Structural Properties of the Female Accessory Gland in the Stable Fly, *Stomoxys calcitrans*[1]

Benjamin J. Cook and Nan W. Pryor

U. S. Department of Agriculture, Agricultural Research Service
Food Animal Protection Research Laboratory, 2881 F&B Road
College Station, TX 77845 USA

J. Entomol. Sci. 30(3): 362-373 (July 1995)

ABSTRACT The accessory reproductive glands of the female stable fly are translucent structures that run parallel to the common oviduct when the ovipositor is extended. The only muscles found associated with the gland were those at either end of the long tube of simple cuboidal epithelial cells. The posterior region of each gland is connected to the anterior vagina by means of a valve of circular muscle. The myofibrils of the valve are separated into sarcomeres of irregular alignment with Z disks that appear as discontinuous rows of dense bodies. Transections through the Z disk region also revealed a perforated character which is common in muscles that have the ability to super contract. The sarcolemma of many cells have tubular invaginations that correspond to the T-system of tubules found in most muscles. Terminal axons with both synaptic vesicles and larger neurosecretory granules were found in close apposition to muscle fibers of the valve. Large vacuoles (with a mean of 26.36 μm and a SD = ± 2.09) were the most prominent structures in the cytoplasm of the glandular epithelium. The fine structure of these vacuoles showed a microvillar border and a central portion that contains clumps of secretory material in a granular matrix. Many vacuoles also contain dense inclusion bodies while other inclusion bodies were observed in apical membranous networks just beneath the cuticular intima. Such ultrastructural features suggest a largely merocrine type secretion for this gland.

KEY WORDS Secretory vacuoles, muscle ultrastructure, synaptic vesicles, neurosecretory granules.

Although the paired female accessory glands of insects have geen given relatively scant attention, such glands can have striking effects on reproductive processes. In the house fly, *Musca domestica* L., it has been shown that the removal of these accessory glands inhibits penetration of the eggs by sperm but the insemination of females without these glands appears to be unaffected (Leopold and Degrugillier 1973). Subsequent studies revealed that the stable fly, *Stomoxys calcitrans* L., and two other calypterate muscoid flies display the same phenomenon (Leopold 1980). Accessory gland secretions also readily dissolve the micropyle cap substance from house fly eggs *in vitro*, suggesting that the gland

[1] Accepted for publication 16 March 1995.

secretion acts indirectly on the acrosome by releasing material from the micropyle cap that in turn liberates the acrosomal contents (Leopold 1980). Further study showed that release of acrosomal material is achieved by treatment of the sperm with a combination of accessory gland secretion and micropyle cap substance but not by gland secretion alone (Degrugillier 1985).

Peptides that inhibit remating behavior and stimulate oviposition have been identified in the accessory reproductive glands of male flies (Chen et al. 1988, Chen and Balmer 1989). More recently a family of peptides has been isolated from the reproductive accessory glands and/or oviducts of *M. domestica, S. calcitrans,* and face fly, *Musca autumnalis* De Geer (Wagner et al. 1993, Wagner et al. 1994). These peptides range from 12 to 20 amino acids in length and are homologous in sequence and structure. Initial studies have shown these peptides to be moderately active on oviduct contraction in the species from which they were isolated.

In spite of these interesting results, no structural data are available on the accessory glands in mucoid flies. Consequently, it has been our intention to determine the main anatomical features of the glands and, if possible, identify the secretory processes that prevail in these tissues.

Materials and Methods

Adult female stable flies were obtained from a laboratory-reared colony at the USDA, ARS, Food Animal Protection Research Laboratory, College Station, TX. In this colony, both male and female flies were held together in screen cages at 27°C and 50-70% humidity. The flies were fed daily by placing cotton pads soaked with citrated (5 gm/L) bovine blood on top of the screen cages. At 7 d after adult emergence, female flies were removed from cages in the colony and dissected. The composition of the saline used for dissection was (in mM) NaCl 105, $KN0_3$ 5, $CaCl_2$ 5, $MgSO_4 \cdot 7H_2O$ 3, L-histidine 10, and glucose 28; pH was adjusted to 6.8 with sodium hydroxide.

Preparation of tissue for microscopic examination. Female flies were immobilized by cooling (4°C) and the head, wings, and legs were removed. The thorax and abdomen were placed ventral side up in a small wax-filled Petri dish, and pinned through the thorax. The retracted ovipositor was pulled out to its full length with forceps and pinned to the wax preparation dish with a minuten pin through the tip. The central portion of the integument on the ventral abdomen was then excised. The body cavity was flooded with stable fly saline and the integument of the abdominal side walls was pinned with a minuten on each side. This procedure was followed by cutting the integument of the extended ovipositor along the midline to fully expose the terminal end of the common oviduct and the branch nerves that run parallel to it.

The digestive tract was carefully removed by severing the hindgut from its close apposition to the oviduct near the spermatheca. The entire oviduct with attached accessory glands was then lifted out of the ovipositor by cutting across the vagina and the tracheal attachments. After the oviduct and accessory glands were thus isolated, the preparation was pinned out on a sylgard coated slide for examination with Nomarski differential interference contrast microscopy (Fig. 1A) or fixation.

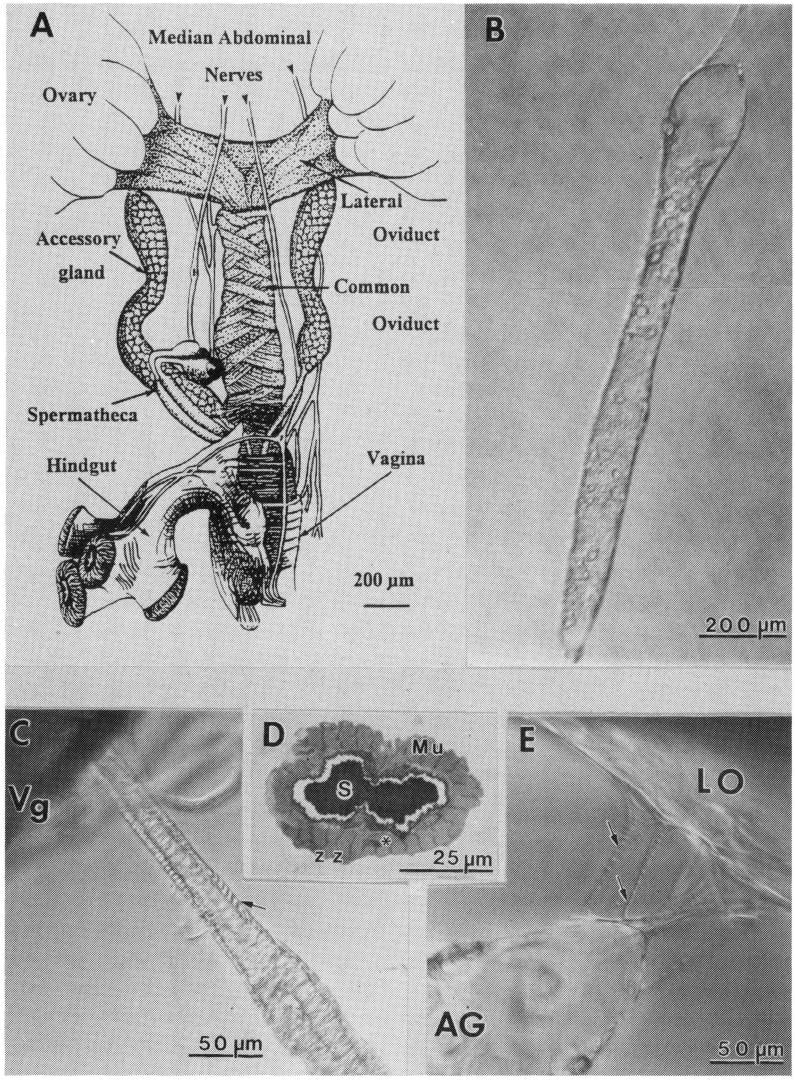

Fig. 1. Basic microscopic features of the female stable fly accessory gland. A) Structural arrangement of accessory glands with respect to the entire reproductive system. B) Differential interference contrast (DIC) micrograph of the secretory portion of the accessory gland which comprises three fourths of the organ. C) DIC micrograph of terminal muscular valve of the accessory gland as it enters the vagina (Vg). Striations (arrow) in these muscles are revealed by Nomarski optics. D) Toluidine Blue stained transection of the muscular valve showing Z bands (Z) in the circular muscles (Mu) and secretion (S) in the lumen. (*) = nucleus. E) DIC micrograph of muscular attachments (arrows) of the accessory gland (AG) to the lateral oviduct (LO).

For observation under transmission electron microscopy, the accessory glands from four insects were fixed in 1% acrolein and 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature or overnight at 4°C. The specimens were then rinsed in the buffer 3 times (10 min each) in the cold and post fixed in 1% OsO_4 buffered with 0.1 M sodium cacodylate for 2 h in the cold. The tissues were dehydrated in a series of aqueous acetone solutions – 30%, 50%, 70%, 95%, and 100% acetone (3 times in 100%) prior to embedding in Spurr's resin as modified by Mollenhauer (1986). Tissues were thin sectioned with a Reichert Ultracut S microtome either at 1 μm and stained with Toluidine Blue, or thin sectioned at 70 nm and stained with lead citrate. Appropriate specimens were viewed either under the light microscope or under a Hitachi H 7000 electron microscope at 75 kV.

Results

The paired accessory reproductive glands of the female stable fly are translucent structures that run parallel to the common oviduct when the ovipositor is extended (Fig. 1A). Under low power Nomarski optics numerous small crater-like structures are evident on the surface of the glands (Fig. 1B). The posterior region of each gland is connected to the anterior vagina by means of a muscular valve approximately 300 μm in length (Fig. 1A & C). Muscle cells of this valve appear striated under differential interference contrast (DIC) microscopy (Fig. 1C) and, in transection under ordinary light microscopy, Z bands can be observed between individual sarcomeres (Fig. 1D). Secretory material is also abundantly visible in the lumen of the valve. The anterior tips of the accessory glands are attached to the lateral oviducts by a cluster of muscle fibers with obvious striations (Fig. 1E).

Ultrastructural details of the circular muscles in the valve region of the distal accessory gland are shown in Fig. 2. Although spontaneous contractions of a myogenic nature occur in these muscles (Cook, unpublished observations), clear evidence of neural regulation of these cells is shown in Fig. 2A. In this electron micrograph terminal axonal profiles are shown in close apposition to muscle fibers. In one axon both synaptic vesicles and larger neurosecretory granules are evident. Both axons are surrounded by glial sheath cells. An epithelial cell with a convoluted system of membranes can also be seen just beneath one of the circular muscle cells. The myofibrils of accessory gland valves are separated into sarcomeres of irregular alignment (Fig. 2B). The Z disk in these muscles consists of discontinuous rows of dense bodies. However, the classical I and A bands are not clearly observed in this micrograph. Tubular invaginations into the cytoplasm are seen along the sarcolemma of many cells. These structures correspond to the T-system of tubules characteristically found in nearly all muscle cells. Such tubules occasionally arise from large clefts in the sarcolemma of muscle fibers, and after coursing their way into the sarcoplasm of cells, they form dyads with another inner series of tubules called the sarcoplasmic reticulum. A transection of circular muscle through the Z-disk region reveals a perforated character (Fig. 2C). Such a feature is common in muscles that have the ability to super contract.

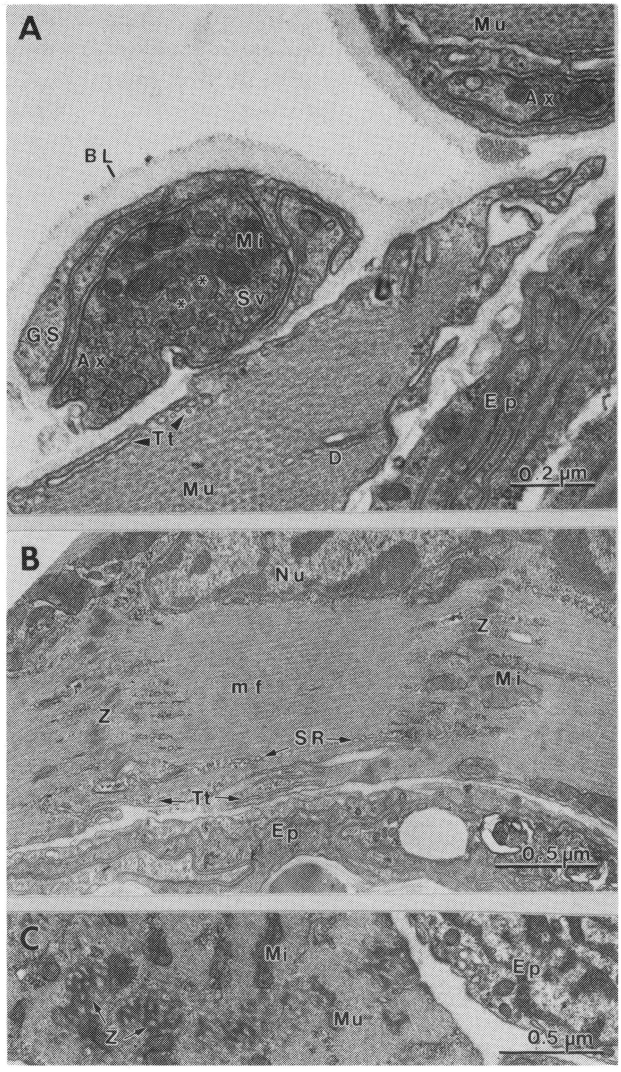

Fig. 2. Nerve-muscle ultrastructure in the terminal valve of the accessory gland. A) Nerve axon terminations (Ax) on muscle fibers (Mu) of the valve showing both synaptic vesicles (SV), secretory granules (*), mitochondria (Mi), and glial sheath cells (GS). D = dyads; T_t = transverse tubule of T-system; Ep = epithelial cell; BL = basal lamina. B) Fine structure of a circular muscle sarcomere showing the irregular distribution of Z bodies (Z). Mi = mitochondria; mf = myofilaments; SR = sarcoplasmic reticulum; T_t = transverse tubule of T-system; Ep = epithelial cell. C) Transection of a muscle fiber (Mu) through the region of the Z-disc (Z) showing perforations (arrows). Mi = mitochondria; Ep = epithelial cell.

The secretory portion of the female accessory gland consists of simple cuboidal epithelial cells with boundaries similar to those shown in Fig. 3A(*). Large vacuoles are the most prominent structures in the cytoplasm of these cells. They have a bubble or crater-like appearance under Nomarski optics (Figs. 3A & B) and they have an average diameter of 26.36 μm (SD = ± 2.09, n = 17). Other vacuoles reveal a more complex inner structure as shown in Fig. 3B (ScV*). The fine structure of a large secretory vacuole in the glandular epithelium is shown in Fig. 3D. Here a microvillar border encloses a number of small clear vacuoles, while the central portion of the large vacuole contains clumps of secretory material in a granular matrix. Many secretory vacuoles also contain dense inclusion bodies which are often visible even under Nomarski optics (Fig. 3A [dots]). Two examples of the fine structure of these inclusion bodies are shown in Figs. 3F & G. Other subcellular components of the glandular epithelium are shown in Fig. 3H. Clusters of mitochondria are evident among much smaller vacuoles and some profiles of rough endoplasmic reticulum.

Two cells of contrasting cytoplasmic properties are illustrated in Fig. 4A. Cell Ep1 contains a large number of vacuoles of varying sizes that extend from the basal limits of the cell wall into the apical region. The large nucleus is situated along the basal margin of the cell and only a small amount of cytoplasm of a granular character is seen near a secretory vacuole. The cytoplasm of cell Ep2 by comparison is granular in appearance and the plasma membrane of the basal region shows extensive infolding. A high magnification of another epithelial cell with a granular cytoplasm like Ep2 is shown in Fig. 4B. Many parallel arrays of rough endoplasmic reticulum are visible. Even vacuoles in the region are surrounded by the rough endoplasmic reticulum and extensions of them can be seen in the infoldings of the plasma membrane along the basal border of the cell.

Several ultrastructural features observed in the glandular epithelium suggest the transport of secretory material. The first example is illustrated in Fig. 4C where a sequential arrangement of secretory granules is found in a membranous channel which in turn is directed toward the lumen of the accessory gland. In the second example (Fig. 4D), a portion of a large secretory vacuole is shown close to the apical surface of the glandular epithelium and secretory material appears to pass through the cuticle into the lumen of the gland. A third possibility is suggested in Fig. 3E where both the cytoplasm and a large secretory vacuole within the cell are observed separating from the cuticular intima that lines the lumen of the accessory gland.

In addition to the inclusion bodies associated with the secretory vacuoles (Fig. 3A & E), other types of inclusion bodies are frequently found in the membranous networks of the apical region (Figs. 4E & F) just beneath the cuticular intima. Many of these membranes in the networks are also septate in character (Fig. 4G).

Discussion

It is generally recognized that most female dipterous insects have paired accessory glands that open to the genital tract behind the spermathecal duct (Matsuda 1976, Kaulenas 1992) and that these glands in *Musca* provide

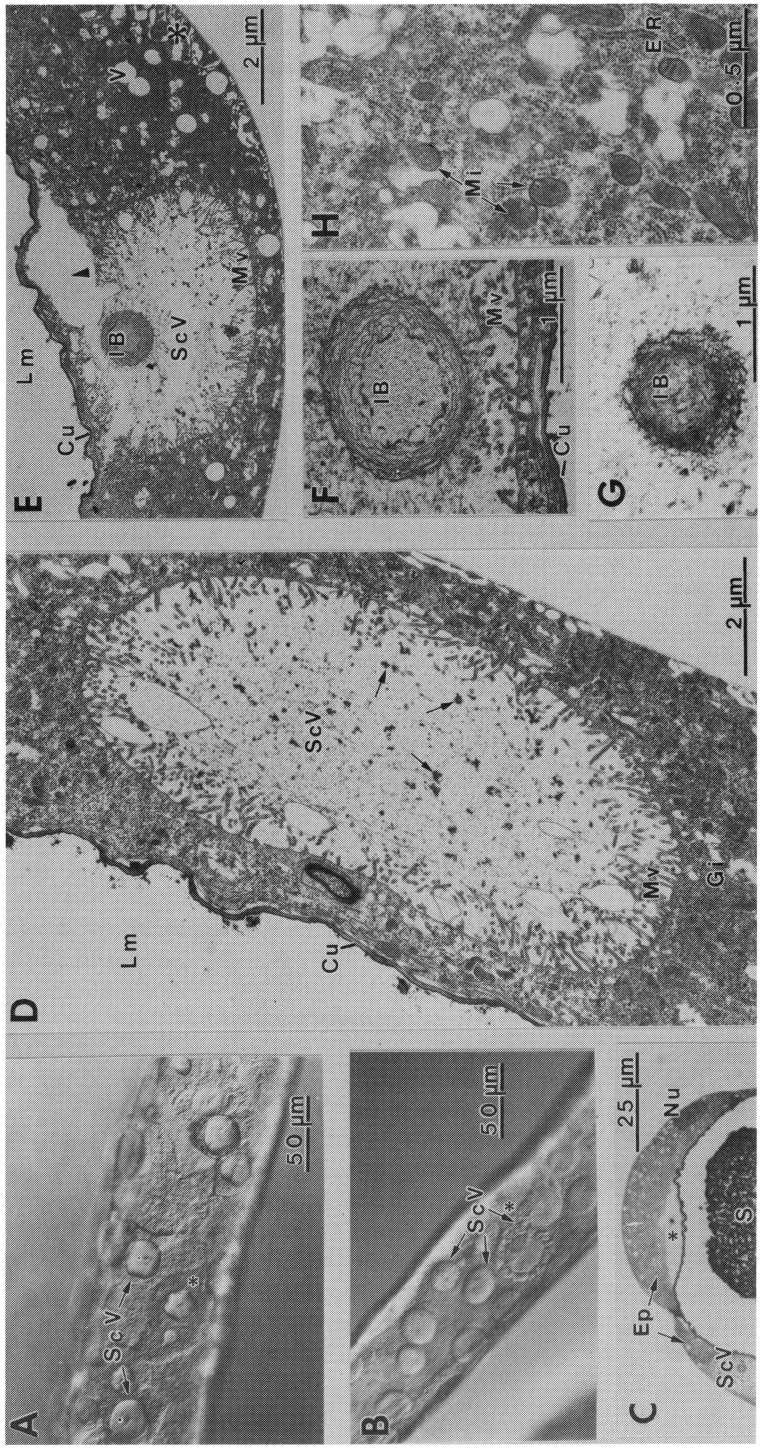

Fig. 3. Principle structural features of the secretory epithelium of the accessory gland. A) DIC micrograph of the mid region of the gland showing large secretory vacuoles (ScV) with inclusion bodies (small dots). The boundaries of one epithelial cell (*) are also evident. B) Another micrograph of the secretory epithelium that shows a large vacuole (arrow marked with an asterisk) with an outer ring of smaller vacuoles within it. C) Transverse section of accessory gland through the secretory cells (Ep and arrows). A nucleus (Nu) is visible in one cell and a secretory vacuole (ScV) in the other. The cuticle that lines the lumen of the gland has separated from the epithelial cytoplasm (*). S = secretion in lumen of gland. D) Fine structure of a large secretory vacuole (ScV) in the glandular epithelium that shows a microvillar border (Mv) which encloses numerous smaller vacuoles. Clumps of secretory granules are also evident (arrows). Cu = cuticle; Lm = lumen; Gi = golgi bodies. E) Many cells with secretory vacuoles contain dense inclusion bodies (IB) of varying structure. In the epithelial cell shown the cytoplasm has broken away from its usual close association to the cuticle (arrowhead). Cu = cuticle; Lm = lumen; Mv = microvilli; V = small vacuoles. F) Higher magnification of an inclusion body (IB) that reveals a laminate structure. This body is close to the apical surface of the glandular epithelium. G) Another type of inclusion body found in secretory vacuoles has a less regular internal structure. H) A cluster of mitochondria (Mi) in the cytoplasm of the glandular epithelium with an occasional example of rough endoplasmic reticulum (ER).

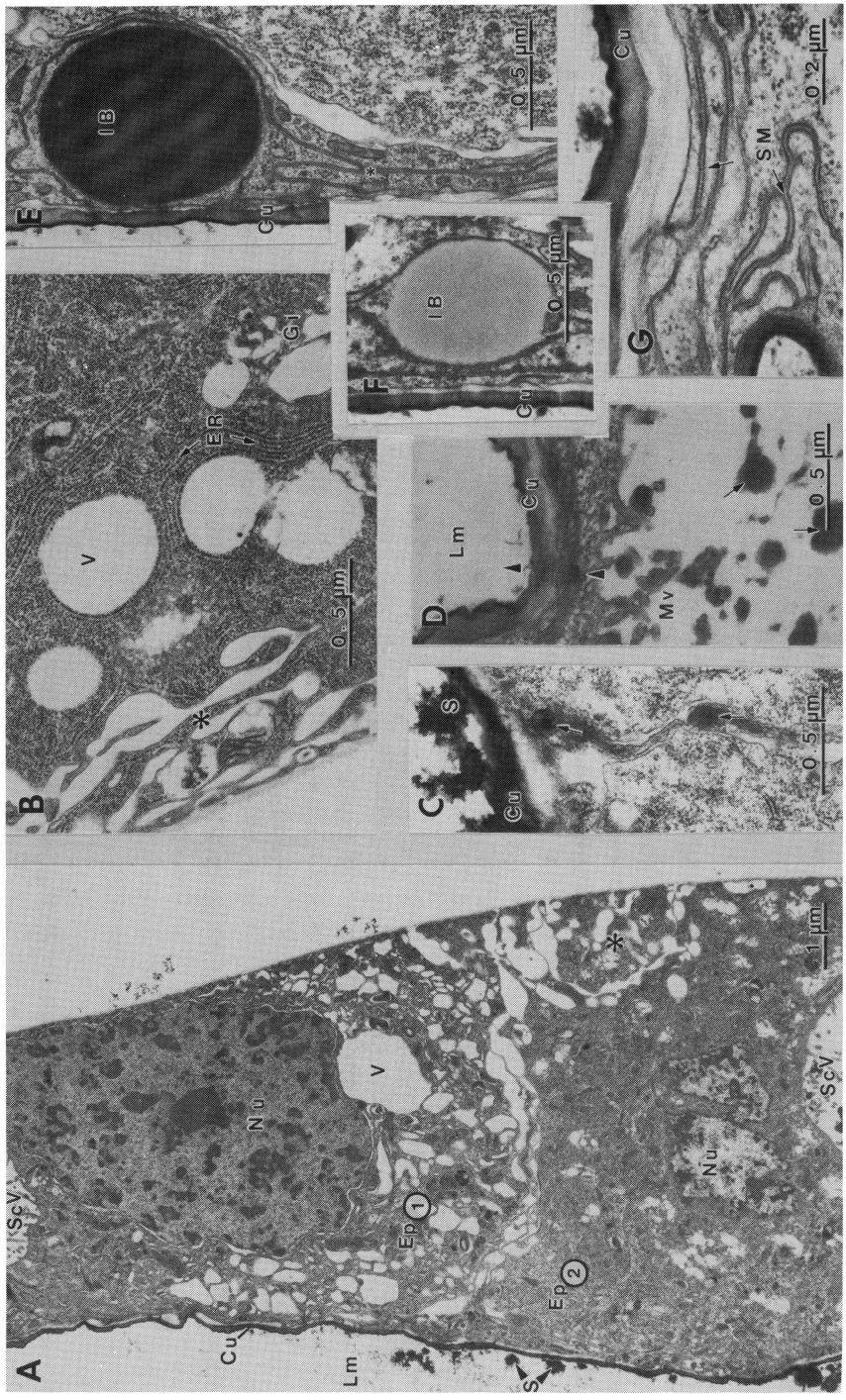

Fig. 4. Additional ultrastructural details observed in the epithelium of the accessory gland. A) Two epithelial cells with different cytological properties are shown. Cell Ep_1 contains a large number of vacuoles (V) of varying size. In cell Ep_2 the cytoplasm is granular in appearance with a much smaller number of vacuoles and the basal region (*) that reveals extensive infolding of the plasma membrane. Cu = cuticle; Lm = lumen of gland; Nu = nucleus; S = secretory granules on surface of cuticle; ScV = secretory vacuoles with microvilli. B) Higher magnification of an epithelial cell with granular cytoplasm like Ep_2 above. Here many profiles of rough endoplasmic reticulum (ER arrows) can be seen even around vacuoles (V). Again the basal region of this cell shows extensive infolding of the plasma membrane (*). C) Secretory granules (arrows) are seen coursing through membrane lined channels toward the lumen of the gland. Cu - cuticle; S = clusters of secretory granules on the lumenal surface of the cuticle. D) Portion of a secretory vacuole close to the apical surface of the glandular epithelium that shows the possible diffusion of secretory material through the cuticle (between arrowheads). Cu = cuticle; Lm - lumen; Mv = microvilli; and arrows = secretory granules in vacuole. E) Large dense inclusion body (IB) enveloped in a membranous network (*) of the glandular epithelium just beneath the cuticle (Cu). F) Another example of an apical inclusion body (IB) of lower density. G) Examples of the septate character of membranes in the apical cytoplasm of the glandular epithelium.

secretions which assist in sperm penetration of eggs by liberating sperm acrosomal contents (Leopold and Degrugillier 1973, Degrugillier 1985). However, further structural details on the accessory glands are not available with the exception of the extensive work that has been done on the "milk glands" of tsetse flies (Tobe et al. 1973, Hecker and Moloo 1983).

The only muscles found associated with the accessory gland were those at either end of the long tube of epithelial cells (Fig. 1). The posterior end of the glandular epithelium is attached to the vagina by a valve of circular muscle approximately 300 μm in length (Fig. 1C), while the anterior end is connected to the lateral oviduct by a dozen or more thin muscular strands (Fig. 1E). Although it is not certain just how secretory material is transferred along the lumen of the gland, it seems clear that the muscular valve is in some way involved. These muscles are spontaneously active (Cook, unpublished observations) and ultrastructural properties of the myofibrils suggest that super contractions can occur (Figs. 3B & C). The myofibrils of all these cells have the characteristic irregular sarcomere alignment and a discontinuous Z band (Hoyle 1983). In addition, the nerve-muscle junctions of the valve (Fig. 3A) indicate that the muscles are regulated from the central nervous system.

Like the secretory cells along the ejaculatory ducts of many male insects (Riemann 1973, Kaulenas et al. 1979, Meola 1982) the glandular epithelium in the accessory gland of the female stable fly shows ample ultrastructural evidence of protein synthesis. The large quantities of rough endoplasmic reticulum and free ribosomes observed in the cytoplasm of most cells (Figs. 3 & 4) confirm the proteinoid nature of the glandular secretion. Although no evidence was found in this study of more than one morphological type of secretory cell in the accessory gland, these cells are capable of secreting a variety of novel peptides (Wagner et al. 1991).

The transport of secretory material in the glandular epithelium appears to be largely merocrine in nature because dense secretory droplets were often observed close to or in a sequential procession toward the cuticular intima of secretory cells (Figs. 4C & D). It also seems possible that the apical inclusion bodies could represent a type of secretory process (Figs. 4E & F). In addition portions of the apical cytoplasm of cells were often observed separating from the rest of the cytoplasm by bulges and outpouchings of the cuticular intima in to the glandular lumen (Fig. 3E). Such a feature could suggest an apocrine mode of secretion as well.

The present study represents one of the first summaries of basic anatomical facts on female accessory glands of a dipterous insect. It should serve as a stimulus to further research on the complex biochemical and physiological events associated with these unique glands.

References Cited

Chen, P. S. and J. Balmer. 1989. Secretory proteins and sex peptides of the male accessory gland in *Drosophila sechellia*. J. Insect Physiol. 35: 759-764.

Chen, P. S., E. Stumm-Zollinger, T. Aigaki, J. Balmer, M. Bienz and P. Bohlen. 1988. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. Cell 54: 291-298.

Degrugillier, M. E. 1985. *In vitro* release of house fly, *Musca domestica* L. (Diptera: Muscidae), acrosomal material after treatments with secretion of female accessory gland and micropyle cap substance. Int. J. Insect Morphol. Embryol. 14: 381-391.

Hecker, H. and S. K. Moloo. 1983. Quantitative morphological changes of the uterine gland cells in relation to physiological events during a pregnancy cycle in *Glossina morsitans morsitans*. J. Insect Physiol. 29: 651-658.

Hoyle, G. 1983. Muscles and their neural control. Wiley, New York.

Kaulenas, M. S., H. E. Potswald, A. L. Burns and R. L. Yenofsky. 1979. Development of structural and functional specializations for export protein synthesis by the accessory gland of the male cricket, *Acheta domesticus* L. (Orthoptera: Gryllidae). Int. J. Insect Morphol. Embryol. 8: 33-49.

Kaulenas, M. S. 1992. Insect accessory reproductive structures. Springer-Verlag, Berlin.

Leopold, R. A. and M. E. Degrugillier. 1973. Sperm penetration of housefly eggs: evidence for involvement of female accessory secretion. Science 181: 555-557.

Leopold, R. A. 1980. Accessory reproductive gland involvement with the sperm-egg interaction in muscoid flies, Pages 253-270. *In* W. H. Clark, Jr. and T. S. Adams (eds.), Advances in invertebrate reproduction. Elsevier North Holland, Inc.

Matsuda, R. 1976. Morphology and evolution of the insect abdomen. Pergamon Press, Oxford.

Meola, S. M. 1982. Morphology of the region of the ejaculatory duct producing the male accessory gland material in the stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae). Int. J. Insect Morphol. Embryol. 11: 69-77.

Mollenhauer, H. H. 1986. Surfactants as resin modifiers and their effect on sectioning. J. Electron Microsc. Tech. 3: 217-222.

Riemann, J. G. 1973. Ultrastructure of the ejaculatory duct region producing the male housefly accessory material. J. Insect Physiol. 19: 213-223.

Tobe, S. S., K. G. Davey and E. Huebner. 1973. Nutrient transfer during the reproductive cycle in *Glossina austeni* newst.: Histology and histochemistry of the milk gland, fat body, and oenocytes. Tissue & Cell 5: 633-650.

Wagner, R. M., J. Kochansky, D. K. Hayes, B. J. Cook, W. R. Lusby, C. W. Woods, D. J. Harrison, D. Landsman, J. Hayes, J. Hill and B. A. Fraser. 1994. A new family of peptides in the reproductive system of female insects. 1st Int. Conf. on Insects, Chemical, Physiological, and Environmental Aspects, Sept. 26-29, Ladek Zdroj.

Wagner, R. M., C. W. Woods, J. A. Hayes, J. P. Kochansky, J. C. Hill and B. A. Fraser. 1993. Isolation and identification of a novel peptide from the accessory sex gland of the female house fly, *Musca domestica*. Biochem Biophys. Res. Commun. 194: 1336-1343.