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S. P. NICHOLLS*

ULTRASTRUCTURAL EVIDENCE FOR PARACELLULAR FLUID FLOW IN THE MALPIGHIAN TUBULES OF A LARVAL MAYFLY

Key words: Malpighian tubules, ultrastructure, paracellular fluid flow.

ABSTRACT. The ultrastructure of the Malpighian tubules of larvae of the Mayfly *Ecdyonurus dispar* (Ephemeroptera) is described. There are about 60 tubules, which consist of four distinct regions. The most proximal section (region I) appears to be responsible for fluid secretion. A unique feature is the presence of channels leading off the main lumen, which end close to the basal border of the cells. Microvilli are confined to these channels in region I. Region II is a short spiral region, the cells of which possess long basal folds and associated mitochondria. Region III is a simple conducting tube leading to one of six collecting ducts (region IV) arranged radially around the gut. In each collecting duct there are two cell types present. Type 2 cells are relatively simple, but give rise to numerous, long, microvilli-like projections. Type 1 cells possess long basal folds, and curious membrane whorls in the apical zone. Evidence is presented which suggest that water movements into region I takes place via the paracellular route. Region II is probably a reabsorptive region, but the function of region IV, based on ultrastructural evidence is more difficult to elucidate.

Introduction

The mechanism whereby cells can generate water movements against an apparent gradient has long been of interest. Most of the suggested mechanisms are based on the creation of some form of osmotic gradient which then serves to drive water flow. Bresler (1978) has implicated organic molecules in these osmotic gradients and Riegel (1970) has described a system of fluid flow based on the secretion of 'formed bodies'. However, the majority of mechanisms suggested are based on gradients created by active ion transport (reviewed by Hill, 1977). Whilst the location and direction of ion pumping can often be determined by either histochemical or electro-physiological techniques (e.g. Maddrell, 1977; Komnick, 1978), the means by which fluid flow is coupled to such movements are not so easy to determine, especially in tissues which can transport iso-osmotic and hypo-osmotic fluids. A number of models to explain this coupling have gained and lost favour over the past two decades. Diamond (1962) and Diamond and Bossert (1967) have developed a theory of standing gradient osmotic flow to explain fluid movements across vertebrate gall bladder. This model has subsequently been applied to a variety of other tissues. Similarly, Curran (1960) has suggested coupling across rat intestine via a three-compartment, two-membrane system, and Hill (1975, 1977) has examined the possibility of electro-osmotic coupling. However, it is often difficult to reconcile these models with the ultrastructural features of the tissues involved. This is especially true of Malpighian tubules in general, where the various channels or compartments required by these models are either too small or absent. The present work describes some unique structural features of the Malpighian tubules of the larvae of the mayfly

Department of Zoology, University of Bristol.
* Present address: H. H. Wills Physics Laboratory, University of Bristol.

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Ecdyonurus dispar, which suggest that fluid flow in this tissue may take place along the spaces between the lateral cell membranes (the paracellular route) in response to localized osmotic gradients developed in the lumen.

Methods

Larvae were collected from a small stream in the Cotswolds, and identified to species using Macan (1979).

For light microscopy, whole larvae were fixed in Bouin's fixative, dehydrated, and embedded in paraffin wax (m.p. 56°C). Rapid penetration of the fixative was ensured by tearing a slit in the dorsal cuticle of the animal. 10 μm sections were cut, and examined after staining in Mallory's triple stain (Pantin, 1969).

For electron microscopy, the Malpighian tubules and associated structures were dissected free under a saline solution based on the haemolymph composition, and fixed in 5% glutaraldehyde in 0·05 M phosphate buffer at 20°C for 1–2 hr. The osmotic pressure of the fixative was adjusted to that of the animals' haemolymph by adding sucrose to a final concentration of 3% by weight. After thorough washing in phosphate buffer, the tissue was post-fixed for 1 hr in phosphate-buffered 1% OsO_4 solution. The tissue was then given a pre-stain rinse in veronal·acetate/HCl and stained *en bloc* for 12 hr in buffered uranyl acetate (Gibbons and Grimstone, 1960). Dehydration was carried out through graded alcohols and epoxypropane, and the tissue was embedded in Araldite. Thin sections were cut on a Porter-Blum ultramicrotome, and stained with lead citrate before examination using a Philips 300 electron microscope.

Results

The gross morphology of the Malpighian tubule system is shown in Fig. 1. Leading from the alimentary canal are six collecting ducts, each of which gives rise to five to ten Malpighian tubules proper. Each tubule consists of three histologically distinct zones, designated regions I–III. The primary tubule (region I) is the most distal zone. This leads into a short spiral region (II) illustrated in Fig. 2. A narrow tubule (region III) connects the spiral section to the collecting duct, the latter being designated region IV. The junction of regions III and IV is illustrated in Fig. 3. Figs. 4 and 5 show the light microscope appearance of the distal and proximal zones of region IV.

The primary tubule (region I) consists of one cell type, about 15 μm high, surrounding a main lumen which is 10–15 μm in diameter (Fig. 6). At the basal edge of these cells the plasma membrane is thrown into a series of folds between 1 and 3 μm long. The cells rest on a thin (70 nm) basement membrane. Mitochondria are numerous and distributed throughout the cell. A unique feature of these tubules is the existence of numerous evaginations leading off from the main lumen (Fig. 6), and which approach to within 1–2·5 μm of the basal membrane (Figs. 7, 8). The lateral cell interspaces invariably open into these evaginations. Apart from a narrow septate junctional zone just prior to this opening, the lateral cell membranes are relatively unspecialized (Fig. 8). Grazing longitudinal sections reveal that the luminal evaginations are much branched (Fig. 7), and are present throughout the primary tubule. Microvilli are confined to the evaginations.

After the junction with the spiral zone (region II), these luminal evaginations are absent, and short microvilli are present around the main lumen (Fig. 10). The lumen may branch in this region, but the tight spiral configuration of this section makes this feature difficult to ascertain from thin sections. The cells of the spiral region have long, tightly packed basal folds which extend almost to the apical border of the cells, and are completely unlike the basal folds of region I (Fig. 9). Elongate mitochondria occur between these folds, but lack mitochondrial–scalariform junctions described from insect recta, etc.

Region II passes abruptly into region III, which appears to be a simple conducting tube (Fig. 11). One cell type is present, which exhibits unspecialized apical and basal plasma membranes. However, the cells are linked by extensive septate junctions on their lateral membranes.

Some five to ten such tubules are joined to each of the six collecting ducts (region IV). The latter is a complex region, with two cell types present (Fig. 12). In the more distal

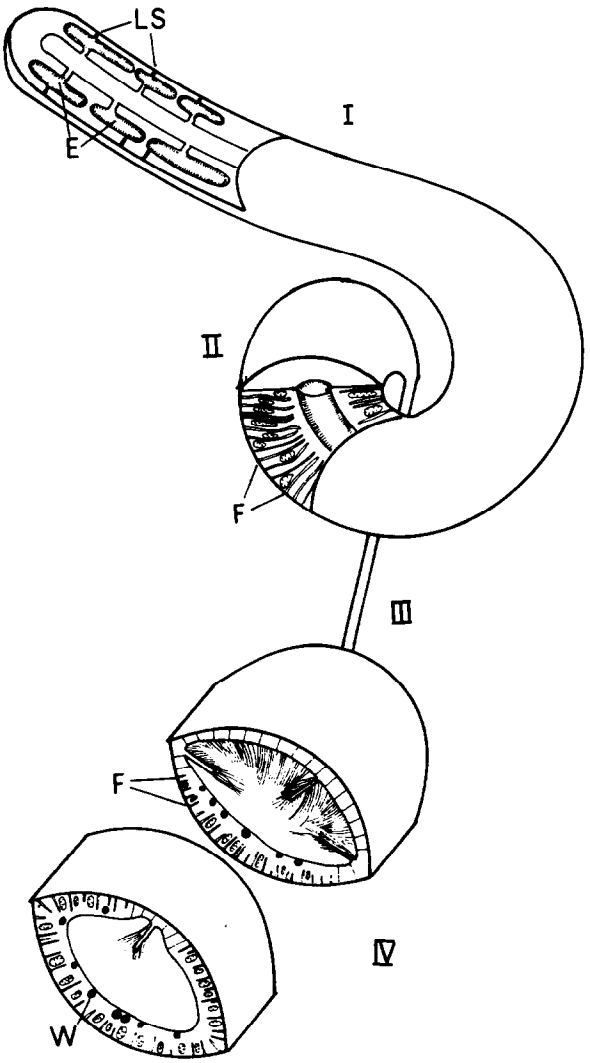
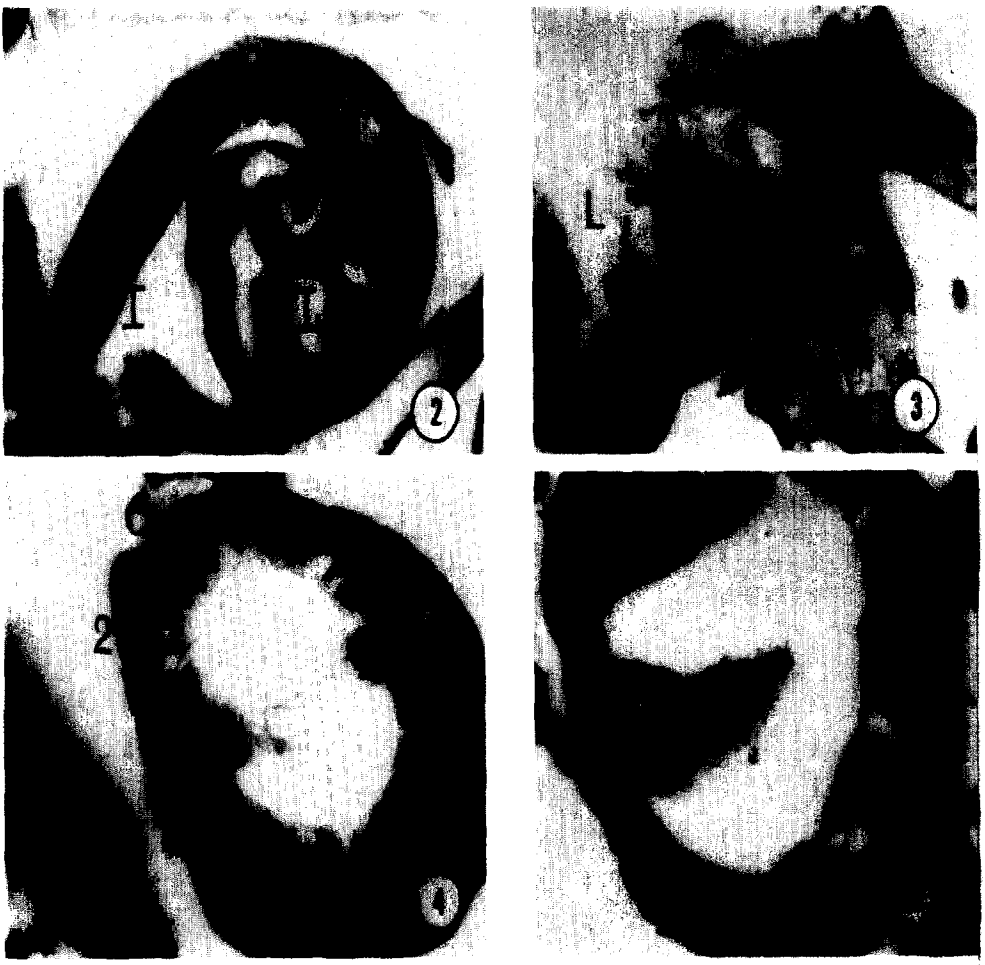


Fig. 1. Summary of the main anatomical and ultrastructural features of the Malpighian tubules of *Ecdyonurus dispar*. The lumen of region I possesses a series of evaginations (E), which end close to the haemolymph side of the cells. Microvilli are confined to these evaginations, into which the lateral cell interspaces (L.S.) also open. Region II is a short spiral region, in which the cells have extensively developed basal membrane folds (F) and associated mitochondria. The lumen is lined with short microvilli. Region III is a simple conducting tube, which leads into one of six collecting ducts (region IV). In this latter region, two cell types are present. Type 1 cells possess long basal folds (F) and membrane whorls (W) in the apical region. Type 2 cells give rise to numerous, long, microvilli-like projections. The distribution of these two cell types varies along the length of each collecting duct.

zones (Fig. 4) each cell type occupies about half of the circumference of the collecting duct. More proximally, one cell type becomes confined to a narrow zone (Fig. 5). Ultrastructurally, these two cell types are very distinct (Fig. 12). Type 1 cells are usually larger than type 2 cells. The basal membrane of type 1 cells is thrown into a



Figs. 2–5. Light micrographs, showing regions I–IV.

Fig. 2. Junction of regions I and II. ×400.

Fig. 3. Junction of regions III and IV. Several tubes of region III can be seen emptying into the lumen (L) of the collecting duct (region IV). ×600.

Fig. 4. Distal section of region IV, in which type 1 (1) and type 2 (2) cells each occupy approximately half of the collecting duct. ×400.

Fig. 5. Proximal section of region IV, in which the type 2 cells, and associated microvilli are confined to a narrow strip. Mallory's triple stain. ×400.

series of folds, which penetrate two-thirds of the way to the apical border. Mitochondria are associated with these folds (Fig. 14). In the apical zone of type 1 cells there are curious 'membrane whorls', which appear to be derived from the apical plasma membrane (Figs. 15, 16). There is an apparent extracellular layer of variable thickness overlying these cells, and penetrating the larger invaginations of the apical plasma membrane. This layer consists of two distinct sections, a thin, outer, densely staining

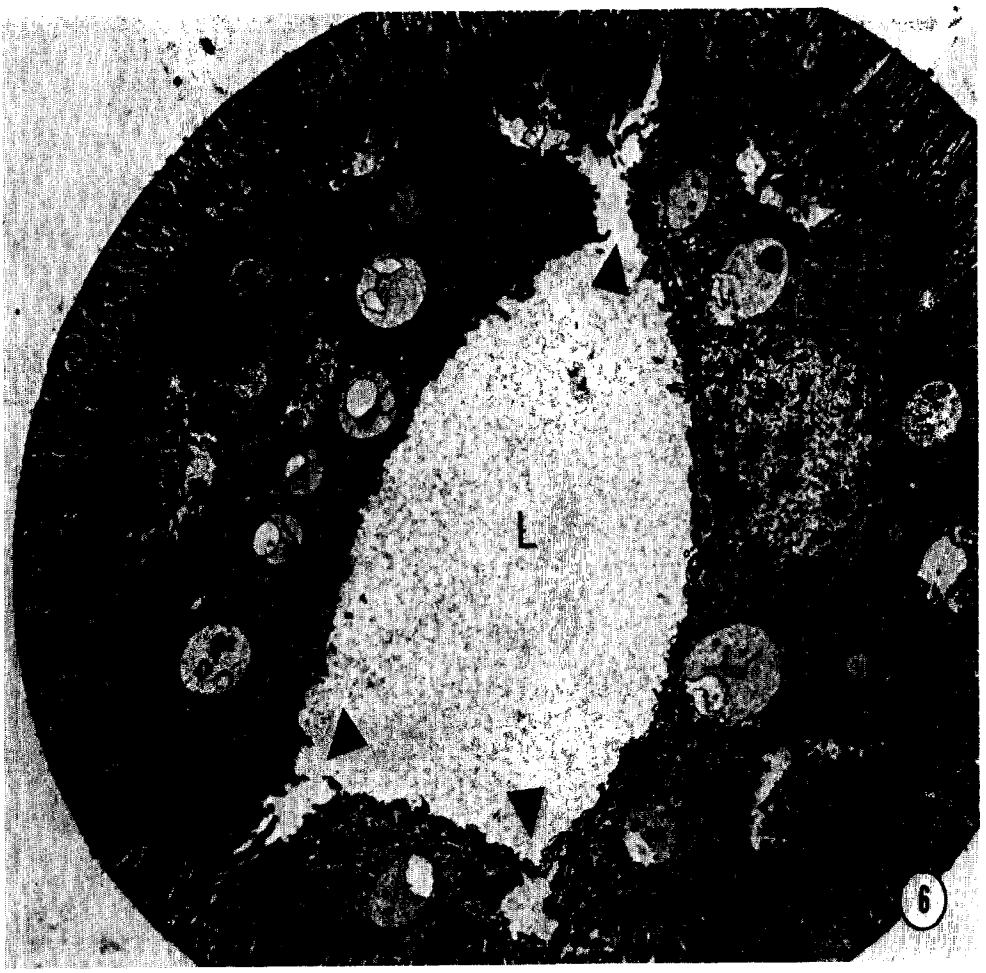


Fig. 6. Transverse section through region I of the tubule. There are numerous evaginations of the main lumen (L). Profiles of these evaginations are numerous throughout the section (stars). In the plane of this section, three of the openings of these evaginations into the main lumen can be seen (arrowed). Microvilli are largely confined to these evaginations. ×5000.

one, and a thicker, amorphous, inner one. In some sections, two such layers were found overlying type 1 cells.

Type 2 cells, by comparison, are relatively unspecialized, although they give rise to numerous, long, microvilli-like structures (Fig. 12) that are clearly visible in the light microscope as well (Figs. 4, 5). These structures contain no organelles, but strands of glycocalyx material can often be seen around them (Fig. 13). Their distribution varies, as does that of type 1 and type 2 cells, along the length of the collecting duct. Thus, they are limited to a narrow band in more proximal sections (Fig. 5), but expand distally so that they come to occupy half of the circumference of region IV (Fig. 4). In some distal sections, the microvilli-like projections

are longer on the lateral sides of the collecting duct, and thus partially divide the collecting duct into two.

Discussion

Work on the structure of the excretory system in the Ephemeroptera is sparse. Henson (1948) has published a general description of the anatomy of the Malpighian tubules of a number of species based on dissections, but other than this little has been written, and there are no ultrastructural accounts. The present work describes a number of features which appear to be unique amongst Malpighian tubules, and which may have a bearing on the contentious problem of solute–solvent coupling. However, it must be remembered that since no physiological data exist as yet for this tissue, some caution is required in interpreting these morphological data.

Much information, both physiological and ultrastructural, is now available for Malpighian tubules of a wide range of species (mostly insects). The tubules are largely concerned with the production of a primary urine, roughly iso-osmotic with the haemolymph, although a variety of reabsorptive processes occur in the more proximal regions of some tubules. However, despite such interspecific differences, there is an underlying similarity in the cells of the secretory portions. These cells characteristically possess a series of membrane folds and long interdigitating processes on their basal borders, and microvilli on their apical borders. Mitochondria are numerous, and in some species are inserted into the microvilli (Messier and Sandborn, 1966; Taylor, 1971) whilst in others they are not (Jarial and Scudder, 1970; Nicholls, 1982). In their basic morphology, the cells of region I of *Ecdyonurus* tubules conform to this general description and, therefore, it seems reasonable to ascribe secretion of the primary urine to this region. Further support for this assumption comes from the distal position of this region. In other insects with regionally differentiated tubules, the secretory portion is usually the most distal.

Fig. 7. Grazing longitudinal section through region I. The luminal evaginations branch throughout the basal half of the cells. ×6000.

Fig. 8. Detail of blind end of luminal evagination, showing its close approach to the haemolymph (H). The direction of opening to the main lumen (L) is indicated. The lateral cell membranes (LM) open into the blind end of the evagination. ×26,000.

Fig. 9. Closely packed basal membrane folds, and associated mitochondria in region II. ×25,000.

Fig. 10. Lumen (L) of region II showing sparse microvilli. ×50,000.

Fig. 11. Transverse section through region III, showing simple cells linked by extensive septate junctions. ×4000.

Fig. 12. Transverse section through region IV. The lumen is largely filled with the microvilli-like projections derived from type 2 cells, which otherwise are relatively simple. Type 1 cells possess a series of haemolymph-directed channels, derived from folds of the basal membrane. At their apical border they possess whorls of membranes. ×3000.

Fig. 13. Detail of microvilli-like projections. Strands of glycocalyx material are visible. ×37,000.

Fig. 14. Detail of basal plasma membrane folds of type 1 cells. BM, basement membrane. ×45,000.

Figs. 15, 16. Detail of membrane whorls present in the apical part of type 1 cells. The cells appear to be overlain by an amorphous extracellular layer, and the whorls derived from the apical plasma membrane. Fig. 15, ×17,500; Fig. 16, ×21,000.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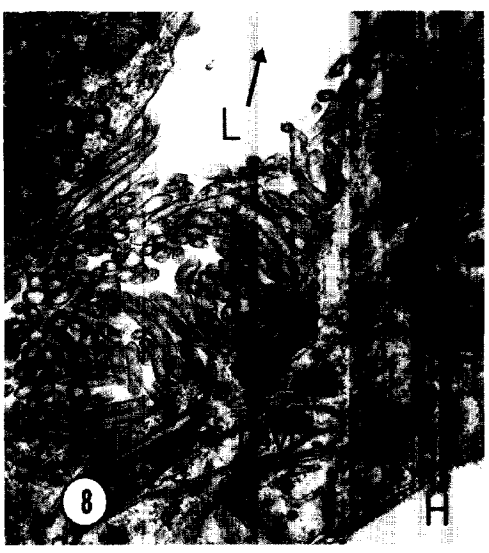
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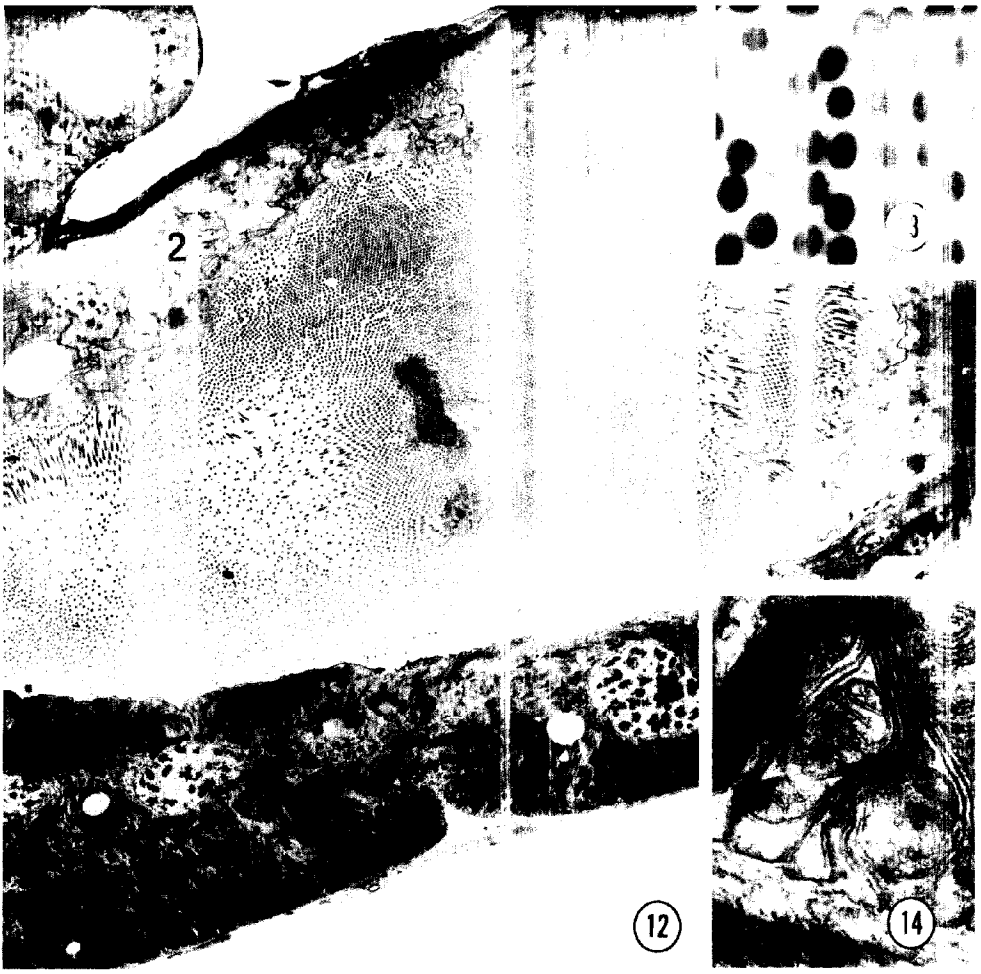
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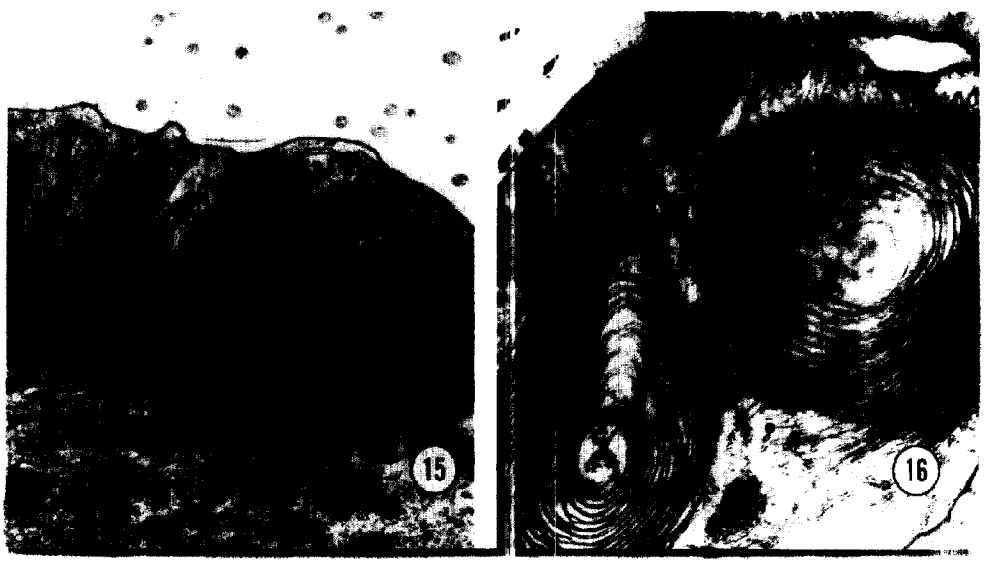
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Fluid flow in Malpighian tubules is driven by active ion movements. In most insects, active potassium movements are involved, although haematophagous insects utilize both potassium and sodium, depending on which ion predominates in the cell (reviewed by Maddrell, 1977). The dragonfly, *Libellula quadrimaculata,* is a further exception, since it is entirely dependent on sodium to generate fluid flow (Nicholls, 1982). However, it is the ionic concentration gradients and their location rather than the specific ions which are important in most models of solute–solvent coupling, and the similarity in basic morphology of the secretory cells in all of these cases suggests that a similar fluid-coupling mechanism may operate. The exact nature of this coupling of fluid movements to active solute movements has been investigated in some detail in a wide variety of fluid-transporting tissues (reviewed by Hill, 1977), but no completely satisfactory model has yet been produced. In the case of Malpighian tubules, Maddrell (1977) has suggested that the ion pumps responsible for driving fluid movement are located on the apical microvilli, and he proposes several possible mechanisms whereby fluid movement could be linked to these ion movements. Ions could be pumped into the spaces between the microvilli, elevating the osmotic pressure here, which then serves to pull water across the apical membrane, creating a standing osmotic gradient along the spaces in the process. Such a mechanism has been invoked by Diamond (1962) and Diamond and Bossert (1967) to explain fluid movements across the vertebrate gall bladder. Alternatively, fluid and ion movements could be linked electro-osmotically, water being carried through membrane pores by ions diffusing down an electro-chemical gradient set up by active transport of the counter-ion. However, Hill (1975, 1977) has shown that it is difficult to account for iso-osmotic fluid flow in Malpighian tubules with these mechanisms.

There is now a growing realization that not all of the junctional complexes seen on the lateral cell membranes are tight (i.e. impermeable to water and ions) (Machen *et al.*, 1972; Lane, 1978, 1979). This knowledge has led to the suggestion that the space between the lateral cell membranes may be important in fluid and solute movements in some tissues. The importance of this paracellular route for passive ion permeation is now generally accepted (e.g. Møllgard and Rostegaard, 1981), but fluid movement via this route is more contentious (Diamond, 1979; Møllgard and Rostegaard, 1981). However, an examination of the ultrastructure of region I of *Ecdyonurus* tubules suggests that, in this tissue at least, fluid flow may take place through the lateral interspaces. The microvilli, which surround the lumen in other species, are confined to luminal evaginations in *Ecdyonurus*. Further, these evaginations closely approach the basal site of the tubule, and the lateral interspaces invariably open into these structures so that the paracellular route thus formed is short. The lateral membranes are also fairly straight. Evidence from the tubules of other species suggests that the apical microvilli house the ion pumps responsible for driving fluid flow (Maddrell, 1977), and further circumstantial evidence for this assertion comes from the observation that the unstimulated secretary rates of the tubules from the larvae of the dragonfly, *Libellula quadrimaculata* are low, whilst those of the adult of the same species are four to five times higher. The main ultrastructural difference between the larval and the adult tubules is the presence of elongate mitochondria inserted into the microvilli of the latter (Nicholls, in press). It therefore seems reasonable to suggest that most lumen-directed ion movement in *Ecdyonurus* tubules takes place into the luminal evaginations. Even if ion pumps are not entirely confined to the microvillar membrane·, the greater surface area of membrane created by the presence of the microvilli means that the absolute number of ion pumps around the evaginations will be greater than around the general lumen. It is suggested therefore that these ion movements elevate ionic concentrations in the evaginations, and that the osmotic gradient so formed serves to drive fluid flow along the lateral interspaces to achieve osmotic equilibration.

From the existing knowledge of the ion-binding properties of the basement membrane (e.g. Dutkowski, 1977) and possibly also of the mucopolysaccharides associated with septate junctions (e.g. Staehelin, 1974), it is likely that fluid moving through the

paracellular route will differ from that bathing the basal side of the cells. This may enhance the ability of such a system to produce an iso-osmotic secretion, by preventing the movement by solvent drag of some ions.

Recent advances in electron-probe X-ray micro-analysis of frozen thin sections have to some extent permitted the localization of ion concentration gradients in fluid transporting tissues, and one such study, on *Calliphora* salivary glands, in particularly relevant here. The cells of this tissue resemble those of Malpighian tubules in having a microvillar border on their apical side, and in generating an iso-osmotic secretion (Gupta and Hall, 1979). However the salivary gland cells differ from those of Malpighian tubules in possessing blind-ended invaginations of the apical surfaces (canaliculi). The electron microprobe reveals that ionic concentrations are high throughout the basal, closed portion of the canaliculus, but fall to iso-osmolarity at the open end. Interestingly, the lateral cell interspace opens into the canaliculus at this point, strongly suggesting that fluid flow via the paracellular route is important in achieving final osmotic equilibration (Gupta and Hall, 1979). The overall geometry of a small semi-isolated portion of the main lumen which serves to prevent dissipation of osmotic gradients, and into which the lateral interspaces open, is similar in both *Calliphora* salivary gland and region I of *Ecdyonurus* tubules, adding further evidence for the importance of paracellular fluid flow in this latter tissue.

Although the primary role of Malpighian tubules is undoubtedly that of secretion of an iso-osmotic urine, in many species regional differentiation of the tubules has resulted in a variety of reabsorption processes occurring in the more proximal sections. Thus, some KCl is reabsorbed in the lower region of *Rhodnius* tubules (Maddrell and Phillips, 1975), and a similar pattern is found in the cryptonephridial tubules of *Calpodes* (Irvine, 1969), although in the latter case there is also some evidence for sodium secretion in the lower portions as well. The ampullae, into which the Malpighian tubules open in *Periplaneta,* are also thought to be involved in modification of the primary urine (Wall *et al.,* 1975).

Regional differentiation is extreme in *Ecdyonurus* tubules, although the cells of the lower regions bear little resemblance to those of the reabsorption regions of other tubules. Again, whilst there are no physiological data for these lower regions, the resemblance of some of the cell types to those of other tissues permits some tentative conclusions to be drawn.

The cells of region II are very different to those of region I. Their dominant feature is the presence of exceedingly long basal membrane folds, associated with mitochondria. The arrangement is strongly reminiscent of that in the rectal pads (or equivalent structures) of aquatic insects (Wall and Oschman, 1975, 1979; Nicholls, 1982). In these latter cases there is evidence that the array of channels thus formed is responsible for the transport of a hyper-osmotic solution in the direction in which the channels open (reviewed by Nicholls, 1982). Their existence in the spiral region of *Ecdyonurus* tubules suggests that this region may be concerned with the removal of ions, etc., from the urine. Such a function would be consistent with the need for fresh-water animals to produce hypo-osmotic urine, but the reason why the structure of such a reabsorptive region should differ so much from that of other tubules remains obscure.

On ultrastructural grounds it would appear that region III is a simple conducting tube, which serves to transfer urine from the spiral region to the collecting duct. The presence of cells with numerous basal channels in this latter region again suggests some form of reabsorptive function. The function of the membrane whorls in type 1 cells is, however, obscure. Although superficially resembling well-developed Golgi complexes they appear to be continuous with the apical membrane and, as such, may functionally resemble the apical folds seen in many ion and water transporting cells (Wall and Oschman, 1975, 1979; Nicholls, 1982). The reason for the elaborate arrangement of type 1 and type 2 cells and associated microvillar-like projections is also obscure, and must await a more detailed physiological study.

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