, 30 min.

9. TB wash, 5 min.

10. Stain: 5-10 min, stirred.

DAB	7.5 mg
TB, pH 7.6	10 ml
30% H_2O_2	3 ul

Make 5-10 min before use.

10. dH_2O wash, 5-10 min and dry grids.

11. Optional stains: 1% OsO_4, 5 min or Lead citrate.

TISSUE FIXATION

I. PB BOUIN'S

A. PHOSPHATE BUFFER

1. make 0.2M monobasic sodium phosphate
   0.69g/25ml
2. make 0.2M dibasic sodium phosphate 7-hydrate
   2.68g/50ml
3. add 50ml dibasic to 16ml of monobasic and adjust the pH to 7.3
4. to make 0.01M PB take 50ml of 0.2M PB and dilute with 1000ml of double distilled water

B. PB BOUINS

1. saturate 0.01M PB with Picric acid (approx. 17g+)
   CAUTION: Picric acid is explosive in contact with metals. DO NOT weigh out using any metal equipment in contact with the Picric acid
   NOTE: Use glassware and containers marked for fixatives and wash these out in the back sink
2. filter the PB-Picric and adjust the pH to 7.3
   (only filter what is needed for the following mixture)
   NOTE: use the electrode marked for fixatives and be sure to rinse before and after use. Also the electrode jacks fit the one large hole and the small hole closest to the large hole
3. mix 750:250 saturate with 37% formalin (in hood)
4. store PB Bouin's fully mixed