FINE STRUCTURE OF THE MALPIGHIAN TUBULES OF MAYFLY NYMPHS, *BAETIS RHODANI* AND *ECDYONURUS VENOSUS* (EPHEMEROPTERA)

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ABSTRACT

The Malpighian tubules (MT) of *Baetis rhodani* and *Ecdyonurus venosus* were studied by light, scanning and transmission electron microscopy. Special attention was paid to the ultrastructural organization of the constitutive cells, which in the secretory regions typically have a microvillated luminal border. Cells rest on a basal lamina, show varying degrees of basal infoldings and a large number of mitochondria. Cells of the collecting ducts show vesicled cytoplasm and have a sponge-like appearance. In *B. rhodani* MT empty individually into the alimentary canal whereas in *E. venosus* they enter through intermediate trunks. Numerous MT proper are attached to each common trunk, mainly in its distal portion. In this species MT are more complex owing to regional specializations. The distal region coils to form an asymmetrical plate, to one side of which the narrow collecting duct adheres before emerging right in the middle of the opposite side. Each duct extends to a certain distance and ends in the trunk. Different cell types are present in the trunk: a ring of cells bordering the duct/trunk junction, cells with apical infoldings and a luminal cuticle intima, and cells whose long thin microvilli almost fill the cavity. The relationship between structural features and known physiological function are discussed.

INTRODUCTION

The comparative anatomy and arrangement of the Malpighian tubules (MT) of Ephemeroptera larvae have been the object of intense scrutiny by Landa (1969), Landa *et al.* (1980), Landa and Soldán (1985), on account of the phylogenetic relevance of these structures. According to these authors, in primitive Ephemeroptera the plesiomorphic arrangement of the MT results from a large number of tubules attached individually to the alimentary canal. The further acquisition is represented by the intermediate stage of buds and their ensuing elongation to form common trunks. Trunks are variable in number and shape, and show a general tendency to reduce the number of their pairs. Numerous interstages characterize mayfly MT system, as does the differentiation of the tubules from straight units to the acquisition of dis-

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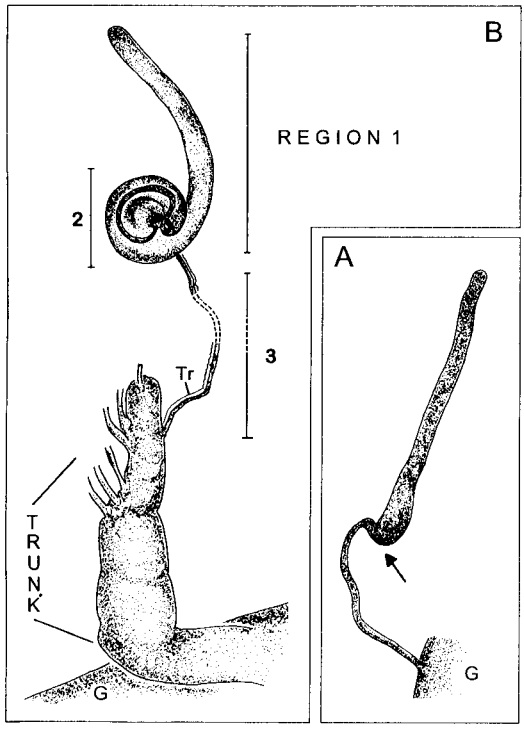


Fig. 1. Sketch of a distinct unit of the Malpighian tubules in *Baetis rhodani* (A) and *Ecdyonurus venosus* (B). In *B. rhodani* the distal portion curves (arrow) at the junction with the proximal portion. Each tubule enters the gut (G) individually. In *E. venosus* each unit achieves a more complex diversification and opens into the gut (G) through an intermediate trunk. Tr= tracheal supply.

coidal plates in the distal region. In short, two main features have to be considered in the anagenesis of the MT: the shape of each tubule and its emptying into the alimentary canal.

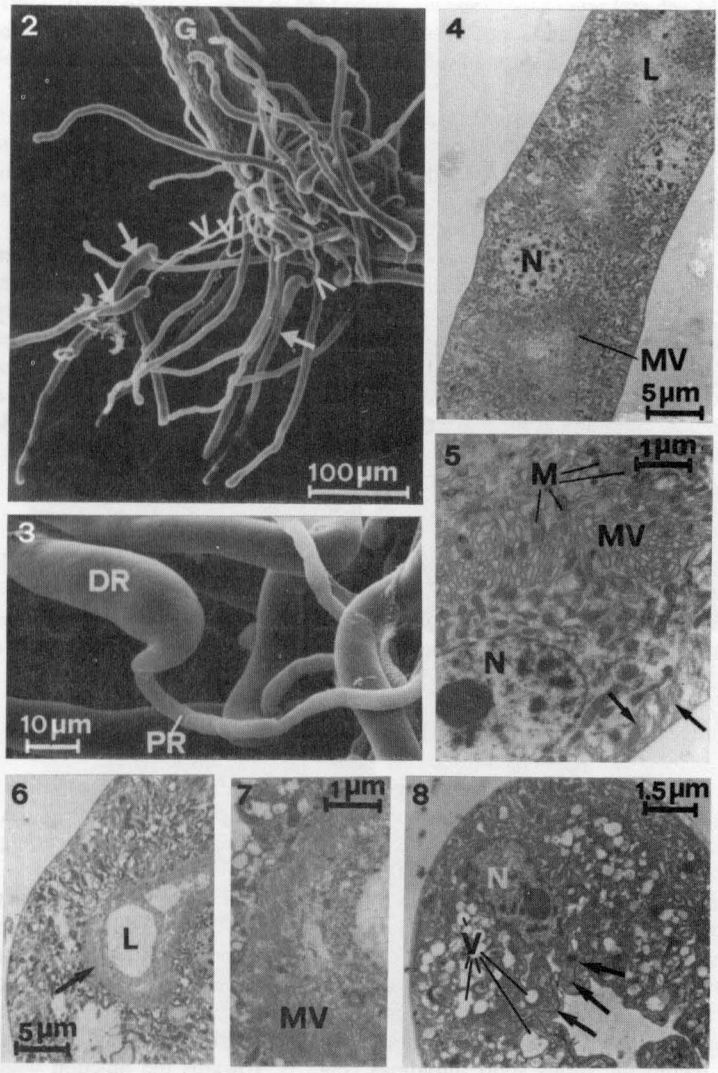
Some information on the morphological and histological organization of MT has been provided for several Italian mayfly species by Grandi (1950). The ultrastructural investigation of Nicholls (1983) on the heptageniid *Ecdyonurus dispar* showed a relationship between regional specialization to the function of cells in each tract.

The present paper describes the ultrastructural organization of Malpighian tubules in the nymphs of *Baetis rhodani* and *Ecdyonurus venosus*.

MATERIAL AND METHODS

Mature nymphs of *Baetis rhodani* and *Ecdyonurus venosus* were collected from the Lemme stream in Alessandria, Piedmont, Italy. The gut and Malpighian tubules were dissected out under a stereo-microscope. The arrangement of the MT was observed by light and scanning electron microscopy (SEM).

Dissected material was fixed in Karnovsky's medium (1965) using cacodylate buffer, pH 7.2, for 1 hour, rinsed several times in the same buffer and postfixed in 1% osmium tetroxide for 1 hour.



Figs. 2-8. Malpighian tubules (MT) of the nymph of *Baetis rhodani* under SEM (2,3) and TEM (4-8). 2- General view of the MT showing their arrangement at their junction with the gut (G). Note the broad (arrows) and the thin (open arrowheads) portions; 3- Detail of a single MT showing the junction between the broad distal region (DR) and the thin proximal one (PR); 4- Closely aligned cells making up the distal region of the MT. L= lumen. MV= microvilli. N= nucleus; 5- Detail of a cell with infoldings (arrows) and microvilli (MV) that include mitochondria (M). N= nucleus; 6- Cross-section of the tubule in the curved region close to the junction with the thin collecting duct. Note the lumen (L) with the microvillated border (arrow); 7- Zoomed view of the microvillated luminal border. Note the absence of mitochondria inside microvilli (MV); 8- Cross-section of the thin collecting duct with cells full of vesicles (V). N= nucleus. Note the interdigitations of the cells joined by extensive junctions (arrows).

For SEM, the specimens were dehydrated in a graded ethanol series, critical-point dried, mounted on stubs with silver conducting paint and sputter-coated with gold-palladium in a Balzers Union Evaporator. Specimens were observed with a Philips EM 515 scanning electron microscope.

For TEM, ethanol processed tissue was embedded in Epon-Araldite mixture resin. Ultrathin sections, obtained with a Reichert ultramicrotome, were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate. Sections were examined under a Philips EM 400 electron microscope.

Immuno-labelling techniques were used to visualize both cytoskeletal actin and nuclei. F-actin was detected by a phalloidin-staining procedure. This consisted of treatment for ten minutes in a mixture of fixative (Triton 0.1%), after fixation of the tissue in a 4% solution of paraformaldehyde for 30 minutes. Afterwards, the material was washed in PBS buffer and stained with phalloidin-rhodamine in a dark oven at 37 °C for 30 minutes.

The fluorescence of the nuclei was detected with 2.8 mM 4', 6-diamino-2-phenylindole 2HCl (DAPI, Sigma), a DNA specific fluorochrome, dissolved in Tris buffer (stock solution) to a final saturating concentration. After a final wash in PBS, differently treated MT were mounted on slides in 60-70% glycerol in PBS. Various controls were performed. Stained slides were examined under a Leika microscope, equipped for epifluorescence in the rhodamine mode. Photographs were taken with a Kodak Ektacrome 400 ASA colour slide film. The terminology in the paper follows that used by Landa and Soldán (1985).

RESULTS

Whatever the organization, the Malpighian tubule system extends out of the alimentary canal at the transition from the midgut to the hindgut. Each tubule floats in the hemolymph and is invested with tracheae. Two main models are considered (see the sketch of figure 1): (a) tubules attached individually to the alimentary canal (Fig. 1A) and (b) tubules with discoidal plate and common trunk (Fig. 1B).

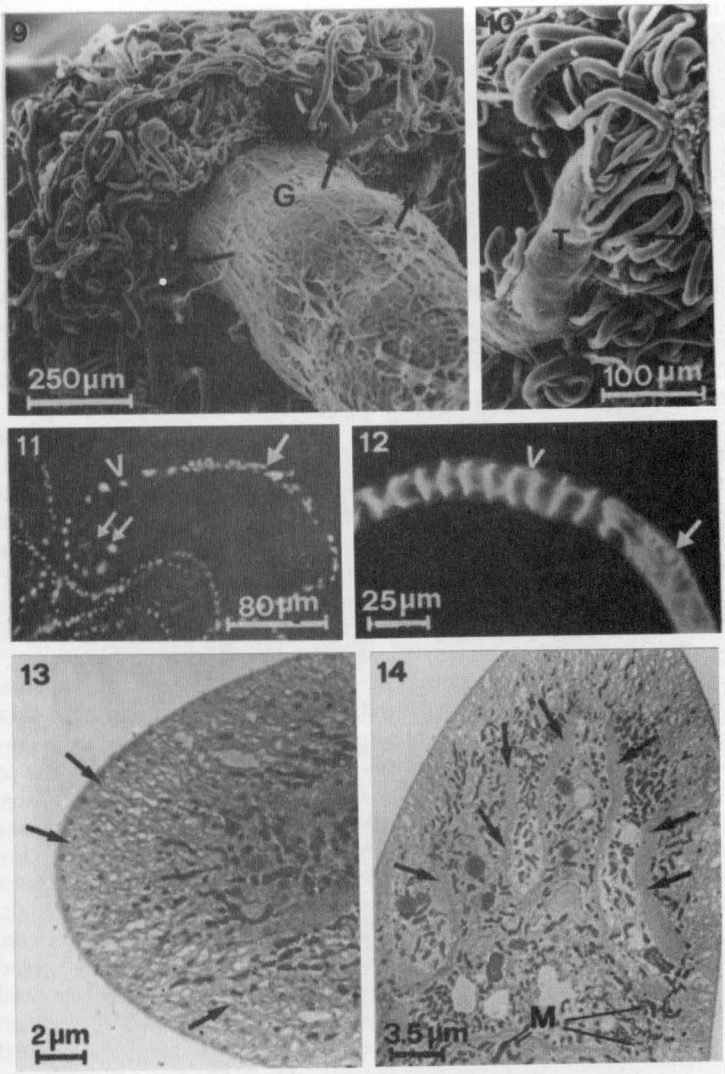
Tubules Attached Individually to the Alimentary Canal

In *Baetis rhodani*, tubules are arranged around the alimentary canal (Fig. 2). Each tubule consists of two regions: a distal portion and a thin proximal one (Fig. 1A). At the junction between the two, the distal portion enlarges and curves (Fig. 3) leading into the proximal portion, or thin collecting duct (Fig. 3).

Ultrathin sections of the distal part show that the epithelial wall is made up of microvillated cells on a thin basal lamina facing an eccentric lumen (Fig. 4). The polytene nucleus occupies the expanded part of the cell body. Malpighian cells have a fusiform shape, each of them extends between the neighbouring ones (Fig. 4). The cell apex protrudes towards the lumen and has numerous microvilli filled with long, thin mitochondria (Fig. 5). The region adjacent to the microvilli also includes mitochondria of varying shape and length. At the basal edge of these cells the plasma membrane forms short infoldings (Fig. 5). Towards the junction with the thin collecting duct, the lumen becomes larger (Fig. 6) and the apical microvillated border is devoid of mitochondria (Fig. 7). The thin collecting duct results from cells delimiting a large lumen (Fig. 8). These cells lie on a basal lamina, have few mitochondria and the cytoplasm is filled with vesicles varying in diameters and containing flocculent material. The adjacent cell membranes are greatly interdigitated and joined by extensive junctions (Fig. 8). Unlike the distal portion of the tubule, the cells lack both microvilli and plasma membrane infoldings (Fig. 8).

Tubules with Discoidal Plate and Common Trunk

Ecdyonurus venosus can represent a typical model where trunks are located around the alimentary canal (Fig. 9) and may branch at their bases. Numerous MT enter each trunk, mainly in its distal portion (Fig. 10).



Figs. 9-14. Malpighian tubules (MT) of the nymph of *Ecdyonurus venosus* under SEM (9,10), light microscopy (11-12) and TEM (13-14). 9- General view of the arrangement of MT showing their junction to the gut (G) by the intermediate trunks (arrows); 10- Detail showing the MT (arrows) at their entry to the trunk (T) in its distal portion; 11-MT whole-mount labelled with DAPI staining. The sequence of large polytene nuclei along the length of the MT reveals a distal tract with packed cells (arrow) and a proximal one with extended cells (open arrowhead). Note the coiled region (double arrow); 12-MT whole-mount with F-actin labelling. Note the marked difference between the distal tract (arrow) and the proximal one with actin bundles arranged perpendicularly to the longitudinal MT axis (open arrowhead); 13- Distal blind portion of MT showing the deep infoldings (arrows). 14- Distal blind portion of the tubule showing cellular borders with short microvilli (arrows) that almost completely occlude the lumen. M= mitochondria.

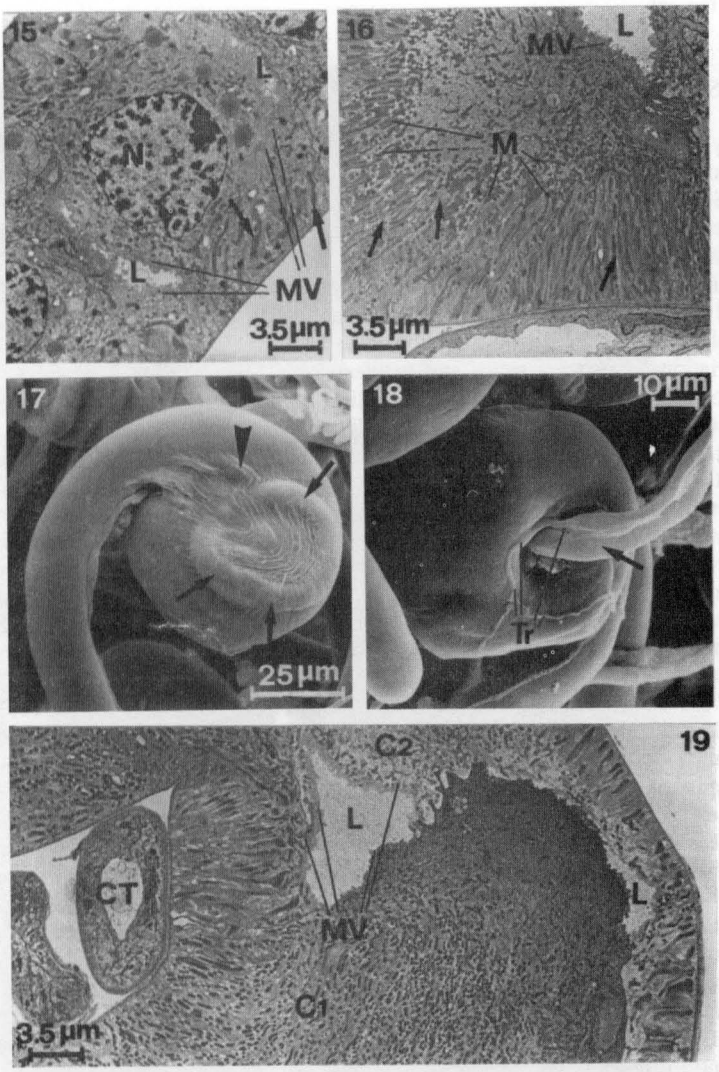
Figure 1B shows several histological distinct regions. The primary most distal region (region 1) leads to a next coiled region (region 2), which acquires the shape of a discoidal plate and leads to a thin collecting duct (region 3). Each duct connects a single plate to a common trunk.

The arrangement and number of the cells in a Malpighian tubule is obtained by DAPI technique (see material and method section), which reveals a sequence of large polytene nuclei along the length of a Malpighian tubule (Fig. 11). This technique coupled with the rodamine-labelled phalloidin staining method and detergent extraction (see material and method section), shows that the primary tubule (region 1) includes two tracts of about equal length (Fig. 12). The blind distal end terminates in a solid rod of tightly packed cells that, after staining for cytoskeletal filamentous actin with rhodamine labelled phalloidine, show a diffuse fluorescence (Fig. 12). This tract leads abruptly into the next tract, whose cells are more elongated and show periphery actin bundles. These bundles are oriented perpendicularly along the axis of the MT (Fig. 12). TEM observations confirm a distinct organization of the two tracts. In the blind distal end, cells rest on a thin basal lamina and show some elaboration of the infoldings (Figs. 13,14). Luminal cells have a border of short microvilli that almost completely fill the lumen (Fig. 14). The majority of mitochondria tend to gather along the luminal side of the cells (Fig. 14).

At a point where the distal end meets the next tract, the lumen is not occluded and mitochondria are sparsely distributed in the infoldings (Fig. 15). Nuclei are located near the apical border of the cells. Moving towards the coiled region, basal infoldings are more extended (Fig. 16). They are regularly spaced and oriented at right angles to the basal lamina. Mitochondria accumulate along the deep infoldings (Fig. 16). The lumen widens and the cell apex of the bordering cells shows an extensive microvillar border of uniform length (Fig. 16).

The coiled region (region 2) is the most complex part and gives rise to a discoidal plate (Figs. 17,18). The discoidal plate has two different sides, one showing a thin raised C-shaped tubular structure (Fig. 17) and the other containing a single emerging thin duct (Fig. 18). The latter is usually associated with a trachea (Fig. 18). Such an organization is the result of an abrupt narrowing of the primary tubule at the end of the coil from which the thin collecting duct departs (region 3). The initial part of the collecting duct adheres by a fibrous sheet to one side of the discoidal plate, and assumes a raised C-shaped configuration (Fig. 17). The collecting duct is kept adherent to the plate by a fibrous sheet (Fig. 17). This duct then emerges in the middle from the opposite side of the discoidal plate (Fig. 18) and, after extending to a certain distance, empties into the common trunk. This arrangement of the duct results in an asymmetry on the sides of the plate. The discoidal plate (about 50 μm in diameter) contains several cavities delimited by large cells (about 5 cells). Ultrastructurally, two cell types are recognizable. The cells located on the upper side of the plate just below the C-shaped collecting duct differ from the remaining ones in many respect. The cells in the upper side have remarkably electron-dense cytoplasm filled with mitochondria that penetrate into the infoldings of the basal plasma membrane (Fig. 19). The remaining cells at the lower end have electron-translucent cytoplasm and a few mitochondria. The apical border of both cell types shows facing microvilli that delimit cavity lumina (Fig. 19). The lumen of the collecting duct (region 3) tends to enlarge towards the trunk and is bordered by cells whose cytoplasm accumulates a large number of vesicles in such a way that it acquires a sponge-like appearance (Fig. 20).

Semithin sections show the junction between trunks and alimentary canal at the transition from the midgut to the hindgut (Fig. 21). Three different cell types are present in the common trunk. In the most distal part, the junction with the collecting duct is bordered by a ring of cells (Fig. 22). The rest of the trunk is constituted of two very distinct cell types delimiting an internal cavity. Type-1 cells are elongated and show deep infoldings along their basal region. They exhibit a more electron-dense cytoplasm around the nucleus whereas the apical region bordering the cavity greatly enlarges to form a lamina from which long microvilli originate (Fig. 23). Microvilli are numerous and form a network in the lumen (Figs. 23,24). Type-2 cells are larger than the previous ones and have apical plasma membrane folds and are bordered by a cuticle intima (Figs. 23,24). The microvilli of the type-1 cells



Figs. 15-19. Malpighian tubules (MT) of the nymph of *Ecdyonurus venosus* under TEM (15,16,19) and SEM (17,18). 15- Longitudinal section of the MT at the transition between region 1 and 2. Note the lumen (L) between microvillated cells (MV) and the mitochondria in the infoldings (arrows). N= nucleus; 16- Cross-section of the tubule close to the coiled region whose cells have very extended basal infoldings (arrows) rich in mitochondria (M). L= lumen bordered by microvilli (MV); 17- Side of the discoidal plate showing the thin raised C-shaped arrangement of the thin collecting duct (arrows). The fibrous sheet (arrowhead) covers the duct; 18- Side of the discoidal plate showing the centrally emerging duct (arrow). Note the associated trachea (Tr); 19- Cross-section of the coiled region showing electron-dense (C1) and electron-translucent (C2) cells whose facing microvilli (MV) delimit cavity lumina (L). CT= section of the thin collecting duct.

may reach the cuticular border of the type-2 cells (Fig. 24). The distribution of the type-1 and type-2 cells may vary along the length of the trunk.

Phalloidin-labelling shows spiral muscle arising from and continuous with the muscle layer of the gut.

DISCUSSION

Physiology and ultrastructure of the Malpighian tubules have been investigated in a wide range of insect species (Wigglesworth and Salpeter, 1962; Berridge and Oschman, 1969; Sohal, 1974; Green, 1979, 1980; Ryerse, 1979; Meyran, 1982; Alkassis and Schoeller-Raccaud, 1984; Satmary and Bradley, 1984; Bradley, 1985; Hazelton *et al.*, 1988; Kukel and Komnick, 1989; Dallai *et al.*, 1991; Kapoor, 1994).

Malpighian tubules are regarded as typical transporting epithelia. They are involved in secretion and reabsorption processes which in some instances occur along successive segments (Maddrell, 1977) and lead to the formation of the primary urine.

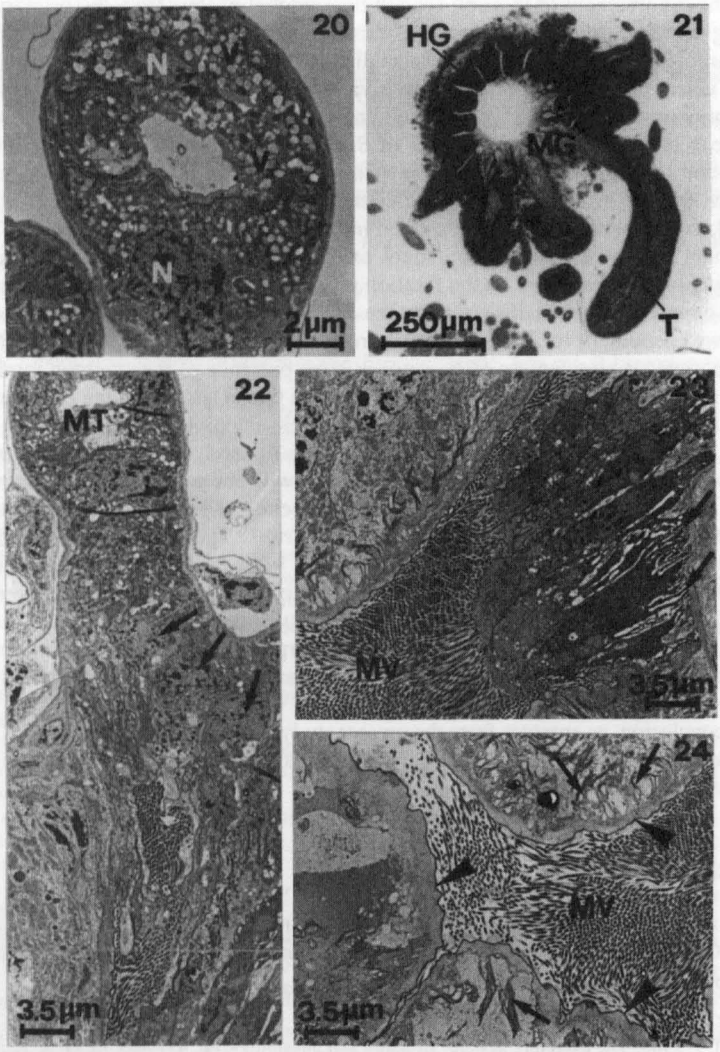
Variations between species and between segments have emerged from comparative studies of cell structure, organizational details, membrane specialization and mitochondrial populations of the constitutive elements (Wigglesworth and Salpeter, 1962; Eichelberg and Wessing, 1975; Wall *et al.*, 1975). In addition, the ultrastructural features may yield information on the activity of the various segments. For instance, the preferential location of mitochondria at the apical or basal cellular pole seems to express a different function of the cells along the tubules (Meyran, 1982). In this regard, mitochondria recruitment from cell cytoplasm and insertion into the microvilli has been experimentally obtained by stimulating a rapid fluid transport (Bradley and Satir, 1977). Likewise, the extension of basal infoldings expresses the cell's ability to create concentration gradients (Berridge and Oschman, 1969; Oschman and Berridge, 1971), and the number of these invaginations seems to be correlated to the activity of the tubule.

MT cell membranes are involved in ion transport (see review in Nicolson, 1993); microvilli and basal infolds are the localization of the ion pump on which fluid secretion depends (Maddrell, 1977; Pannabecker *et al.*, 1992).

In spite of the different physiological activity performed by MT in the various insect species examined, there is a surprising morphological similarity in the primary cells: extensive membrane infoldings along their basal region, microvillar apical border and a remarkable number of mitochondria. The present investigation visualise that in their basic morphology the cells of the distal part of the tubules of *B. rhodani* and *E. venosus* conform to this general description. Other well-known features in insects, such as polyploidization and regional differentiation, were highlighted in *E.venosus* by MT whole-mounts utilising DAPI and F-actin labelling techniques respectively. Cytoskeletal actin patterns provided a clear insight into the differentiation of the successive segments in region 1 of *E. venosus* MT, thus confirming the close relationship between actin filament arrangement and cell shape (Kukel and Komnick, 1989; Meulemans and De Loof, 1990,1992).

Extreme regional specializations have been observed in *Ecdyonurus dispar* by Nicholls (1983), who suggested that in the distal part of the MT the fluid flow takes place via the paracellular route at the interspace between lateral cells membranes. Our TEM images of *E. venosus* do not show similar specialization, thus highlighting the controversy regarding fluid movement via this route (Diamond, 1979; Møllgard and Rostegaard, 1981).

Like *E. dispar*, the MT of *E. venosus* are divided into distinct regions and, in comparison with those of *B. rhodani*, they have a more complex organization, as proved by the ultrastructural investigation. Even though in both species the general morphological features of the cells in the solid terminal segment of the MT differ from those of the luminated lower segment, there is no doubt that the model of *B. rhodani* is far simpler. Indeed, in this latter species, the distal portion of each MT curves at its junction with the proximal duct, without however reaching the regional specialization of the discoidal plate of



Figs. 20-24. Malpighian tubules (MT) of the nymph of *Ecdyonurus venosus* under TEM (Figs. 20, 22-24) and light microscope (Fig. 21). 20- Cross-section of a collecting duct bordered by cells full of vesicles (V). N= nucleus; 21- Semi-thin section showing the transition from the midgut (MG) to the hindgut (HG). T= trunk; 22- MT proper at its junction with a trunk. Note a ring of cells (arrows); 23- Elongated type-1 cells of the trunk showing deep infoldings along the basal region (arrows). Note the electron-dense cytoplasm around the nucleus and their long microvilli (MV); 24- Type-2 cells with folded apical plasma membrane (arrows) and cuticle intima (arrowheads). MV= microvilli of type-1 cells.

E. venosus. In the discoidal plate, two different cell types are easily recognizable on the basis of their morphology. The reason for such a difference is so far obscure, as is the occurrence of the thin collecting duct adherent to one side of the plate, and must await a more detailed physiological study. We can speculate that the dominant feature consisting of electron-dense cytoplasm, deep basal membrane infoldings and associated mitochondria may reflect the involvement of this cell type in the removal of ions from the primary urine, thus recalling special structures supporting this function in other aquatic insects (Wall and Oschman, 1975). Indeed, regional specializations of the tubules have been observed in various insect groups and correlated to a variety of reabsorptive processes able to modify the primary urine (Irvine, 1969; Maddrell and Phillips, 1975; Wall *et al.*, 1975).

The thin collecting duct of the MT of *B. rhodani* and *E. venosus* appears similar in both species and its organization is coherent with the function of a simple conducting device. However, the constitutive cells, characterized by a rich amount of vesicles with flocculent material, recall mucosubstances described in the cells of MT collecting ducts of a dragonfly (Kukel and Komnick, 1989). We cannot exclude that these vesicles might have included some components dissolved by the fixation procedure. Indeed, it is rather surprising that the mineral concretions normally found in the MT of insects were not evidenced inside cells, nor did we encounter profiles indicating extrusion of such concretions into the lumen.

The most striking feature of *E. venosus* is the presence of the common trunk into which collecting ducts open separately. The trunk is more than a simple bridge between MT proper and the hindgut, as it consists of different cell types, as pointed out in *E. dispar* by Nicholls (1983). In particular, the cells with long microvilli and deep basal infoldings may be extensively modified midgut elements. The cells with the apical cuticular intima show the general profile of the hindgut cells. As a consequence, it seems acceptable that cells from different stem lines cooperate in building up the trunk. The function of these different cell types is obscure. We can speculate that mayflies need to eliminate excess fluid from the hemolymph owing to a continuous dilution in the fresh-water environment. These microvillated cells may compensate for loss of ions as is presumed to occur in the discoidal plate. An active ion uptake together with a reabsorption of substances by the cells bordered by the cuticle may allow the insect to adjust its hemolymph content, thereby recalling the hindgut-cell activity.

In conclusion, as emerges from the present study, the MT of *B. rhodani* and *E. venosus* express two different levels of complexity and may be extreme models among numerous interstages of increasing diversification.

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