The Journal of Experimental Biology 212, 363-372
Published by The Company of Biologists 2009
doi:10.1242/jeb.023739

Review

Too much of a good thing: how insects cope with excess ions or toxins in the diet

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Accepted 18 September 2008

Summary

Much of our understanding of the ionoregulatory and excretory physiology of blood-feeding insects can be traced to a series of papers by Simon Maddrell and colleagues in the 1970s and 1980s. These studies of the Malpighian (renal) tubules of *Rhodnius prolixus* revealed a number of physiological adaptations to the short-term and long-term stresses associated with blood feeding. More recent electrophysiological studies using voltage- and ion-selective microelectrodes have extended our understanding of the mechanisms and control of ion transport by the secretory and reabsorptive segments of the *Rhodnius* Malpighian tubule. The discovery that the rates of transport of organic anions, urates and Ca^{2+} are synchronized to coincide with the appearance of the products of blood meal digestion in the haemolymph of *Rhodnius* has stimulated parallel studies in *Drosophila*. This recent research has examined how excretory mechanisms for organic cations and organic anions are altered by exposure to such compounds in the diet. These studies also show that the *Drosophila* Malpighian tubule provides a useful model for analysis of the roles of transporters such as P-glycoproteins and multidrug resistance-associated proteins in the excretion of toxins.

Key words: insect, Malpighian tubule, midgut, ion-selective microelectrodes, excretion, dietary toxins, haematophagy.

Introduction

When a blood-feeding insect such as *Rhodnius* ingests a meal that may equal 10 times its unfed weight, it has manifestly solved the problem of obtaining the necessary nutrients and energy required for growth and reproduction. But ingestion of food, particularly in blood feeders or insect larvae which feed at high rates to maintain high growth rates, creates a number of challenges for the maintenance of homeostasis. In particular, toxins present in the diet or produced by metabolism need to be excreted. Much of our understanding of the mechanisms of excretion of excess ions or toxins in insects can be traced to a number of seminal studies by Simon Maddrell and coworkers in the 1970s and 1980s. These papers dealt with *Rhodnius prolixus* which faces both short-term and long-term stresses following the blood meal. In the short term, the insect must eliminate large quantities of Na^+, Cl^- and water, in essence excreting the nutrient-poor plasma fraction of the blood meal, thereby concentrating the nutrient-rich blood cells and reducing the insect's size so that it is more mobile and less susceptible to predation. In the long term, the insect is faced with excesses of ions, such as K^+ and Ca^{2+}, derived from digestion of the blood cells, as well as nitrogenous waste in the form of uric acid and organic anions resulting from catabolism of blood meal proteins. In addition, reactive oxygen species (ROS) are generated from some of the breakdown products of haemoglobin.

Short-term mechanisms for ionic and osmotic homeostasis in *Rhodnius*

Rhodnius feeds on avian and mammalian species whose blood osmolality (~320 mosmol l^{-1}) is lower than that of its own haemolymph (370 mosmol l^{-1}). It must therefore secrete a hypo-osmotic urine in order to preserve homeostasis, and this process involves two steps, as first outlined by Maddrell and Phillips (Maddrell and Phillips, 1975). Fluid secreted by the upper (distal) Malpighian tubule is approximately iso-osmotic with the insect's haemolymph and contains high levels of both Na^+ (125 mmol l^{-1}) and K^+ (70 mmol l^{-1}). As fluid passes through the downstream lower (proximal) Malpighian tubule, K^+ and Cl^- but not water are reabsorbed, so that a hypo-osmotic urine (250 mosmol l^{-1}) is eliminated (Fig. 1A).

Fig. 1B shows the transporters involved in the secretion of ions; measurements with double-barrelled ion-selective microelectrodes (ISMEs) have provided the thermodynamic evidence supporting this model. During diuresis, ion secretion is driven by an apical V-type H^+-ATPase (Maddrell and O'Donnell, 1992). The electrochemical potential established for H^+ across the apical membrane by the H^+-ATPase is sufficient to drive either Na^+ or K^+ from cell to lumen through an amiloride-sensitive H^+/alkali cation exchanger (Ianowski and O'Donnell, 2006). A Na^+/H^+ exchanger (*Ae*NHE8) that is a molecular candidate for this transporter has been identified from the Malpighian tubules of the mosquito *Aedes aegypti* by Kang'ethe and colleagues (Kang'ethe et al., 2007). Na^+ and K^+ movements across the apical membrane thus represent forms of secondary active transport. In turn, transport of Na^+ from cell to lumen establishes an electrochemical gradient for Na^+ across the basolateral membrane that is sufficient to drive coupled entry of Na^+, K^+ and Cl^- through a bumetanide-sensitive $Na^+/K^+/2Cl^-$-cotransporter. This cotransporter is thus a form of tertiary active transport, two steps removed from ATP hydrolysis by the apical H^+-ATPase (Ianowski et al., 2002). Proton transport by the H^+-ATPase is strongly electrogenic, and the consequent lumen-positive apical membrane potential favours Cl^- movement from cell to lumen through conductive pathways (i.e. channels).

Water movements during diuresis are a passive osmotic consequence of active transepithelial ion secretion. Early measurements of osmotic permeability of up to 4.3×10^{-3} cm s^{-1} osmol^{-1}, equivalent to 5.8×10^{-3} cm s^{-1} osmol^{-1} after

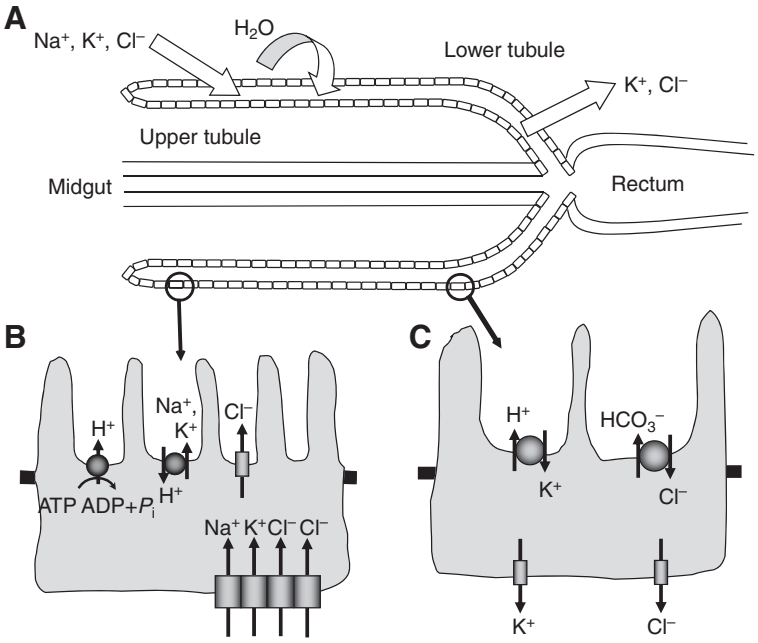

Fig. 1. Schematic diagrams showing (A) the excretory system in *Rhodnius prolixus* and current models of (B) Na^+, K^+, Cl^- and H_2O secretion across the upper Malpighian tubule (UMT) and (C) K^+ and Cl^- reabsorption across the lower Malpighian tubule (LMT). P_i, inorganic phosphate.

correction for unstirred layer effects (O'Donnell et al., 1982), indicated that osmotic gradients of as little as 0.7 mosmol l^{-1} across the basolateral membrane and a further 2.6 mosmol l^{-1} across the apical membrane are sufficient to account for the observed fluid secretion rates (O'Donnell and Maddrell, 1983). Thus, an increase in luminal osmolality of 3.3 mosmol l^{-1} (=0.7 mosmol l^{-1}+2.6 mosmol l^{-1}) is sufficient to account for the observed rates of fluid secretion. This value is within 1% of iso-osmolarity, close to the value (1.3%) actually measured. Osmotic permeability is increased 35% in the upper Malpighian tubule (UMT) by the diuretic factor 5-hydroxytryptamine (5-HT) (O'Donnell et al., 1982), probably as a consequence of the insertion of aquaporins (Martini et al., 2004). Aquaporin functions in insect Malpighian tubules are reviewed by Spring and colleagues in this review volume (Spring et al., 2009).

It is worth emphasizing the very high rates of fluid and ion secretion by the UMTs of *Rhodnius* during diuresis. Maddrell (Maddrell, 1991) estimated that each cell secretes its own volume of near iso-osmotic fluid every 10 s. Given that the levels of Cl^- in the cell are less than one-third of those in the secreted fluid (Ianowski et al., 2002), rates of transepithelial Cl^- secretion are even more impressive, equivalent to exchange of the entire cellular content of Cl^- every 2 to 3 s!

The maintenance of K^+ homeostasis during diuresis requires reabsorption of K^+ from the near-equimolar NaCl and KCl secreted by the UMT. Simon Maddrell and John Phillips first demonstrated that reabsorption of KCl is accomplished by the lower Malpighian tubule (LMT) (Maddrell and Phillips, 1975) and that almost all of this reabsorption is accomplished by the lower third of the LMT (Maddrell, 1978). In tubules of fifth instar *Rhodnius*, fluid moves along the LMT at 0.6 mm s^{-1} (at 22°C) and is in contact with the reabsorptive region for less than 10 s. The K^+ concentration falls from 80 mmol l^{-1} to less than 5 mmol l^{-1} during this time, meaning that a decline of 1 mmol l^{-1} K^+ takes only 130 ms and a decline in osmolality of 1 mosmol l^{-1} takes only 80 ms (Maddrell, 1978). Almost no water is reabsorbed during KCl reabsorption. The osmotic permeability (P_{os}) of the upper two-thirds of the LMT is 3×10^{-3} cm s^{-1} osmol^{-1}, similar to the value of 4.3×10^{-3} cm s^{-1} osmol^{-1} for the UMT. Osmotic permeability (P_{os}) decreases along the lower third of the LMT to 0.4 cm s^{-1} osmol^{-1} (O'Donnell et al., 1982). Moreover, P_{os} declines further over much of the lower third of the LMT in response to 5-HT. P_{os} is thus reduced when rates of K^+ and Cl^- reabsorption are highest, thereby further minimizing water reabsorption and contributing to the production of hypo-osmotic urine.

The importance of rapid K^+ reabsorption by the LMT can be appreciated by considering the consequences of its failure. Given the high rate of fluid secretion by the UMT and the high (~80 mmol l^{-1}) concentration of K^+ in the fluid, the entire haemolymph K^+ content would be lost in <60 s if K^+ was not reabsorbed downstream (Maddrell et al., 1993). It is thus important that the ion reabsorption mechanism of the LMT be activated prior to the onset of fluid and ion secretion by the UMT. The mean time for K^+ reabsorption to reach 50% of the maximum rate in the LMT of fifth instar *Rhodnius* is 2.7 min. By contrast, the UMT does not begin to secrete fluid until 3 min after addition of 5-HT and the mean time for secretion to reach 50% of the maximum rate is 4.1 min (Maddrell et al., 1993).

The transporters involved in K^+ and Cl^- reabsorption by the LMT differ fundamentally from those involved in the secretion of these ions and Na^+ by the UMT (Fig. 1C). The results of a variety of pharmacological and ion-substitution experiments suggest the involvement of an omeprazole-sensitive P-type H^+/K^+-ATPase and a stilbene-insensitive Cl^-/HCO_3^- exchanger in the apical membrane of the LMT. There are separate conductive pathways (i.e. channels) for both ions in the basolateral membrane (Haley and O'Donnell, 1997; Haley et al., 1997). The effects of changes in bathing saline K^+ and Cl^- indicate that as much as 61% of the membrane potential is attributable to a Cl^- conductance and 29%–52% is attributable to a K^+ conductance. Cl^- makes a larger contribution to basolateral membrane potential when the bathing saline K^+ concentration is reduced. A role for basolateral Cl^- channels is further suggested by the finding that Cl^--dependent changes in basolateral membrane potential and KCl reabsorption are inhibited by Cl^- channel blockers such as diphenylamine-2-carboxylate and 5-nitro-2(3-phenylpropylamino) benzoic acid. The effects of a variety of pharmacological agents rule out the involvement of K^+/Cl^-- or

$Na^+/K^+/2Cl^-$-cotransporters in KCl reabsorption. The contribution of basolateral K^+ channels is suggested by the finding that K^+ reabsorption and K^+-dependent changes in membrane potential are inhibited by the K^+ channel blocker Ba^{2+}.

Maintenance of haemolymph K^+ homeostasis during diuresis involves autonomous regulatory mechanisms in the UMT and LMT. As K^+ concentration in the haemolymph declines, the UMT secretes fluid more slowly and less K^+ is secreted. It now appears that the replacement of K^+ by Na^+ in the secreted fluid reflects competition between Na^+ and K^+ for entry into the cell through the bumetanide-sensitive Na^+:K^+:2Cl-cotransporter in the basolateral membrane (Ianowski et al., 2004). Dose–response curves of secretion rate *versus* bumetanide concentration are identical for tubules bathed in K^+-free saline and control saline with IC_{50} values of 2.6×10^{-6} mol l^{-1} and 2.9×10^{-6} mol l^{-1}, respectively. Kinetic analyses using double-reciprocal plots of K^+ secretion rate *versus* bathing saline K^+ concentration show that increasing Na^+ concentration in the bathing saline increases the Michaelis–Menten parameter K_t but has no effect on maximum flux, J_{max}, consistent with competitive inhibition of K^+ transport by Na^+. The $Na^+/K^+/2Cl^-$-cotransporter thus appears to operate as a bumetanide-sensitive $2Na^+/2Cl^-$-cotransporter under some conditions. The net effect of these changes in K^+ secretion by the UMT is that the LMT has more time to reabsorb K^+, because fluid moves down the lumen more slowly, and that there is less K^+ to reabsorb. In addition, the LMT reabsorbs more K^+ from the lumen as haemolymph K^+ declines, presumably reflecting more favourable gradients for K^+ to move from cell to lumen through the basolateral channels.

Long-term threats to homeostasis following the blood meal

In the days following the blood meal the Malpighian tubules of *Rhodnius* and other blood feeders play important roles in the elimination of the potentially toxic products of blood meal digestion. These include excess Ca^{2+}, since mammalian blood contains at least 1.5 mmol l^{-1} Ca^{2+} and the blood of avians may contain higher levels, particularly during periods of egg laying. K^+ derived from the breakdown of the blood cells must also be eliminated, albeit slowly. During diuresis, ion transport is dependent almost entirely upon the actions of the V-type H^+-ATPase and there is no effect of adding the Na^+/K^+-ATPase blocker ouabain. In the days following the blood meal, however, a basolateral ouabain-sensitive Na^+/K^+-ATPase enhances the entry of K^+ into the cells at the expense of Na^+, thus increasing the secretion of K^+ (Maddrell and Overton, 1988). As the protein of the blood meal is broken down to amino acids, both nitrogenous waste and organic anions resulting from the metabolism of aromatic amino acids (Phe, Trp, Tyr) are produced. In addition, both iron and haem derived from the catabolism of haemoglobin in the blood cells contribute to the generation of ROS.

Nitrogenous waste excretion by blood feeders

Fifth instar *Rhodnius* excretes 1.2 mg of urate per day by 3–4 days after the blood meal, equivalent to more than 14% of unfed body mass per day. Similarly, the tsetse fly *Glossina morsitans* excretes nearly 50% of the dry mass of ingested blood as nitrogenous waste to eliminate surplus nitrogen (Bursell, 1965). Tsetse flies excrete mainly uric acid, but also arginine and histidine. Surprisingly, mosquitoes excrete mostly ammonia (Scaraffia et al., 2005), somewhat at odds with the expectation that terrestrial insects are uricotelic. However, cockroaches have long been known to excrete ammonia as the primary nitrogenous waste (Mullins and Cochran, 1972; Mullins and Cochran, 1973) and it appears that ammonia excretion in many studies of insects may have been underestimated, either because NH_3 volatilized from the collected waste, particularly if samples were oven dried, or because dilution of the waste pellets during preparation for analysis was insufficient to release ammonium from the precipitated form (Harrison and Phillips, 1992).

Another surprise is that only 3% of the amino acids derived from the blood meal proteins are used for egg production in the mosquito *Aedes aegypti* (Scaraffia et al., 2005). By contrast, 60% of the amino acids are oxidized to provide energy. Ammonia toxicity from amino acid metabolism is avoided by high rates of ammonia excretion in the faeces but also through the synthesis of glutamine and proline. Ammonia derived from amino acid deamination is temporarily stored in a non-toxic form as proline. The ammonia can subsequently be recovered for excretion and the carbon skeleton used for synthesis of various compounds or for energy production (Goldstrohm et al., 2003). Glutamine plays an important role in shuttling ammonia from the flight muscle to the fat body.

Increases in the rates of transport of Ca^{2+}, urate and organic anions after the blood meal

Simon Maddrell and colleagues were the first to demonstrate that the rates of excretion of Ca^{2+}, uric acid and organic anions vary with time after the blood meal. Simply put, the insect appears to develop the necessary excretory capacity in response to increases in the levels of these ions in the haemolymph. Although this work was done using *Rhodnius*, the results have broad implications for phytophagous or omnivorous insects, as discussed below in the section on *Drosophila*.

Malpighian tubules isolated from unfed *Rhodnius* secrete organic anions such as para-aminohippurate (PAH) at very low rates. PAH secretion rates rise sharply after feeding, reaching peak values 3–4 days after the ingestion of a blood meal (Fig. 2). The induction is produced by the continued presence in the haemolymph of a product of digestion of a protein rich meal (Maddrell and Gardiner, 1975). A similar pattern is seen for the rates of excretion of uric acid (Fig. 2). Urate secretion increases dramatically in isolated tubules dissected from animals 2–3 days after feeding. Injection into the haemolymph of an amount of urate equivalent to the amount eliminated in 2 h is sufficient to induce a doubling of the rate of urate transport by the Malpighian tubules. *In vitro* experiments with the tsetse fly *Glossina* suggest that increases in the rates of urate secretion may be a direct response of the tubule cells to an increase in urate concentration in the surrounding fluid (O'Donnell et al., 1983).

High rates of Ca^{2+} uptake by isolated tubules of *Rhodnius* also appear to be a response to excess Ca^{2+} ingested in the blood meal. In this case, Ca^{2+} is not secreted into the lumen but remains within non-crystalline membrane-bound concretion bodies within the Malpighian tubules (Maddrell et al., 1991). Deposit excretion, rather than secretion into the tubule lumen, may be an adaptation to avoid interference with the transport of water and solutes downstream in the rectum. As for uric acid and organic anions, rates of Ca^{2+} uptake peak 3–4 days after the blood meal at a value 7 times that of unfed insects and rates remain elevated for 9 days.

ROS in blood feeders

Blood feeders are exposed to high levels of haem during hydrolysis of host haemoglobin in the gut. Degradation of haem by haem oxygenase produces iron, CO and biliverdin. Both iron and haem can lead to the formation of ROS, which can damage a variety of biological molecules. Although biliverdin has antioxidant

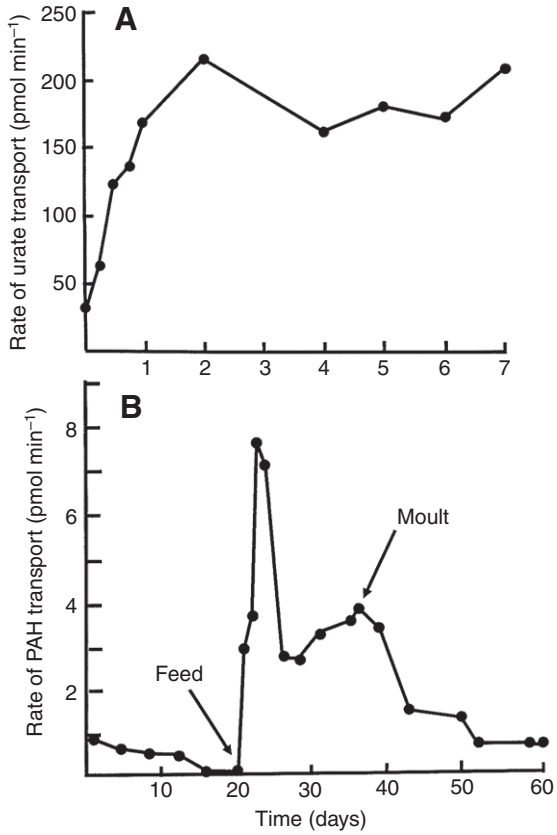

Fig. 2. Rates of urate and para-aminohippurate (PAH) transport by isolated Malpighian tubules of fifth instar *Rhodnius prolixus* at the times indicated after the blood meal on day 0 (A) or day 20 (B). Data replotted from Maddrell and Gardiner and from O'Donnell et al. (Maddrell and Gardiner, 1975; O'Donnell et al., 1983).

properties it must be excreted to avoid cytotoxicity (Graca-Souza et al., 2006). The precise role of CO derived from haem in blood-feeding arthropods is unknown, although Graca-Souza and colleagues speculate that it may act in an immunomodulatory capacity, preventing an excessive immune response to the haem derived from digestion of the host's blood (Graca-Souza et al., 2006). There are multiple pathways that lead to the production of ROS from iron and haem. Hydrogen peroxide (H_2O_2) produced by mitochondria can combine with iron to produce hydroxyl radicals (OH*) *via* the Fenton reaction: $Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+OH^*$. Hydroxyl radicals then enter into a multistep pathway of lipid peroxidation that leads to the production of organic hydroperoxides (ROOH). These, in turn, react with haem-Fe^{2+} and haem-Fe^{3+} to produce alkoxyl (RO*) and peroxyl (ROO*) radicals.

The potential for severe oxidative stress in blood-feeding insects has resulted in the evolution of multiple lines of defence (Graca-Souza et al., 2006). Firstly, insoluble aggregates of haem (such as haemozoin in *Rhodnius*) may form inside the gut. Secondly, antioxidant enzymes such as superoxide dismutase and catalase act to reduce the levels of ROS. Thirdly, haem-binding proteins and ferritins (iron-binding proteins) may minimize the formation of ROS from haem and iron. Lastly, blood feeders such as *Rhodnius* maintain high levels of urate in the haemolymph by balancing the rates of urate formation and excretion. High levels of urate are produced experimentally in response to the injection of haemin (the Fe^{3+} oxidation product of haem) into the haemolymph or exposure to high ambient O_2 levels. Urate is thus produced in response to oxidative stress and its concentration, up to 5 mmol l^{-1} *in vivo*, may account for almost all of the free radical scavenging activity (Souza et al., 1997).

Excretion of organic cations and organic anions in *Drosophila* and other species

Early studies of the haematophagous species *Rhodnius prolixus* by Simon Maddrell set the stage for current studies on the excretion of dietary toxins by omnivorous and phytophagous insects. Increases in rates of secretion of organic anions by the Malpighian tubules of *Rhodnius* are a response to the appearance in the haemolymph of the products of protein digestion. By contrast, secretion rates of alkaloids such as nicotine, atropine and morphine are unaffected by blood feeding and digestion (Maddrell and Gardiner, 1976). Secretion of nicotine by the Malpighian tubules of the tobacco hornworm, *Manduca sexta*, indicated a role for P-glycoprotein-like transporters (Gaertner et al., 1998). P-Glycoproteins are the products of expression of multidrug resistance (MDR) genes and have been extensively studied in mammalian tumour cells (Leslie et al., 2005) and in the defence against xenobiotics by aquatic organisms (Bard, 2000). P-Glycoproteins are often implicated in the transport of what are termed type II organic cations (OCs) (Wright and Dantzler, 2004). Whereas type I OCs are relatively small (<400 Da) and monovalent, type II OCs are larger (>500 Da) and frequently polyvalent compounds. Endogenous type I OCs include *N*-methyl-nicotinamide, choline and catecholamines, and exogenous type I OCs include tetraethylammonium and tetrabutylammonium. Endogenous type II OCs include corticosterone and exogenous type II OCs include many alkaloids and quinones.

Organic anions (OAs) are similarly classified as type I or type II. Endogenous organic anions include mono-, di- and tricarboxylates, folates, ascorbate, cyclic nucleotides and the metabolites of the ring-containing amino acids Phe, Trp and Tyr. Exogenous OAs include fluorescein, para-aminohippuric acid, salicylate, herbicides such as 2,4-D and metabolites of insecticides such as malathion. Transport of type I OAs by insect Malpighian tubules is strongly Na^+ dependent (Linton and O'Donnell, 2000; Ruiz-Sanchez and O'Donnell, 2007a), whereas transport of type II OAs such as Texas Red and methotrexate is sodium independent and appears to involve transporters which are similar to multidrug resistance-associated protein 2 (MRP2). MRP2-like transporters have been identified in the Malpighian tubules of cockroaches, crickets and fruit flies (Karnaky et al., 2003; Leader and O'Donnell, 2005; O'Donnell and Leader, 2006).

Methods for analysis of toxin transport in insects

The small size of insects has necessitated the modification or development of micro methods for measuring the concentrations of toxins, including some organic anions and organic cations, in cells, fluid samples or the unstirred layer near the surface of isolated tissues such as the gut and the Malpighian tubules. Specifically, those methods that have had the greatest impact are: radio- and fluorescence-labelled probes, ISMEs and the scanning ion-selective electrode technique (SIET). These will be addressed in turn in this section.

Radiolabelled probes have long been used to measure the concentration of inorganic ions and organic compounds in fluid samples collected from Malpighian tubules *in vitro* (e.g. Maddrell and Gardiner, 1974). Recently, fluorescent probes and either conventional fluorescence microscopy or confocal laser scanning

microscopy (CLSM) techniques have been developed for use with insect preparations and collected fluid samples (Karnaky et al., 2003; Neufeld et al., 2005; Leader and O'Donnell, 2005).

ISMEs have been used extensively for analysis of physiological ions (H^+, Na^+, K^+, Ca^{2+}, Cl^-) inside cells of the Malpighian tubules or in samples of secreted fluid (O'Donnell and Maddrell, 1995; Ianowski et al., 2002). More recently, a Cd^{2+}-selective microelectrode originally developed for analysis of Cd^{2+} uptake by plant roots (Piñeros et al., 1998) has been applied to the analysis of Cd^{2+} transport by isolated tissues of the larvae of the midge *Chironomus riparius*, a species known to be extraordinarily tolerant of toxic metals (E. M. Leonard, P. L. Gillis, C. M. Wood and M.J.O'D., unpublished observations). ISMEs have also been used to study transport of the prototypical organic cation tetraethylammonium (TEA) and the organic anion salicylate. In addition, we have applied the non-invasive SIET to measure the fluxes of TEA and salicylate across isolated tissues such as the gut and the Malpighian tubules of insects (Rheault and O'Donnell, 2004; O'Donnell and Rheault, 2005).

SIET exploits ionic concentration gradients created in the unstirred layer by ion transport across cell membranes or epithelial layers (Fig. 3). The microelectrode is positioned by an orthogonal array (*X*, *Y*, *Z*) of computer-controlled stepper motors and is moved between two points at each measurement site. The first point is close (within 5 to 10 μm) of the cell surface and the second point is 30–100 μm farther away, at right-angles to the surface. The voltage difference between the two limits of the microelectrode excursion (ΔV) is used to calculate a corresponding concentration difference (ΔC) using the electrode calibration curve. The concentration difference is then converted to net flux (mol cm^{-2} s^{-1}) using Fick's Law: $J=D\Delta C/\Delta X$, where D is the diffusion coefficient of the ion of interest and ΔX is the excursion distance. SIET is particularly useful for spatial and temporal analysis of ion transport. As discussed below, the role of different segments of the gut and the Malpighian tubules in ion transport can be determined even for small insects such as fruit flies and mosquitoes that are not amenable to the Ussing chamber studies used for gut segments of larger species such as locusts and *Manduca sexta*.

We have also developed methods involving ISMEs to measure the concentrations of ions in the fluid secreted by isolated Malpighian tubules set up in the Ramsay assay (Fig. 4). Transepithelial flux (pmol min^{-1}) for each tubule is calculated as the product of fluid secretion rate (nl min^{-1}) and ion concentration (mmol l^{-1}) determined by the ISME. Multiple droplets can be collected from 20 or more tubules set up in the Ramsay assay.

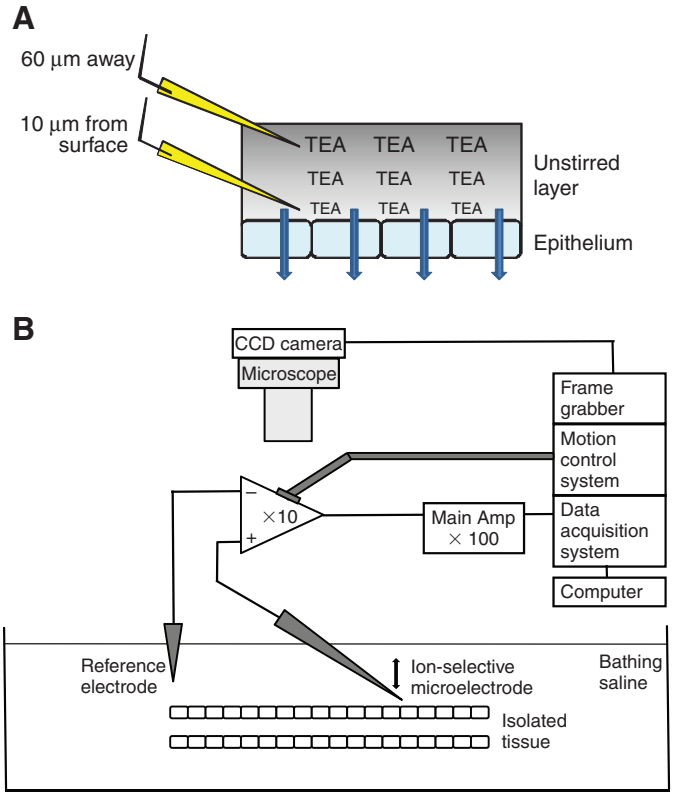

Fig. 3. Non-invasive measurement of ion fluxes by the scanning ion-selective electrode technique (SIET). (A) The technique exploits gradients in ion activity within the unstirred layer that are created by ion transport across cells or epithelia. In this example, secretion of tetraethylammonium (TEA) across the epithelium in the direction of the arrows reduces the concentration of TEA close to the epithelial surface relative to that farther away. The size of the labels and the density of the shading correspond to the concentration of TEA. (B) Schematic diagram of equipment used for SIET measurements. The isolated tissue and the ion-selective microelectrode (ISME) are observed through a microscope equipped with a CCD camera. A computer-controlled motion control system drives an orthogonal (*X*, *Y*, *Z*) array of stepper motors which move the amplifier headstage and attached ISME to sites along the tissue and then at two points orthogonal to the tissue surface, as indicated in A. Voltage differences between the two limits of excursion are recorded by the data acquisition system. Voltage gradients at different sites can be overlaid as vectors on an image of the tissue captured by the frame grabber connected to the CCD camera (as shown in Fig. 5A).

Organic cation transport by insect Malpighian tubules and gut

The prototypical organic cation TEA is transported from haemolymph to lumen across the main and lower but not the distal segments of the Malpighian tubules of *Drosophila*. TEA is also transported across the posterior midgut and the ureter, which connects each of the two pairs of Malpighian tubules to the gut (Rheault and O'Donnell, 2004). TEA influx is relatively constant along the length of the main segment (~1 pmol cm^{-2} s^{-1} for Malpighian tubules bathed in saline containing 0.1 mmol l^{-1} TEA) and then increases along the LMT, reaching values as high as 6 pmol cm^{-2} s^{-1} close to the ureter (Fig. 5). The LMT shows the highest area-specific rate of TEA transport and the highest affinity for TEA (i.e. lowest K_t), relative to the main segment of the tubule and the posterior midgut (Fig. 6). The main segment nonetheless plays an important role in TEA secretion by virtue of its greater length. TEA influx across the main segment of tubules bathed in saline containing 0.1 mmol l^{-1} TEA is equal to 72% of the flux across the whole tubule (main plus lower) (Bijelic and O'Donnell, 2005).

TEA transport by the LMT increases when the basolateral membrane potential (V_{bl}) is hyperpolarized and decreases when V_{bl} is depolarized, consistent with entry through a potential-dependent mechanism. Importantly, blockade of K^+ channels with Ba^{2+} does not block either TEA uptake or the effects of TEA on V_{bl}, indicating that electrogenic, carrier-mediated TEA uptake does not occur through K^+ channels (Rheault et al., 2005).

The mechanisms involved in the movement of TEA from cell to lumen across the apical membrane of the tubule are unclear. In vertebrate models of OC transport, exchange of cellular organic cations for luminal H^+ has been proposed (Pritchard and Miller, 1991). The LMT of *Drosophila* acidifies the lumen (O'Donnell and Maddrell, 1995), so OC/H^+ exchange would be a feasible

mechanism. TEA transport across the LMT is sensitive to the P-glycoprotein inhibitor verapamil, suggesting that a P-glycoprotein-like mechanism may also be involved in secretion of TEA by this segment. By contrast, TEA secretion along the main segment is unaltered by verapamil (Rheault and O'Donnell, 2004).

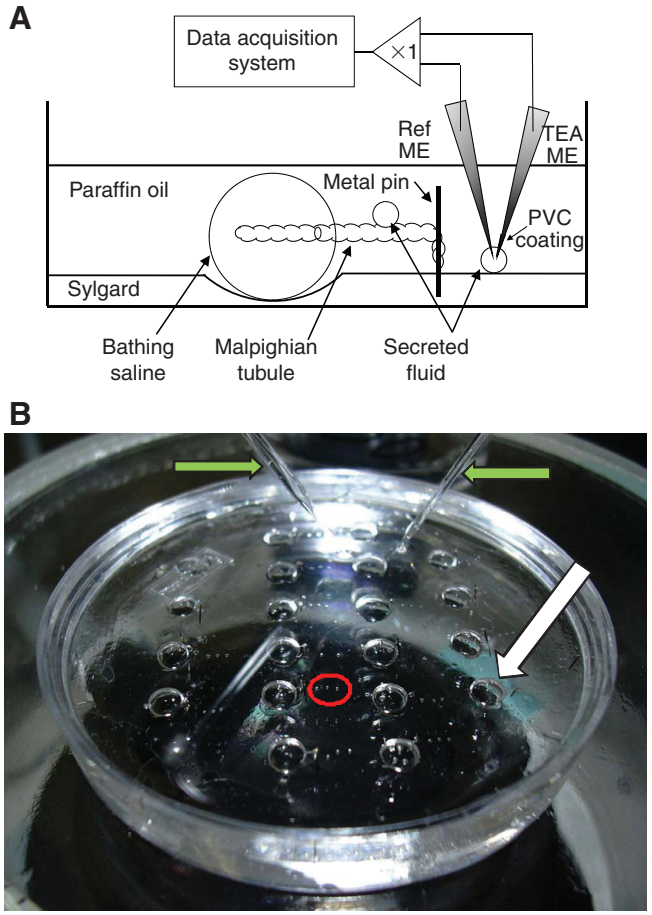

Fig. 4. Measurement of the concentration of TEA in droplets of fluid secreted by isolated Malpighian tubules in the Ramsay assay. (A) An isolated pair of *Drosophila* Malpighian tubules is placed in a droplet of bathing saline under paraffin oil. One tubule remains in the saline, and the other is pulled out and wrapped around a stainless steel pin embedded in the Sylgard-line base of a Petri dish. Secreted fluid droplets which form at the ureter, positioned just outside the bathing saline droplet, are collected on glass rods and placed on the bottom of the dish adjacent to calibration droplets containing known concentrations of TEA in *Drosophila* saline. For each droplet, the potential difference between the TEA-selective microelectrode (TEA ME) and the reference microelectrode (Ref ME) is measured by a high impedance (>10^{15} Ω) operational amplifier and recorded on a PC-based data acquisition system. TEA concentration in the secreted droplets is calculated from the voltage difference between the secreted droplet and the calibration droplets, as described by Rheault and O'Donnell (Rheault and O'Donnell, 2004). The tip of the TEA-ME is coated with a solution of ~10% polyvinyl chloride (PVC) in tetrahydrofuran to avoid capillary rise of the paraffin oil into the silanized microelectrode. (B) Photograph showing 20 *Drosophila* tubules set up in a Ramsay assay in a 55 mm diameter Petri dish. One of the 20 µl bathing droplets is indicated by the white arrow. Each tubule is secured to a black minutien pin anchored in the Sylgard to the right of each bathing saline droplet. Three secreted droplets collected at 30 min intervals are shown within the red ellipse. Reference and TEA-selective microelectrodes (green arrows) are positioned in a bathing saline droplet.

Dietary loading with organic cations: effects on mortality and excretion in *Drosophila*

Drosophila larvae are extraordinarily tolerant of diets containing high levels of TEA, as indicated by the LC_{50} value of 158 mmol l^{-1}. TEA concentration in the haemolymph is ~3% of that in the diet over the range 0–300 mmol l^{-1} TEA. Mortality increases if competitive inhibitors of organic cation transporters are also included in the diet: mortality increases from 24% with diets containing 100 mmol l^{-1} TEA to 83% and 61% when the diet contains 100 mmol l^{-1} TEA and 10 mmol l^{-1} quinidine or 10 mmol l^{-1} cimetidine, respectively (Bijelic et al., 2005). Mortality in larvae exposed to 10 mmol l^{-1} quinidine or cimetidine alone is <20%.

Feeding *Drosophila* larvae a diet enriched in TEA is associated with dramatic increases in TEA secretion by isolated Malpighian tubules. Acute (24 h) exposure of the larvae to diets containing 50 mmol l^{-1} or 100 mmol l^{-1} TEA is correlated with an increase in TEA secretion by isolated tubules of 37% and 77%, respectively (Bijelic et al., 2005). There was no effect on TEA transport by the gut. The effects on the tubules are more pronounced in response to chronic exposure to TEA in the diet. For larvae raised from egg hatching to third instar (~10 days) on a diet containing 10 mmol l^{-1} or 50 mmol l^{-1} TEA, secretion of TEA by isolated tubules increases by 114% and 158%, respectively (S. Weerawardane and M.J.O'D., unpublished observations). These findings suggest that even acute exposure to a toxin may result in enhancement of the capacity of the excretory system to eliminate the toxin.

Although TEA transport by the gut does not appear to be altered in response to acute exposure to TEA in the diet, the gut nonetheless plays an important role in the elimination of TEA from the haemolymph. Active, saturable transport of TEA by the posterior midgut and Malpighian tubules can account for much of the observed rate of clearance of TEA from the haemolymph when the diet contains less than 20 mmol l^{-1} TEA, corresponding to haemolymph TEA concentrations of ~0.5 mmol l^{-1}. However, for very high concentrations of haemolymph TEA, the combined rates of saturable transport of TEA by the posterior midgut and Malpighian tubules can account for only ~10% of the observed rate of decline in haemolymph TEA levels both in larvae switched from a TEA-rich to a TEA-free diet and after injection of TEA into the haemolymph of animals maintained on TEA-free diet (Bijelic and O'Donnell, 2005). It appears that passive diffusion of TEA from the haemolymph into the gut lumen may augment TEA clearance. Ingestion of TEA-free food not only clears the gut lumen of TEA but also creates a TEA-free compartment into which TEA may diffusive passively from the haemolymph. This raises the possibility that an insect exposed to a toxin such as TEA may be able to clear that toxin from the haemolymph much more rapidly simply by ingesting toxin-free food. The large volume and surface area of the gut in insects, particularly in larvae, may thus allow it to act as a passive sink for toxins.

P-Glycoproteins and alkaloid transport in insect Malpighian tubules

Pharmacological evidence supports the view that nicotine and TEA are transported by different transporters in the Malpighian tubules of insects (Rheault et al., 2006). As noted above, a role for a P-glycoprotein-like transporter for nicotine transport by insect Malpighian tubules was first proposed for the tobacco hornworm *Manduca sexta* by Gaertner and colleagues (Gaertner et al., 1998). In addition to excretion of nicotine to reduce nicotine levels in the haemolymph, sensitive tissues such as the nervous system are

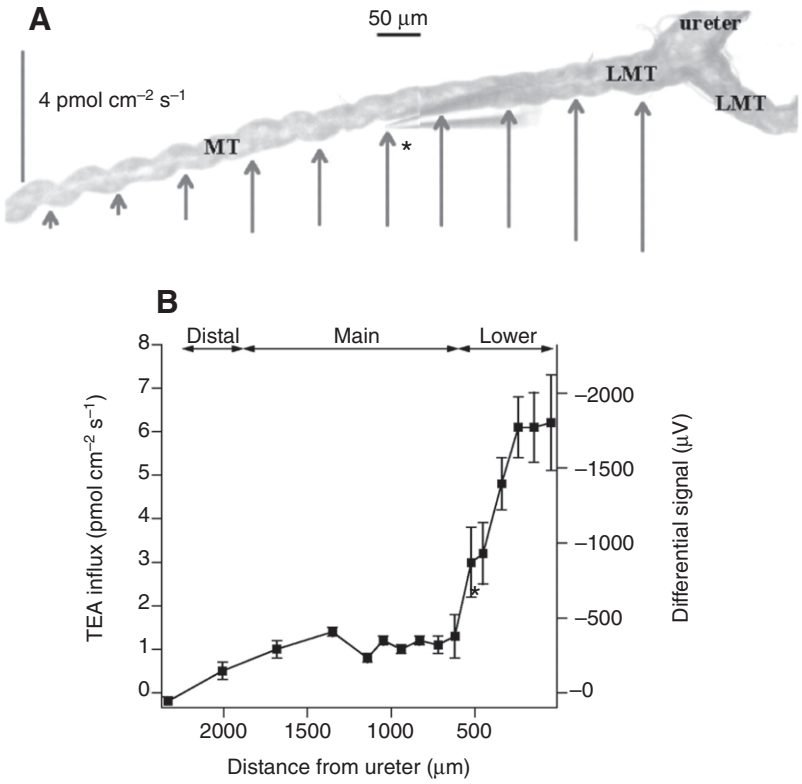

Fig. 5. (A) Representative scan of TEA flux at locations along the secretory main segment of the Malpighian tubule (MT) and the LMT. Tubules were bathed in saline containing 100 μmol l^{-1} TEA. Part of the other LMT of the pair of tubules and the common ureter are shown. The tip of the TEA-selective microelectrode is located just above the asterisk. At each site, indicated by arrows, the software calculated the TEA-specific signal differences (ΔV; μV) between the two limits of microelectrode excursion by subtracting the voltage at the outer limit of the excursion from that measured at the inner limit. The length of each arrow corresponds to the magnitude of TEA influx. (B) TEA influx is plotted as a function of distance from the ureter along lower, main and distal segments of the MT. Each point is the mean ± s.e.m. of four tubules. Influx of TEA reduces TEA concentration in the unstirred layer adjacent to the surface of the tissue, and the corresponding voltage difference is therefore negative. Distance 0 on the abscissa corresponds to the junction of the ureter and the LMT. Both the differential signal recorded by the TEA-selective microelectrode (right ordinate) and the calculated TEA influx (left ordinate) are shown. Figure is replotted from Rheault and O'Donnell (Rheault and O'Donnell, 2004).

further protected by P-glycoprotein-like transporters in the blood–brain barrier (Murray et al., 1994). Clues to the molecular nature of the P-glycoprotein-like pump have been provided by analysis of *Drosophila*. Three MDR genes (*MDR49*, *MDR50*, *MDR65*) are found in *Drosophila* and there is expression along much of the length of the gut and the Malpighian tubules, again pointing to a significant role for the gut in the transport of P-glycoprotein substrates. MDR expression is enhanced by treatments such heat shock (Tapadia and Lakhotia, 2005).

Secretion of type I organic anions

Early work by Simon Maddrell and colleagues demonstrated that rates of secretion of organic anions by the Malpighian tubules of *Rhodnius* increase in response to the appearance in the haemolymph of the digestion products of proteins in the days following a blood meal (Maddrell and Gardiner, 1975). This finding set the stage for experiments with other species to test the hypothesis that loading the diet with organic anions modulates the capacity of the excretory organs to eliminate organic anions.

Secretion of PAH is sodium dependent in vertebrate renal tissues, where renal secretion of OAs involves tertiary active transport. The Na^+/K^+-ATPase generates a Na^+ gradient favouring entry of Na^+ into the cell. This Na^+ gradient is utilized to drive secondary active uptake of dicarboxylic acids such as α-ketoglutarate into the cell. In turn, cellular α-ketoglutarate is exchanged for extracellular organic anions such as PAH. Preloading the cells with dicarboxylic acids such as α-ketoglutarate tends to stimulate uptake of PAH into the cells (*trans*-stimulation), whereas addition of α-ketoglutarate to the external medium along with PAH reduces PAH uptake into the cells (*cis*-inhibition). Although basolateral uptake of organic anions such as PAH or salicylate is strongly Na^+ dependent in *Drosophila* Malpighian tubules, there is no evidence for *cis*-inhibition or *trans*-stimulation (Ruiz-Sanchez and O'Donnell, 2006). Current models propose that salicylate entry across the basolateral membrane of *Drosophila* Malpighian tubules involves Na^+/salicylate-cotransport. This mechanism is sensitive to the organic anion transport inhibitor probenecid, as well as to the monocarboxylic acid transport inhibitor α-cyano-4-hydroxycinnamic acid. Although some monocarboxylate transporters involve cotransport of H^+ with the monocarboxylate and are stimulated by acidic external pH values, there is no effect of pH on salicylate uptake by *Drosophila* tubules over the pH range 5 to 7. We have therefore proposed that a direct coupling of salicylate entry to that of Na^+ mediates salicylate entry across the basolateral membrane.

Chronic exposure (10 days) of *Drosophila* larvae to dietary salicylate is associated with dramatic effects on fluid and salicylate secretion by isolated Malpighian tubules (Fig. 7). Unexpectedly, there is a 3-fold increase in the basal rate of fluid secretion. This increase appears not to be mediated by the release of diuretic factors into the haemolymph because full responsiveness to first messengers such as the diuretic peptide leucokinin or intracellular second messengers such as cAMP is retained (Ruiz-Sanchez and O'Donnell, 2007b). There are no large changes in the concentration of salicylate in the secreted fluid (Fig. 7B) and the salicylate flux (the product of fluid secretion rate and salicylate concentration in the secreted fluid) increases 3- to 5-fold over the range of 0.05 to 0.5 mmol l^{-1} salicylate in the bathing saline (Fig. 7C).

One consequence of the elevation of fluid secretion rate is that there is less tendency for passive diffusive back-flux of organic anions from the lumen to the haemolymph. Malpighian tubules of *Drosophila* produce very high concentrations of organic anions in the secreted fluid (S) relative to those in the bathing medium (M), so the S/M ratio can exceed 100. It was first pointed out by Simon Maddrell that the Malpighian tubules of *Rhodnius prolixus* are relatively impermeable to organic anions, so there is little diffusion

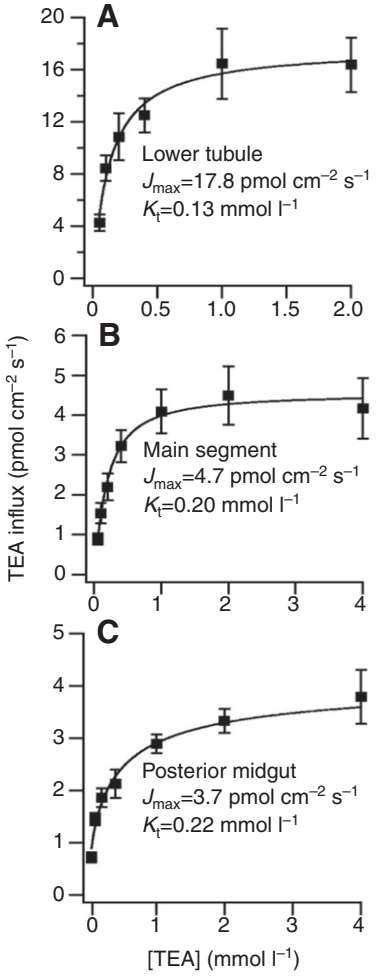

Fig. 6. Plots of TEA influx as a function of bathing saline TEA concentration for (A) the LMT, (B) the main segment and (C) the posterior midgut. Each point is the mean ± s.e.m. of N=4–7 preparations. Values for J_{max} and K_t were determined by non-linear regression analysis as described in Rheault and O'Donnell (Rheault and O'Donnell, 2004), from which this figure is taken.

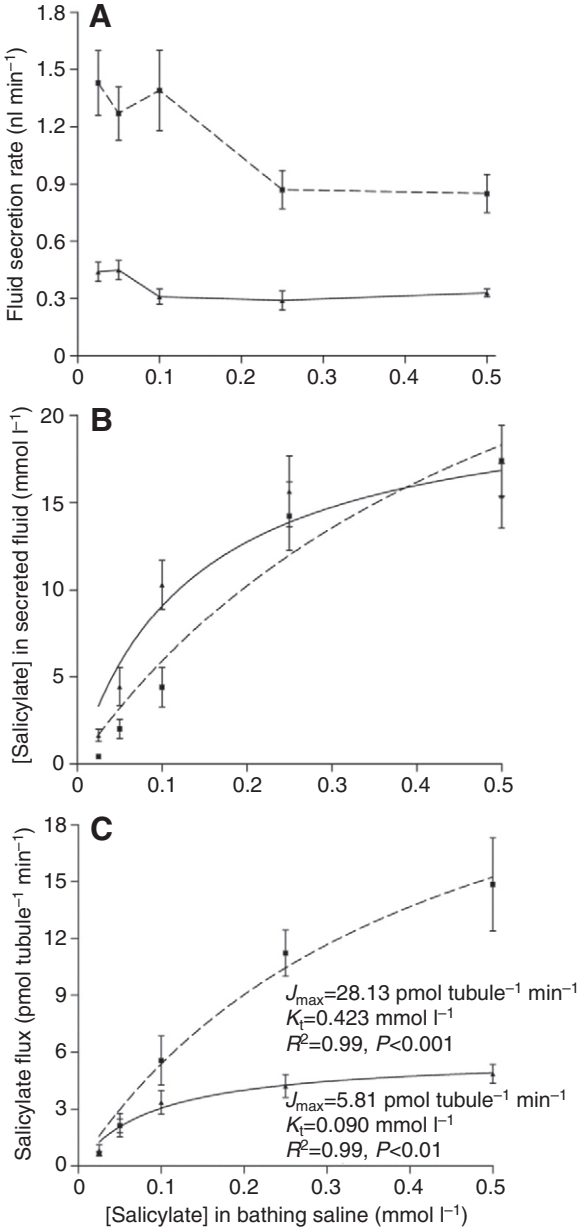

Fig. 7. Effects of chronic exposure of *Drosophila* larvae to dietary salicylate on (A) fluid secretion rate, (B) salicylate concentration in the secreted fluid and (C) transepithelial flux of salicylate across the main segment of isolated Malpighian tubules set up in the Ramsay assay. Each point represents the mean ± s.e.m. of 7–10 tubules. Solid and broken lines indicate the control and experimental group, respectively. Experimental larvae were raised for 10 days on a 10 mmol l^{-1} salicylate-enriched diet. Control larvae were raised on a salicylate-free diet. Figure replotted from Ruiz-Sanchez and O'Donnell (Ruiz-Sanchez and O'Donnell, 2007b).

of these molecules back into the cell after transport into the lumen (Maddrell et al., 1974). As consequence, there is little effect of fluid secretion rate on the net secretion of organic anions. By contrast, secretion of organic anions across the relatively 'leaky' tubules of dipterans such as *Calliphora* is strongly dependent upon fluid secretion rate. Dilution of the organic anions in the lumen by high rates of fluid secretion maintains a lower concentration of solutes in the lumen and minimizes diffusive back flux of an actively transported solute down its concentration gradient. Importantly, for Malpighian tubules isolated from *Drosophila* larvae raised on salicylate-enriched diets, the increase in fluid secretion rate will enhance the elimination not just of salicylate but of any toxin which is actively accumulated in the secreted fluid.

MRPs in insect Malpighian tubules

The MRPs are a group of proteins that appear to be involved in the secretion of compounds such as methotrexate and Texas Red by insect Malpighian tubules. As for P-glycoproteins, MRPs are integral membrane proteins that act as ATP-dependent efflux pumps in tissues such as the gut, kidney, liver and blood–brain barrier. MRPs have extraordinarily broad substrate specificities but are most often associated with the transport of anionic compounds. They also transport some neutral or cationic compounds and also anionic conjugates of amphiphilic compounds with glutathione, glucuronide or sulphate (Bard, 2000).

Evidence for MRP2-like transporters in insect Malpighian tubules was first presented by Karnaky and colleagues (Karnaky et al., 2000; Karnaky et al., 2001; Karnaky et al., 2003) using the fluorescent MRP2 substrate Texas Red (sulphorhodamine 101).

Accumulation of Texas Red in the cells and lumen of the cockroach *Periplaneta americana* and the cricket *Acheta domesticus* is ATP dependent and is reduced by the presence of the non-fluorescent MRP2 substrate chlorodinitrobenzene. The presence of MRP2-like transporters in insect Malpighian tubules is further supported by staining of the apical surface of cricket (or cockroach) MTs with an antibody to a sequence of rat MRP2 (Karnaky et al., 2003).

Isolated Malpighian tubules of the cricket *Teleogryllus commodus* accumulate the MRP2 substrate Texas red in the cells and lumen at concentrations up to 20 and 40 times, respectively, those in the bathing medium (Leader and O'Donnell, 2005). However, quantitative CLSM analysis of fluorochrome transport is not practical for some cricket tubules and most *Drosophila* tubules because opaque concretions that can block or interfere with laser light transmission are present in the cells and lumen. Instead, nanolitre samples of fluid secreted by tubules set up in Ramsay assays can be collected in hollow rectangular glass capillaries. Dye concentration in the samples within the optically flat capillaries can then be measured by CLSM and transepithelial dye flux is calculated as the product of the fluid secretion rate (measured in the Ramsay assay) and dye concentration (Leader and O'Donnell, 2005). The latter study also noted that high concentrations of MRP2 substrates such as Texas Red or inhibitors such as MK571 inhibit fluid secretion by isolated Malpighian tubules. It can therefore be difficult to determine from concentration measurements alone whether an observed increase in luminal dye concentration reflects a change in dye transport *per se* or simply a decrease in the rate of fluid secretion. Calculation of dye flux as the product of fluid secretion rate and dye concentration in collected fluid samples thus provides an independent measurement of the non-specific toxicity of transporter substrates or inhibitors.

In *Drosophila* Malpighian tubules, transporters implicated in Texas Red secretion show a 3-fold lower capacity (J_{max}= 118 fmol min^{-1} tubule^{-1}) but 4-fold higher affinity (K_t=7.1 µmol l^{-1}) than the transporters implicated in secretion of the type I organic anion fluorescein (J_{max}=299 fmol min^{-1} tubule^{-1}, K_t=31.9 µmol l^{-1}) (Leader and O'Donnell, 2005). Nonetheless, the Malpighian tubules can play an important role in eliminating MRP2 substrates from the haemolymph. For example, Texas Red at a concentration of 20 µmol l^{-1} can be cleared from the haemolymph in ~6 min in adult *Drosophila* (Leader and O'Donnell, 2005).

Effects of fluid secretion rate on the transport of organic anions and organic cations

Secretion of MRP2 substrates is enhanced by diuretic factors such as tyramine or their intracellular second messengers such as cAMP or by increasing fluid secretion rates by bathing tubules in hypo-osmotic saline. Significant increases in transepithelial flux are seen only when the dye is present at concentrations close to or greater than the Michaelis–Menten parameter K_t (O'Donnell and Leader, 2006). Regression analysis indicates that 57%–88% of the change in Texas Red flux can be attributed to the change in fluid secretion rate. Tyramine depolarizes the transepithelial potential whereas cAMP produces a hyperpolarization, so the stimulation of dye transport is not dependent upon changes in electrical potential. Nor is there evidence for a direct effect of the stimulants upon MRP2, since dye flux in hyperosmotic saline is associated with a reduction in flux even in the presence of cAMP. Rather, stimulation of Texas Red flux by cAMP or hypo-osmotic saline is an indirect effect, and it is proposed that increases in fluid secretion rate minimize diffusive back flux of the dyes.

A similar relationship is seen for the type I organic anion salicylate; 64% of the change in salicylate secretion in response to stimulation with cAMP, cGMP or leucokinin can be explained on the basis of changes in fluid secretion rate (Ruiz-Sanchez and O'Donnell, 2007a). By contrast, only 7% of the increase in secretion of the organic cation TEA by the Malpighian tubules can be explained on the basis of the increase in fluid secretion rate when the tubules are stimulated with cAMP, cGMP, leucokinin or tyramine (Bijelic and O'Donnell, 2005). For TEA, higher rates of proton transport by the apical V-type H^+-ATPase in stimulated tubules may enhance the availability of H^+ for a process of TEA/H^+ exchange.

Future research

The ground-breaking research on Malpighian tubules initiated by Simon Maddrell more than 40 years ago has had far-reaching implications for our current understanding of epithelial transport mechanisms and detoxification in insects. Recent genetic studies have led to the development of extraordinarily useful tools such as FlyAtlas for studies of *Drosophila* Malpighian tubules (Chintapalli et al., 2007). In conjunction with the use of knockouts for specific transporters available through the Vienna Drosophila RNAi Centre, these resources should allow us to identify more clearly which transporters are involved in the elimination of specific toxins. Dow and Davies (Dow and Davies, 2006) have highlighted the abundance of solute transporter genes which are expressed in the tubules. For example, it will be of interest to knock out expression of genes for specific transporters such as *Drosophila* MRP in the tubules and examine the effects on the transport of MRP2 substrates, with a view to determining which transporters are most important.

It will also be important to examine the links between phase I detoxification pathways (such as P450 enzymes), phase II conjugation reactions (such as those mediated by glutathione-S-transferases) and phase III elimination pathways. It will be of interest, in particular, to test the hypothesis that exposure to a toxin leads to a coordinated up regulation of both detoxification pathways and phase III elimination pathways. An understanding of these processes may contribute to the design of novel and environmentally benign control measures for pest species of insect.

Research in the author's laboratory is supported by the Natural Sciences and Engineering Research Council (Canada).

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