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Formation and Ultrastructure of the Peritrophic Membrane in Larval Midge *Chironomus tentans* (Diptera: Chironomidae)

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ABSTRACT—The ultrastructural features of the cardia from fourth instar larvae of the midge *Chironomus tentans* are consistent with it being the source of the peritrophic membrane. The group of anterior epithelial cells display short basal membrane infoldings, abundant rough endoplasmic reticulum, Golgi complexes, mitochondria, free ribosomes, vesicles and electron-dense secretory granules. The secretory material is secreted into the narrow luminal cleft between the evenly spaced microvilli and the apposing non-secretory esophageal valve. The valve and apices of secretory cells act as a press to mold the secretory product into the definitive peritrophic membrane. The single 255–488 nm thick uniform peritrophic membrane is devoid of holes and is composed of three layers in the posterior cardia, two layers in the rectum but only one 110 nm thick discernable layer in the midgut. Since the inner loosely arranged third layer is absent in the midgut and the rectum, it is postulated that it is incorporated into the electron-dense second layer. The second or middle layer which represents the whole peritrophic membrane in the midgut displays unique vertical striations that enclose 6.5 nm diameter channels. It is suggested that these newly described open-ended channels facilitate passage of water, salts, digestive enzymes and digested food material in either direction.

INTRODUCTION

The peritrophic membrane is a cylindrical membranous sheath which encloses the solid food in the midgut in most insects (Snodgrass, 1935). Since the midgut is not internally protected by cuticle, it is lined by the peritrophic membrane which protects its epithelium from the abrasive action of solid, and rough food material that passes into it from the foregut (Wigglesworth, 1930; 1972). It is apparently absent in insects that feed on liquid diets (Imms, 1957; Waterhouse, 1953). The peritrophic membrane is formed by secretion from the cardia and/or midgut epithelium and is composed of both chitin and proteins (Chapman, 1985; Lehane, 1976; Peters, 1976; Richards and Richards, 1971, 1977; Wigglesworth, 1972). In blowfly larvae (*Lucilia* and *Calliphora*), the peritrophic membrane is continuously produced at a rate of 5–10 mm/day (Waterhouse, 1954).

Two types of peritrophic membranes occur in insects. In type I which is found in Ephemeroptera, Coleoptera, Hymenoptera, Lepidoptera and Odonata, the peritrophic membrane arises by delamination from the entire surface of the midgut epithelium. In type II which is best developed in Diptera, it is secreted by a band of specialized cells in the anterior end of the cardia or proventriculus which encircles the base of the esophageal valve (Waterhouse, 1954; Wigglesworth, 1930, 1972).

Earlier electron microscopic studies by Huber and Haasser (1950), Mercer and Day (1952) revealed that the intact peritrophic membrane is composed of a fibrillar network in a hexagonal or square lattice with a thin film stretching over the holes. It has been suggested that the holes in the fibrillar network correspond to the spaces occupied by the individual microvilli of the midgut cells around which the fibrils are formed and oriented (Chapman, 1985; Mercer and Day, 1952; Peters *et al.*, 1979). This hypothesis has been disputed by Platzer-Schultz and Welsch (1969, 1970) and Richards and Richards (1977).

A number of ultrastructural studies have shown that the peritrophic membrane consists of one to five layers, each produced by a different region of the folded epithelial of the cardia or by the midgut epithelium (Becker *et al.*, 1976; Bertram and Bird, 1961; Binnington, 1988; King, 1988; Lehane, 1976; Peters, 1976; Peters *et al.*, 1979; Platzer-Schultz and Welsch, 1969, 1970; Richards and Richards, 1971, 1977; Smith, 1968).

The aim of the present study was to further elucidate the mechanism of peritrophic membrane formation and examine its fine structure in full-grown larva of the midge *Chironomus tentans*. This insect is one of the few insects that possesses functional hemoglobin in its hemolymph (Wigglesworth, 1972).

MATERIALS AND METHODS

Full-grown, fourth-instar larvae of *Chironomus tentans* FABRICIUS were collected from a local creek, transported to the lab in a thermos jug with mud, pebbles, and creek water, and processed immediately.

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For transmission electron microscopy dissected tissue was fixed at room temperature with 2.5% glutaraldehyde and 2% paraformaldehyde (1:1) in 0.1 M cacodylate buffer at pH 7.4 (Millonig, 1976), and postfixed in 1% osmium tetroxide in the same buffer. Tissue was dehydrated in an ethanol series, transferred to propylene oxide, and embedded in Epon 812 (Luft, 1961). Polymerization was carried out at 60°C, overnight. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 or HU-11A transmission electron microscope. Thick sections (2 µm) were also cut, stained with azure II, and examined with an American Optical Series 20 light microscope. Similarly fixed tissues were also dissected to remove the tubular peritrophic membrane intact or in halves, then chemically dried in hexamethyl-disilazane (Polysciences), coated with gold/palladium, and examine with a Cambridge S-90 scanning electron microscope.

Some cardia and midguts of the larvae were dissected into insect Ringer (Hoyle, 1953), fixed in Bouin's fluid and embedded in paraplast. Sagittal sections (6 µm) were cut and stained with Harris hematoxylin/eosin and/or with aqueous periodic acid-Schiff (PAS) solutions (Humason, 1979) and examined with the light microscope.

RESULTS

General morphology and histological observations

The midgut of full-grown *Chironomus tentans* larva consists of a distinct, short, pear-shaped cardia or proventriculus, anterior to the straight, tubular midgut proper. The cardia exhibits several outward projecting short caeca that are devoid of muscular coats. The esophagus with its highly folded epithelium and intima protrudes into the cardia as an inner tube which reflects upon itself to form the esophageal or cardiac valve (Figs. 1a, c, 2a). The highly folded intima of the reflected esophageal valve is attached to the anterior end of the cardia (Fig. 1b). The group of large deeply staining epithelial cells at the anterior end of the cardia measure about 62 µm in height and 12 µm in width and contain many clear vacuoles in the cytoplasm and large basally placed nuclei with prominent nucleoli. They are covered by a distinct basal lamina

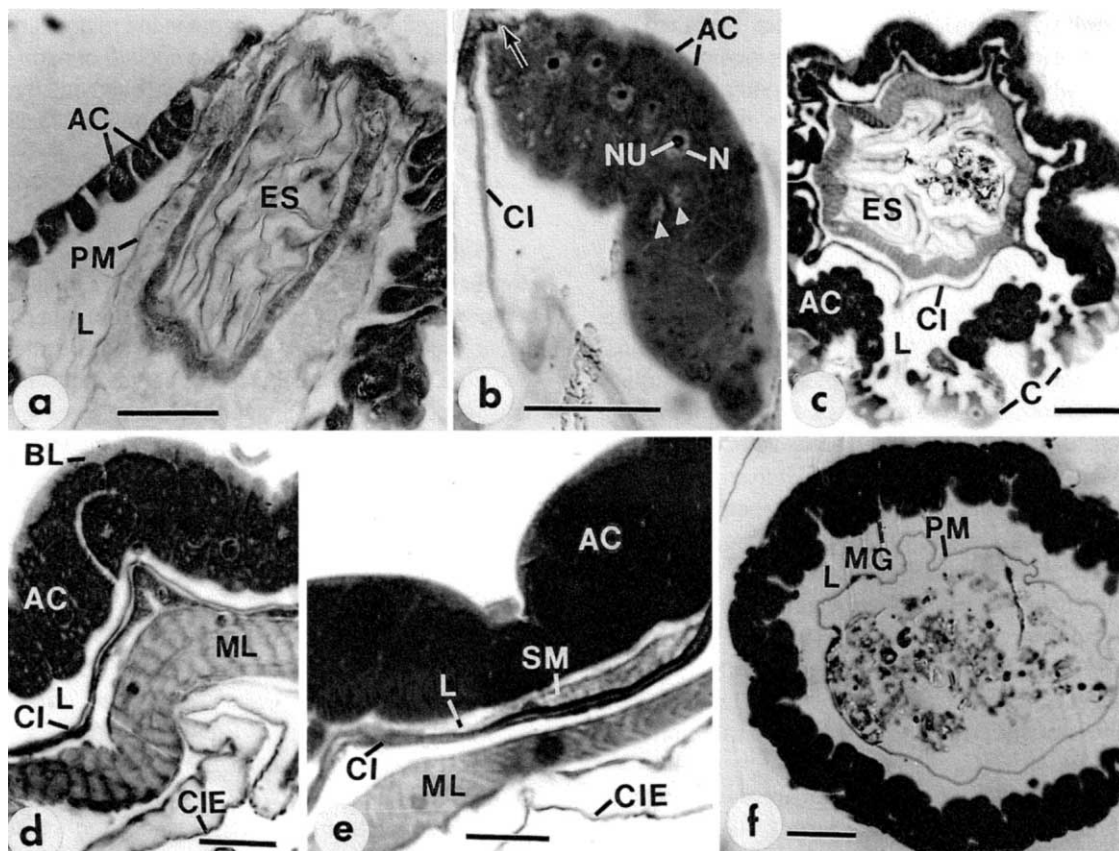


Fig. 1. Light micrographs of cardia and midgut. (a) Sagittal paraplast section (6 µm) of cardia stained with hematoxylin/eosin, showing esophagus (ES) extending into it. AC, anterior cells of cardia; L, lumen; PM, peritrophic membrane. Scale bar, 0.05 mm. (b) Sagittal plastic section (2 µm) of the right half of cardia stained with azure II showing large anterior cardia cells (AC) and reflected cuticular intima of the esophageal valve (CI) attached anteriorly to the cardia (arrow). Note large nuclei (N) with well-developed nucleoli and several clear vacuoles in the cytoplasm (arrow heads). Scale bar, 0.05 mm. (c) Transverse section (2 µm) through anterior cardia showing anterior cells (AC), caeca (C), reflected cuticular intima of the esophageal valve (CI) and esophagus (ES) in the lumen (L). Scale bar, 0.05 mm. (d) Portion of a transverse section (2 µm) through anterior cardia showing large cells (AC) covered externally by a thick basal lamina (BL), reflected cuticular intima of esophageal valve (CI), muscle layer (ML) and cuticular intima of the esophagus (CIE). L, lumen of cardia. Scale bar, 0.02 mm. (e) Transverse section (2 µm) through anterior cardia showing densely stained cells (AC), secretory material (SM) in the lumen (L) and in contact with reflected intima of the esophageal valve (CI), muscle layer (ML) and cuticular intima of the esophagus (CIE). Scale bar, 0.02 mm. (f) Transverse section (2 µm) through midgut (MG) showing peritrophic membrane sheath (PM) filled with food material. L, lumen of midgut. Scale bar, 0.05 mm.

(Fig. 1a-e). These cells secrete amorphous material into the narrow lumen between their apices and the reflected cuticular intima of the esophageal valve (Fig. 1e). The single uniform peritrophic membrane forms a delicate sheath around the ingested food as it moves posteriorly in the lumen of the cardia and the midgut. The peritrophic membrane is stained positively by the Periodic-Acid Schiff (PAS) reagent for polysaccharides (not shown). It is separated from the epithelium of the cardia and the midgut by a clear space (Fig. 1a, f).

Scanning electron microscopic observations

In scanning micrographs, the external surface of pear-shaped cardia is easily distinguished from the tubular midgut proper. The cardia displays three tiers of short caeca (Fig. 2a). The outer surface of the midgut shows numerous round prominences which most likely represent the bases of the epithelial cells bulging out between the loosely arranged fibers of the visceral muscles. A pair of muscles is inserted at the anterior end of the cardia which apparently suspends it to the body wall (Fig. 2a). The outer surface of the peritrophic membrane sheath appears rather smooth and is devoid of holes (Fig. 2b, c). The inner surface of the sheath is corrugated, and also lacks holes (Fig. 2d).

Transmission electron microscopic observations

The peritrophic membrane-secreting anterior epithelial cells of the cardia are covered by a 0.1-0.3 μm wide basal

lamina (Fig. 3a) which in turn is surrounded by a thin coat of visceral muscles (Fig. 3a, inset). The epithelial cells are of one type and exhibit short (1.2 μm) basal membrane infoldings that are not associated with mitochondria (Fig. 3a). The cytoplasm contains abundant rough endoplasmic reticulum with small rounded cisternae, and Golgi complexes consisting of clusters of vesicles. Mitochondria, free ribosomes, vesicles, lysosomes, and electron-dense granules are scattered throughout the cytoplasm (Figs. 3a, b, 4a). The large spherical nuclei located in the basal halves of the cells, contain prominent nucleoli (Fig. 3b). The lateral cell membranes enclose narrow intercellular spaces and are united by intercellular junctions which presumably contain septate desmosomes (Figs. 3a, 4a). The luminal surface of the anterior cardia cells display short, well-separated 85 nm diameter microvilli projecting into the lumen (Fig. 4a). The apical plasma membrane along with cytoplasmic fragments are occasionally being seen sluffed from the apical surface (Fig. 4a). In addition, membrane-bound cytoplasmic fragments, organelles, and amorphous material are observed near the peritrophic membrane in the lumen of cardia suggesting that at least some of the secretory material is released by apocrine secretion (Fig. 5a, b). The lumen adjacent to junctional cells that are located between the cardia and the midgut proper, contains fibrillar material which is also present between the microvilli (Fig. 4b).

The secretory product of the anterior epithelial cells of the cardia appears to be released by apocrine and/or mero-

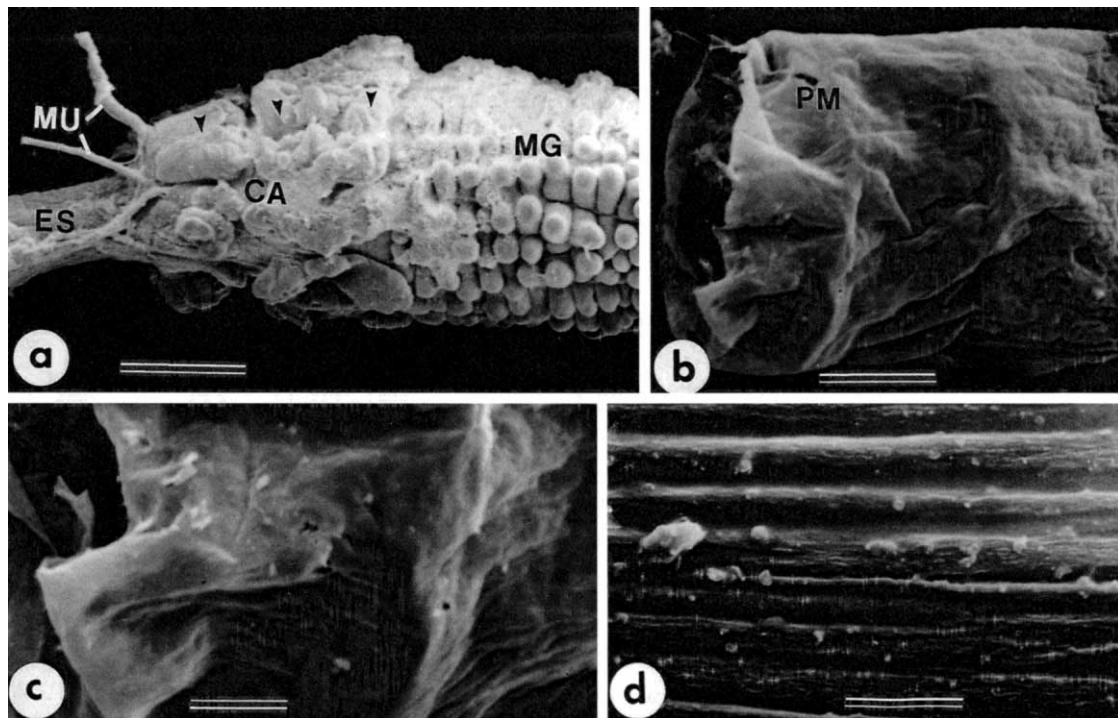
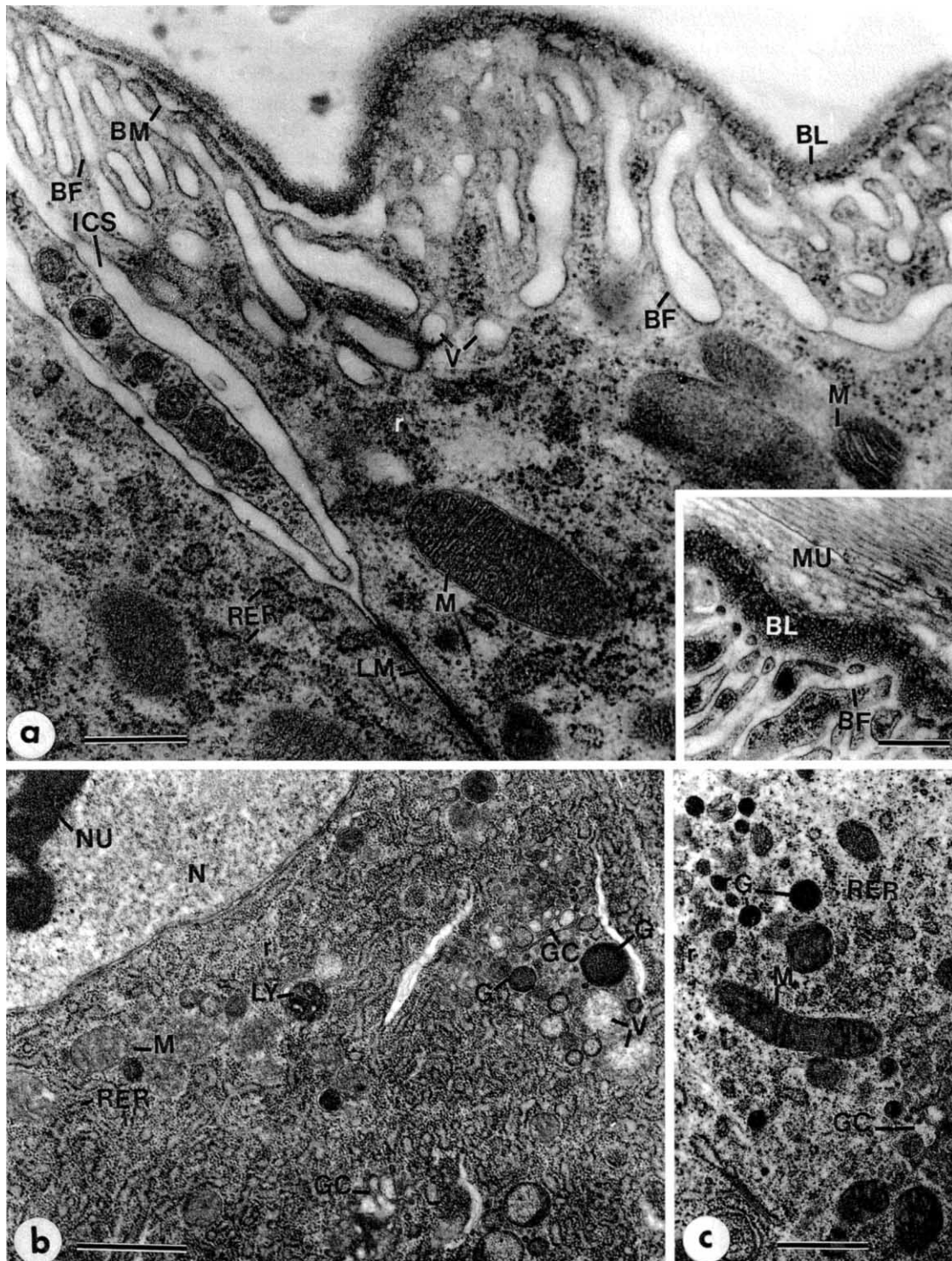


Fig. 2. Scanning electron micrographs of cardia, midgut and peritrophic membrane. (a) Intact gut of the full-grown larva of *Chironomus tentans* showing esophagus (ES), cardia (CA) and midgut (MG). Note three tiers of caeca of cardia (arrow heads) and a pair of muscles (MU) inserted at its anterior end. Scale bar, 0.2 mm. (b) External surface view of intact peritrophic membrane sheath. Note smoothness of the external surface. Scale bar, 0.1 mm. (c) Magnified image of a portion of external surface of peritrophic membrane. Note absence of holes. Scale bar, 0.03 mm. (d) View of the internal corrugated surface of peritrophic membrane sheath. Scale bar, 0.01 mm.



Figs. 3-7. Transmission electron micrographs of anterior cardia cells, peritrophic membrane and esophageal valve.

Fig. 3. (a) Basal region of a secretory anterior cardia cells displaying basal lamina (BL) covering basal plasma membrane (BM), short basal membrane infoldings (BF) and cytoplasm containing mitochondria (M), ribosomes (r) and rough endoplasmic reticulum (RER). ICS, intercellular space; LM, lateral membrane, V, Vesicles. Scale bar = 0.5 μ m. Inset shows visceral muscle (MU) external to the thick basal lamina (BL). BF, basal membrane infoldings. Scale bar, 0.5 μ m. (b) Nuclear region of a secretory anterior cardia cell showing a portion of nucleus (N) and nucleolus (NU) and perinuclear cytoplasm containing abundant rough endoplasmic reticulum (RER). G, secretory granules; GC, Golgi complexes; LY, lysosome; M, mitochondrion; V, vesicles. Scale bar, 1 μ m. (c) A portion of cytoplasm of a secretory cell containing secretory granules (G) Golgi complex (GC), mitochondria (M), ribosomes (r) and, rough endoplasmic reticulum (RER). Scale bar, 1 μ m.

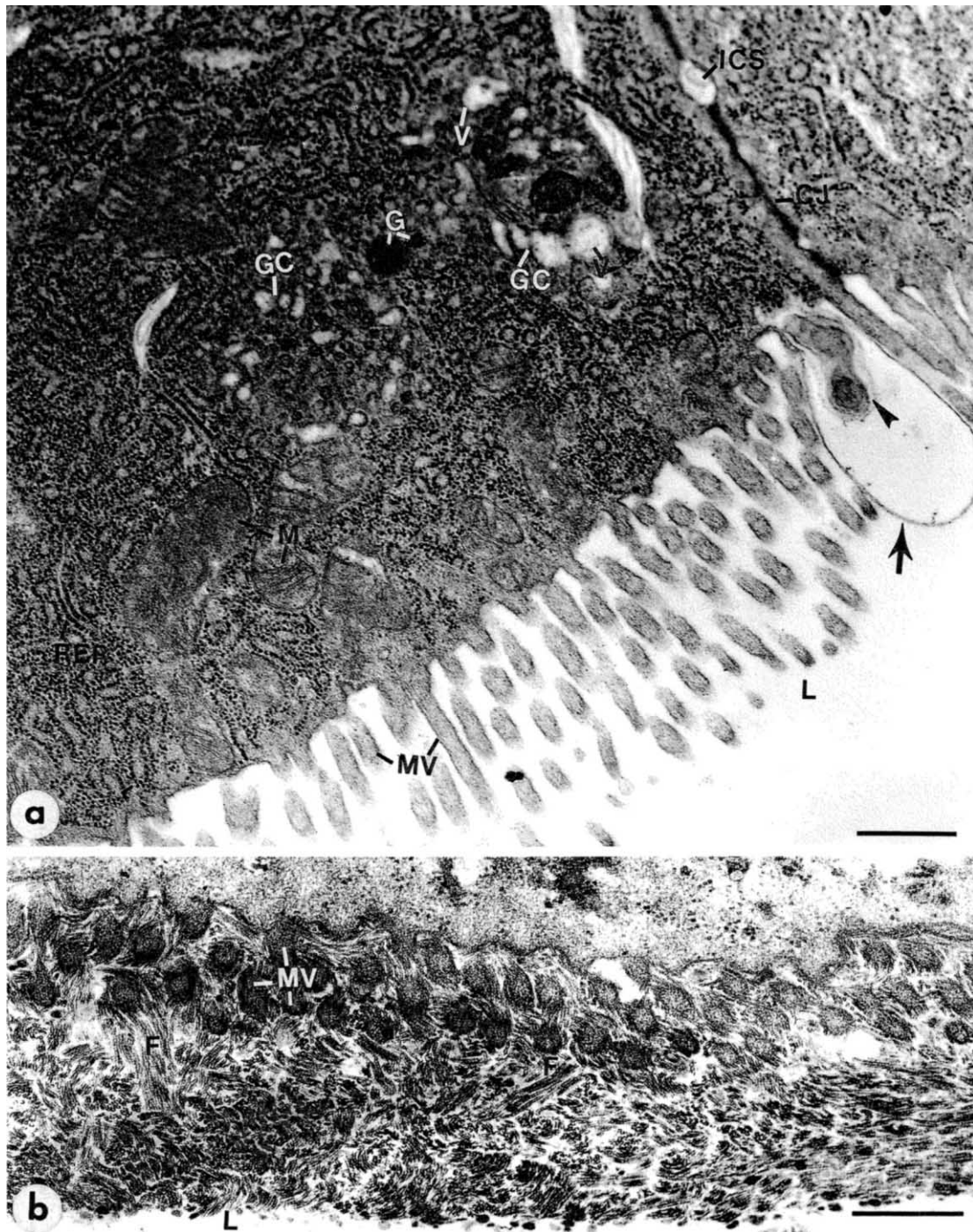


Fig. 4. (a) Apical region of a secretory anterior cardia cell showing secretory granules (G), Golgi complexes (GC), mitochondria (M), abundant rough endoplasmic reticulum (RER) and vesicles (V) in the cytoplasm and microvilli (MV) projecting into the lumen (L). Note apical plasma membrane (arrow) and cytoplasm (arrow head) being sluffed from the apical surface. CJ, intercellular junction; ICS, inter-cellular space. Scale bar, 0.5 μ m. (b) Luminal surface of a cell from specialized midgut epithelium which separates the anterior secretory cells of cardia from the midgut proper. Note fibrillar material (F) between the microvilli (MV) extending into the lumen (L). Scale bar, 0.5 μ m.

crine secretion to accumulate in the lumen, and then becomes organized into a single peritrophic membrane adjacent to the reflected intima of the esophageal valve (Figs. 5a, b, c) as it moves toward the midgut. The peritrophic membrane in the cardia has a total thickness of 255-488 nm, thus being of the

same thickness as that of *Chironomus strenzkei* (Platzer-Schultz and Welsch, 1969) but much thinner than the 1 μ m thick peritrophic membrane reported in *Aedes aegypti* larva (Richards and Richards, 1971). In the posterior cardia, the peritrophic membrane becomes thinner (255 nm) and is com-

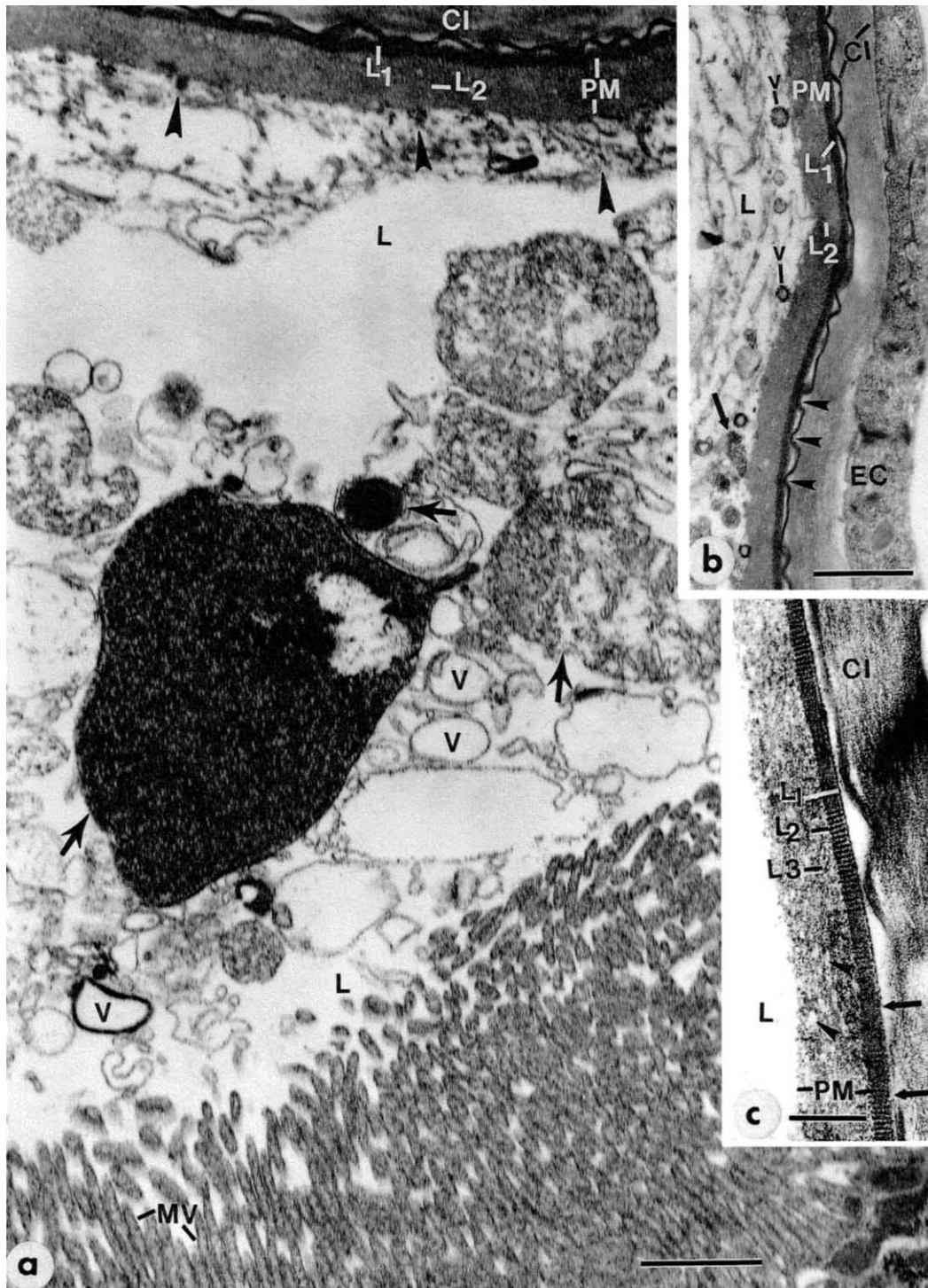


Fig. 5. (a) Luminal surface of a secretory anterior cardia cell displaying membrane-bound portions of cytoplasm (arrows), empty vacuoles (V) and vesicles (v) lying free in the cardia lumen (L) near the microvilli (MV). The peritrophic membrane (PM) is displaying two layers L₁, L₂. Note amorphous material (arrowheads) near L₂ layer. CI, Cuticular intima of the esophageal valve. Scale bar, 1 μ m. (b) Peritrophic membrane (PM) in the anterior cardia lumen (L) consisting of two layers (L₁, L₂) in contact with small epicuticular pleats (arrowheads) of reflected intima (CI) and thin epithelial cells (EC) of the esophageal valve. Note empty vesicles (v) and amorphous material (arrow) in contact with L₂ layer of the peritrophic membrane. Scale bar, 1 μ m. (c) Peritrophic membrane (PM) in close association with reflected cuticular intima (CI) near the lower end of the esophageal valve displaying three layers (L₁, L₂, L₃). Note vertical striations in the middle electron-dense layer (L₂), spaces (arrowheads) in the inner layer (L₃) and breaks in outer layer L₁ (arrows). L, lumen of posterior cardia. Scale bar, 0.25 μ m.

posed of three layers (Fig. 5c). These component layers have been named according to the nomenclature of Lehané (1976). The very thin outer layer 1 facing the lumen is 20 nm thick. Its outer edge is bounded by a very delicate electron-dense line which at places appears broken (Fig. 5c). In the anterior cardia layer 1 is indistinguishable from the adjoining layer 2. The electron-dense middle layer 2 is thicker (39 nm) than layer 1 and displays vertical striations (Fig. 6b). Similar striations are not apparent in this layer in the anterior cardia. The inner layer 3 facing the endoperitrophic space is 196 nm thick and contains loosely arranged amorphous material that surrounds small spaces of varying size (Fig. 5a, b).

In the midgut, the peritrophic membrane is composed of a single 104-110 nm thick layer which represents electron-dense layer 2 of the cardia peritrophic membrane. The unique electron-lucent vertical striations in it, enclose 6.5 nm diameter electron-dense channels which provide communication between the midgut lumen and the endoperitrophic space (Fig. 6a, b). The presence of such channels containing electron-dense material in the peritrophic membrane is a finding that to our knowledge, has not been reported before. The peritrophic membrane in the rectal lumen is composed of two

layers: The outer thin layer 1 which appears broken at places, and the inner electron-dense layer 2 displaying vertical striations. The endoperitrophic space here is filled with bacteria, some of them escaping into the rectal lumen through the open-ended and free-floating peritrophic membrane (Fig. 7a).

The flat epithelial cells of the esophageal valve are lined by a 0.7 μm thick cuticular intima, which consists of two layers: inner endocuticle (0.66 μm) separated from the apical membrane by subcuticle and electron-dense epicuticle (0.04 μm) which displays longitudinal folds and smaller pleats (Fig. 7b). The epithelial cells have a simple ultrastructure. The basal and the apical membranes are fairly straight and do not exhibit infoldings. The lateral membranes are folded and closely apposed. The cytoplasm contains mitochondria, free ribosomes, but rough endoplasmic reticulum and Golgi complexes are sparse (Fig. 7b).

DISCUSSION

This study has shown that the pear-shaped cardia in *Chironomus tentans* larva is distinctly different from the midgut, and it possesses three sets of short caeca. The esoph-

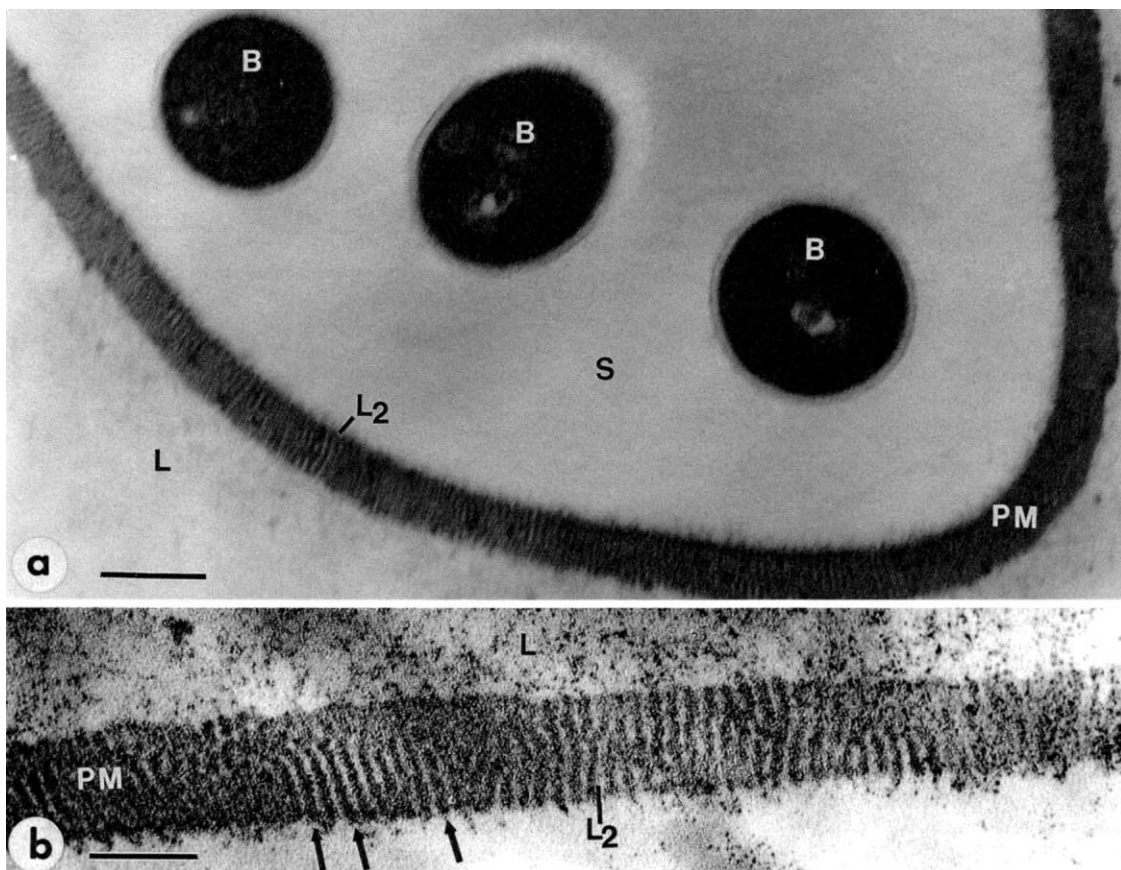


Fig. 6. (a) Peritrophic membrane (PM) in the midgut consisting of only one layer (L_2) which display vertical striations. Note three bacteria (B) in the endoperitrophic space (S). L, lumen of midgut. Scale bar, 0.2 μm . (b) A higher magnification field of peritrophic membrane (PM) in the midgut displaying electron-dense channels containing fine particles (arrows) between electron-lucent vertical striations in its single layer (L_2). The particulate material in the lumen (L) of midgut is food debris. Scale bar, 0.1 μm .

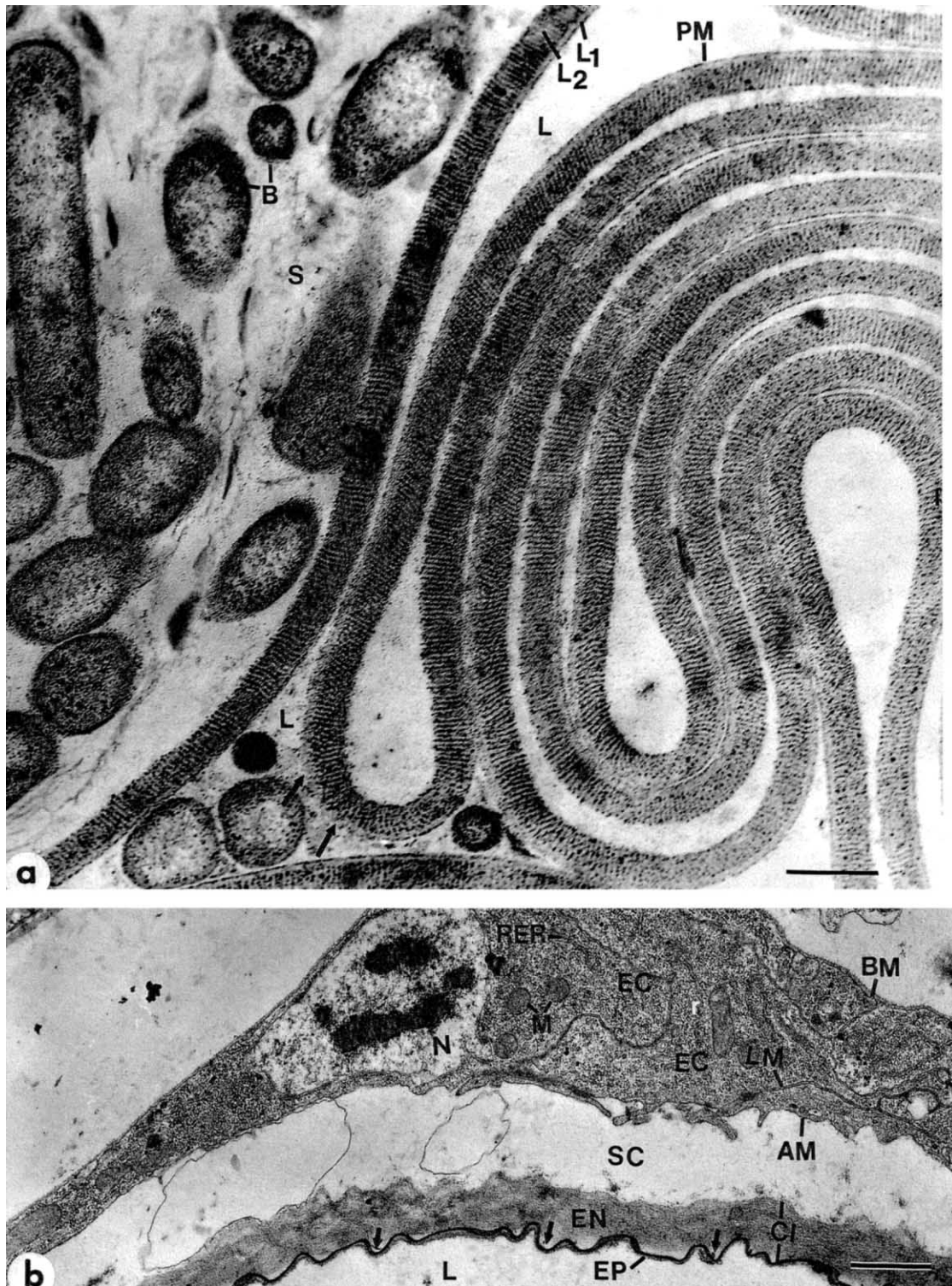


Fig. 7. (a) Free-floating, highly coiled peritrophic membrane (PM) consisting of two layers (L_1 , L_2) in the rectal lumen (L). Outer thin layer (L_1) displays breaks (arrows). Note bacteria (B) in the endoperitrophic space (S) some of which are also seen in the rectal lumen (L). Scale bar, 0.5 μm . (b) A section through the posterior end of esophageal valve showing its epithelial cells (EC) lined by cuticular intima (CI) composed of endocuticle (EN) and epicuticle (EP), the latter displaying folds with smaller pleats (arrows). AM, apical plasma membrane; BM, basal plasma membrane; L, lumen of cardia; LM, lateral cell membrane; M, mitochondria; N, nucleus; r, ribosomes; RER, rough endoplasmic reticulum; SC, subcuticle. Scale bar, 1 μm .

ageal valve which protrudes into the cardia as an inner tube has a highly folded epithelium and cuticular intima. This inner tube is reflected back upon itself; its anterior end joins the cardia.

The anterior epithelial cells of the cardia have been recognized for a long time to play a role in the formation of the peritrophic membrane in the larvae and adult Diptera (Miall and Hammond, 1900; Rizki, 1956; Wigglesworth, 1929, 1972). The existence of basal membrane infoldings that are not associated with mitochondria apparently provide increased surface area for the transport of substances from the hemolymph into epithelial cells where the substances are utilized in the synthesis of the materials which form the peritrophic membrane. The presence of extensive rough endoplasmic reticulum, Golgi complexes, abundant free ribosomes, vesicles and electron-dense secretory granules in the anterior epithelial cells of the cardia in *C. tentans* larva suggest that they are involved in the synthesis of proteins that are found in the peritrophic membrane. Such structural features are classically associated with cells that are engaged in secretory activity (Palade, 1975). Sluffing of the apical plasma membrane and cytoplasmic fragments and the presence of membrane-bound cytoplasmic pieces, organelles and amorphous material near the tips of the microvilli indicates that at least some of the secretory granules are released by apocrine secretion into the lumen of the cardia where they transform into an amorphous product. The secretory cells apparently remain healthy during this process. The presence of typical secretory granules suggests merocrine secretion may also contribute to the amorphous material. In *Chironomus thummi* the peritrophic membrane is also formed by apocrine secretion (Platzer-Schultz and Welsch, 1970). This secretory product apparently is forced through the narrow space between the apices of the anterior epithelial cells and the closely apposed ruffled cuticular intima (of the esophageal valve); in essence they may act as a press to mold the amorphous secretion into a smooth, thin peritrophic membrane. Even though the peritrophic membrane here does not display honey-comb pattern on its luminal aspect, its uniform structure supports the "annular press" mechanism of peritrophic membrane formation proposed by Wigglesworth (1929, 1972). A similar mechanism of peritrophic membrane formation has been observed in *Drosophila* (Dimitriadis, 1991; King, 1988; Rizki, 1956), *Calliphora* (Becker *et al.*, 1976; Smith, 1968), *Stomoxys* (Lehane, 1976) and *Lucilia* (Binnington, 1988). The likelihood of the esophageal valve epithelium secretes the peritrophic membrane-forming material is ruled out because of its simple ultrastructure; its cells have few rough endoplasmic reticulum, Golgi complexes, and they lack secretory granules. In sharp contrast, Smith (1968) and King (1988) describe the origin of part of the peritrophic membrane from the epithelial cells of the esophageal valve in the cardia.

Regarding the peritrophic membrane's extension into the alimentary canal, Rizki (1956) has suggested that the peristaltic movements of the gut tend to pull the peritrophic membrane downwards into the midgut and toward the rectum rather

than it being pushed by its continued secretion.

The peritrophic membrane in the posterior cardia displays three layers. In the rectum it is composed of two layers, but in the midgut only one layer (L_2) is discernable. The very thin layer 1 appears structurally similar to the outer epicuticle of the ion-transporting anal papillae of *C. tentans* larva (Jarial, 1995); its thickness is comparable to the similarly-placed layer in the larval peritrophic membrane of *Drosophila* (Dimitriadis, 1991). The electron-dense Layer 2 (middle layer) is the prominent component of the peritrophic membrane of *C. tentans* larva. It displays unique vertical striations which were described as rod-like structures in the analogous layer in the peritrophic membrane of *Chironomus thummi* (Platzer-Schultz and Welsch, 1970). In the midgut, the vertical striations enclose 6.5 nm wide channels that are open at both ends and contain particulate material. To our knowledge the presence of such channels in an insect peritrophic membrane has not been reported previously. These channels may allow the passage to water, salts, disaccharides, amino acids, polypeptides, digestive enzymes and digested food materials in both directions (Wigglesworth, 1929; Zhuzhikov, 1964). Since the inner layer 3 is absent in the midgut and rectal peritrophic membrane, it is suggested that the loosely arranged amorphous material in its matrix may coalesce and contribute to the formation of electron-dense Layer 2.

The peritrophic membrane, is composed of chitin and proteins (Chapman, 1985; Peters and Latka, 1986; Richards and Richards, 1977). This study suggests that the protein component is synthesized in the extensive rough endoplasmic reticulum and packaged in the Golgi complexes of the anterior epithelial cells and released into the cardia lumen. Smooth endoplasmic reticulum is usually not observed, but, it is assumed that these cells also produce PAS staining polysaccharides that contribute to its chitinous component. In a recent study Lehane *et al.* (1996) have shown histochemically that the peritrophic matrix in the tsetse fly, *Glossina*, contains chitin, glycoproteins and glycosaminoglycans. The sulfated sites in the glycosaminoglycans were shown to be 53 nm apart, a distance similar to the spacing between the charged sites in the basement membrane of renal glomerulus, suggesting to these authors a filtration role for these sites in the peritrophic membrane. The ultrastructure of the peritrophic membrane of *C. tentans* larva suggests that it plays a role in selective permeability, allowing small and medium size molecules to pass through it. In addition, it protects the epithelium of the cardia and the midgut from abrasion by rough and sharp food particles and bacteria by confining them to the endoperitrophic space.

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