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Ion-selective microelectrode analysis of salicylate transport by the Malpighian tubules and gut of *Drosophila melanogaster*

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Summary

Transport of the organic anion salicylate by the Malpighian tubules and gut of larval and adult fruit flies was studied using two salicylate-selective microelectrode methods. The first method combined the high selectivity of tridodecylmethylammonium-based electrodes for salicylate with the self-referencing ion-selective microelectrode technique for non-invasive spatial and temporal analysis of salicylate flux. Measurements with this technique revealed secretion of salicylate across the main and distal segments of the Malpighian tubule as well as the midgut, ileum and rectum. The second method used a salicylate-selective microelectrode to measure the concentration of salicylate in fluid droplets secreted by isolated *Drosophila* Malpighian tubules set up in a Ramsay secretion assay. Transepithelial salicylate flux was calculated as the product of fluid secretion rate and secreted fluid salicylate concentration. Measurements with this method revealed that salicylate transport was active and saturable; the kinetic parameters J_{max} and K_t were 2.72 pmol min^{-1} tubule^{-1} and 0.046 mmol l^{-1}, respectively. Measurements of transepithelial salicylate flux determined by both microelectrode methods were in good agreement. Transepithelial flux measurements measured by microelectrodes were also validated by comparing them with measurements of radiolabelled salicylate levels in secreted droplets. Salicylate concentrations in haemolymph samples were measured with salicylate-selective microelectrodes after injection of salicylate into the haemocoel or after insects were fed salicylate-rich diets. The rate of salicylate secretion by Malpighian tubules *in vitro* was sufficient to account for the measured rate of decline of salicylate concentration in the haemolymph *in vivo*.

Key words: salicylate, ion-selective microelectrode, organic anion transport, Malpighian tubule.

Introduction

A wide range of charged, potentially toxic xenobiotics as well as endogenous organic ions are transported into the lumen by renal tissues of vertebrates and invertebrates. Organic anions include endogenous compounds such as bilirubin, cyclic nucleotides, folates and prostaglandins. In addition, many plant secondary chemicals or anthropogenic compounds such as salicylate, indigo carmine and the herbicide 2,4,5-trichlorophenoxyacetic acid are organic anions. Excretion of xenobiotics in insects is accomplished primarily by the Malpighian tubules (MTs). Two organic anion transporters, one that transports carboxylate compounds, such as para-aminohippuric acid (PAH) and fluorescein, and another that transports sulphonates, such as amaranth and indigo carmine, have been identified on the basis of competition experiments in MTs of the blood-feeding hemipteran *Rhodnius prolixus* (Maddrell et al., 1974) and the fruit fly *Drosophila melanogaster* (Linton and O'Donnell, 2000). However, in tubules of the cockroach *Blaberus giganteus* it has been suggested that there is either one common carrier for organic acids or two carriers with overlapping substrate affinities (Bresler et al., 1990). The possible role of the gut in transport of organic anions has not been examined in any of these species.

The present study addresses three questions concerning organic anion transport by insect epithelia. (1) Do Malpighian tubules of the fruit fly *Drosophila melanogaster* transport the organic anion salicylate? Salicylate is of particular interest for studies of organic anion transport by insect tissues because it is a plant-produced signal that activates plant defence genes after herbivory or pathogen attack. In insects, salicylate is also known to activate cytochrome P450 genes that are associated with detoxification, thereby protecting the insect against toxins produced by host plants (Li et al., 2002). (2) Does the gut of the fruit fly transport salicylate? We have recently shown that organic cations, such as tetraethylammonium, are transported from the haemolymph to the lumen not only by the Malpighian tubules but also across the posterior midgut (Rheault and O'Donnell, 2004). It is therefore of interest to determine if regions of the gut contribute to elimination of salicylate from the haemolymph. (3) Can transport of salicylate be measured using ion-selective microelectrodes? Radiolabelled or fluorescent compounds have been widely used in studies of

organic anion transport by tissues, such as the vertebrate kidney, crustacean antennal gland, and insect Malpighian tubule (Bresler et al., 1990; Pritchard and Miller, 1991; Linton and O'Donnell, 2000). Our paper describes measurement of organic anion transport using rapid and low-cost microelectrode methods that exploit the high selectivity of the anion exchanger tridodecylmethylammonium (TDMA) for the organic anion salicylate. Macroscopic (~1 cm diameter) electrodes based on lipophilic quaternary ammonium salts such as TDMA chloride have previously been used to measure the concentrations of salicylate (Rover et al., 1998; Creager et al., 1995), penicillin (Yao et al., 1989) and heparin, a negatively charged polysaccharide (Ma et al., 1993). These electrodes are typically >2000 times more selective towards salicylate than to Cl^- (e.g. Rothmaier et al., 1996), the predominant inorganic anion in intracellular and extracellular fluids.

Our first method combines the high selectivity of TDMA-based electrodes for salicylate and the self-referencing ion-selective microelectrode technique for non-invasive spatial and temporal analysis of ion flux (Smith et al., 1994; Piñeros et al., 1998). A salicylate-selective self-referencing microelectrode is moved between two positions within the unstirred layer adjacent to the surface of a tissue. Salicylate transport by the tissue perturbs the salicylate concentration in the unstirred layer, and the difference in salicylate concentrations measured between the two microelectrode positions can be converted into a corresponding salicylate flux using the Fick equation. We have used this method to assess spatial and temporal variations in salicylate flux in different regions of the MTs and gut.

Our second method uses a salicylate-selective microelectrode to measure the concentration of salicylate in fluid droplets secreted by isolated insect Malpighian tubules set up in a Ramsay secretion assay. Flux across the entire tubule can be calculated from the product of secretion rate and secreted droplet salicylate concentration. We have also used salicylate-selective microelectrodes to measure changes in salicylate concentration in haemolymph collected after animals have been fed salicylate-enriched diets or after salicylate has been injected into the haemocoel.

Materials and methods

Animals

Oregon R strain of *Drosophila melanogaster* Meigen were maintained in laboratory cultures according to procedures described by Ashburner (1989). All experiments were carried out at room temperature (21–25°C) and ambient humidity using female flies, 3–7 days post-emergence or 3rd instar larvae.

Dissection and Ramsay assay

Malpighian tubule dissection and application of the Ramsay assay for measurement of fluid secretion rates have been described previously (Dow et al., 1994). The four Malpighian tubules are arranged as an anterior and posterior pair. Each tubule in the anterior pair consists of a distal segment, a secretory main segment and a reabsorptive proximal segment. The lumen of the distal segment is filled with Ca^{2+}-rich concretions (Dube et al., 2000) but does not secrete fluid or K^+ (Dow et al., 1994; Rheault and O'Donnell, 2001). When bathed in saline containing 20 mol l^{-1} K^+ and 132 mmol l^{-1} Na^+ the main segment secretes a near-isoosmotic fluid containing ~120 mmol l^{-1} K^+ and ~30 mmol l^{-1} Na^+ (O'Donnell and Maddrell, 1995). The proximal (lower) segment secretes Ca^{2+} into the lumen, acidifies the lumenal fluids and reabsorbs K^+, Cl^- and water (O'Donnell and Maddrell, 1995). The posterior tubules are identical except that they lack a distal segment. The anterior and posterior pairs of MTs are each connected to the hindgut through a short ureter. Each pair of Malpighian tubules joined by a common ureter was dissected out under *Drosophila* saline consisting of (in mmol l^{-1}): 117.5 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 20 glucose, 10 L-glutamine, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4 and 8.6 Hepes. The saline was titrated with NaOH to pH 7. The addition of glutamine has been found to maintain higher and stable rates of fluid secretion for prolonged periods (>2 h; Hazel et al., 2003). Pairs of isolated tubules were transferred on fine glass probes from the dissecting saline to 20 µl droplets of saline under paraffin oil. One tubule of each pair was pulled out of the bathing droplet and wrapped around a fine steel pin. The lower tubule and ureter were positioned outside of the bathing saline, so that the composition of the secreted fluid was determined by transport activity of the main segment only. The lower tubule was readily identified by the absence of stellate cells. Secreted droplets formed at the end of the ureter and were collected with a fine glass probe. Droplet diameters (d) were measured using an ocular micrometer, and droplet volume (nl) was calculated as $\pi d^3/6$. Secretion rate (nl min^{-1}) was calculated by dividing the droplet volume by the time (min) over which the droplet formed.

To collect fluid from tubules in which the lower tubule was also positioned inside the bathing droplet, an alternative preparation was used (O'Donnell and Maddrell, 1995). By dissecting out all four tubules plus a very short connecting section of gut, it was possible to collect fluid from two whole tubules, including both the main segments and the lower Malpighian tubule. One pair of tubules was positioned inside a 40 µl bathing saline droplet. One tubule of the other pair was removed and discarded, and the remaining tubule was pulled out into the paraffin oil and used to anchor the preparation. Fluid was thus collected after it had passed through the entire length of two tubules upstream of their common ureter.

Salicylate-selective self-referencing (Sal-SeR) microelectrodes

Technical and theoretical aspects of self-referencing microelectrodes have been described previously (Smith et al., 1994; Piñeros et al., 1998). Applications of the technique to the study of K^+ and tetraethylammonium transport by isolated insect Malpighian tubules have been described previously in

Rheault and O'Donnell (2001) and Rheault and O'Donnell (2004), respectively.

Procedures for microelectrode construction were similar to those described previously for measurement of K^+ flux using valinomycin-based K^+-selective microelectrodes (Rheault and O'Donnell, 2001). Micropipettes were pulled to tip diameters of ~5 μm on a programmable puller (P-97 Flaming-Brown, Sutter Instrument Co., Novato, CA), silanized by treatment with N,N-dimethyltrimethylsilylamine (200°C, 30 min), cooled and then stored in an air-tight chamber over desiccant until use. Immediately prior to use, microelectrodes were back-filled with 150 mmol l^{-1} KCl. The KCl solution was forced to the tip by positive pressure and the microelectrode tip was then front-filled with a short column length (~100 μm) of the ion exchanger cocktail, which consisted of 9% (w/v) TDMA Cl (Fluka, Buchs, Switzerland) in 2-nitrophenyl octyl ether. The reference electrode consisted of a 10 cm long, 1.5 mm diameter glass capillary tube (TW150-4) filled with a mixture of 3 mol l^{-1} KCl and 1% agar inserted into a microelectrode holder Ag-AgCl half-cell filled with 3 mol l^{-1} KCl.

Selectivity coefficients for TDMA-based microelectrodes were calculated by the separate solutions method (Ammann, 1986) for salicylate relative to the anions found in *Drosophila* saline (Cl^-, HCO_3^-, $H_2PO_4^-$). Selectivity coefficients were also determined for two substrates of organic anion transport systems (PAH and cyclic adenosine monophosphate). All solutions were prepared at 0.1 mol l^{-1} and neutral pH. All salicylate calibration solutions were made up in *Drosophila* saline containing 158.5 mmol l^{-1} Cl^-. This method is referred to as the 'unorthodox' (Thomas, 1978) or 'empirical' method (Vaughan-Jones and Aickin, 1987) of correction for the effects of interfering ions on electrode response.

The development of the Sal-SeR microelectrode system has been referred to in a recent review of insect epithelial anion transport (O'Donnell et al., 2003) but the details of the system's use for analysis of salicylate flux have not been described previously. The arrangements of the electrode, positioning system, signal amplifier and data acquisition are shown in schematic form in Fig. 1. The Sal-SeR microelectrode was connected through a chlorided silver wire to the amplifier head stage, which was mounted on a set of translator stages (Newport Corp., Fountain Valley, CA, USA). An orthogonal array of computer-controlled stepper motors connected to the translator stages (CMC-4, Applicable Electronics Inc., Forrestdale, MA, USA) moved the microelectrode in three dimensions with submicron accuracy and repeatability. At each measurement site, the electrode was moved perpendicular to the tissue surface between two positions separated by 100 μm. Voltage measurements taken at the limits of the excursion were amplified 1000-fold (IPA-2 ion/polarographic amplifier, Applicable Electronics Inc.) and used to calculate a voltage difference over the excursion distance of the microelectrode. This differential signal was then converted into a salicylate concentration difference using a standard microelectrode calibration curve that related voltage output to salicylate concentration in saline. The highly sensitive self-referencing system resolves voltage differences as small as 10 μV, corresponding to differences in salicylate concentration (in bathing saline containing 500 μmol l^{-1} salicylate) as small as 0.04%. The Sal-SeR microelectrode was viewed using an inverted microscope equipped with a video camera and the 'move, wait and sample' protocol at each measurement site was controlled through a PC running Automated Scanning Electrode Technique (ASET) software (Sciencewares, East Falmouth, MA, USA). The Sal-SeR microelectrode tip was first 'moved' to a site 10 μm from the tissue surface. The microelectrode then remained stationary during the 9 s 'wait' period to allow ion gradients near the tubule to re-establish after the localized stirring during the movement period. No data were collected during the wait period. The microelectrode voltage was recorded and averaged for 1 s during the 'sample' period. The probe was then moved to the other extreme of the 100 μm excursion, followed by another wait and sample period. Each move, wait and sample cycle at each extreme of probe excursion was complete in 10 s. Each flux determination required measurement of the concentration difference between the two extremes of probe excursion, for a total of 20 s. Fluxes are reported as an average of 3–5 repetitive measurements at each site.

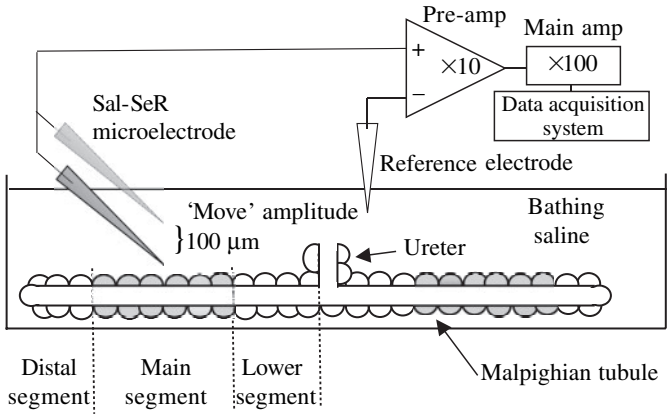

Fig. 1. The arrangements for recording salicylate flux using salicylate-selective microelectrodes based on the ion exchanger tridodecylmethylammonium chloride. (A) The tip of a salicylate self-referencing microelectrode (Sal-SeR) microelectrode is moved from a position 10 μm away from the surface of the Malpighian tubule epithelium to a position 100 μm farther away by computer-controlled stepper motors. The differential signal between the two positions is amplified 10× in the head stage and a further 100× in the connected amplifier for a total voltage amplification of 1000×. An associated PC-based data acquisition system running ASET software records the voltages and controls the stepper motors for microelectrode positioning and movement. The relative locations of the three segments of an anterior pair of Malpighian tubules and the connecting common ureter are indicated. Further details in text.

For salicylate flux measurements with Sal-SeR microelectrodes, tissues were transferred after dissection to 35 mm diameter Petri dishes filled with 4 ml of saline. Dishes were pre-coated with 100 μl droplets of 125 μg ml^{-1} poly-L-lysine and air dried before filling with saline to facilitate

adherence of the tubules to the bottom of the dish. Fluxes were measured typically at 3–8 sites in the field of view (550 μm at 10× magnification). The preparation was then moved so that sites in an adjacent region of the gut or MT could be scanned.

Voltage differences (ΔV, μV) obtained over the amplitude of the Sal-SeR microelectrode excursion were converted to a salicylate concentration difference (ΔC, μmol cm^{-3}) using the equation:

$$\Delta C = 2.3\ (\Delta V C_B) / S\ ,$$

where ΔV is the differential signal measured over the amplitude of electrode excursion, C_B is the background concentration of salicylate in the medium at a distance of >1000 um from the preparation (μmol cm^{-3}) and S is the slope (μV) of the electrode measured in response to a 10-fold change in salicylate concentration. Derivations of this equation are given by Kuhtreiber and Jaffe (1990) and Smith et al. (1994). Except where noted, C_B was 0.5 μmol cm^{-3} in all experiments. Values of ΔC were converted into corresponding fluxes by substitution into the Fick equation:

$$J_{Salicylate} = D\ (\Delta C / \Delta r)\ ,$$

where $J_{Salicylate}$ is the flux of salicylate (mol cm^{-2} s^{-1}), D is the diffusion coefficient for salicylate at 25°C (0.959×10^{-5} cm^2 s^{-1}; Lide, 2002) and Δr is the amplitude of electrode excursion (cm).

Measurement of salicylate flux in Ramsay secretion assays

Secreted fluid droplets were collected under paraffin oil using the Ramsay assay and the concentration of salicylate was determined using either salicylate-selective microelectrodes or liquid scintillation counting of ^{14}C-labelled salicylate.

The arrangements of the electrodes and recording system are shown in schematic form in Fig. 2. Micropipettes were silanized, backfilled with 150 mmol l^{-1} KCl and front filled with TDMA Cl as described above for Sal-SeR microelectrodes. Displacement of the ion exchanger cocktail by paraffin oil was prevented by coating the tip of each microelectrode with a thin (~1 μm) layer of poly vinyl chloride (PVC), as described previously for use of tetraethylammonium-selective microelectrodes under paraffin oil (Rheault and O'Donnell, 2004). The reference microelectrode electrode had a tip diameter of ~1 μm and was filled with 150 mmol l^{-1} KCl. Both the salicylate-selective microelectrode and reference microelectrode were connected through chlorided silver wires to a high impedance (>10^{15} Ω) electrometer, which in turn was connected to a computerized data acquisition and analysis system (Axotape, Burlingame, CA, USA).

For experiments with [^{14}C]-labelled salicylate (31 mCi mmol^{-1}), isolated tubules were set-up in 40 μl droplets of *Drosophila* saline containing salicylate at a final concentration of 33 μmol l^{-1}. Secreted fluid droplets were collected after 60 min, secretion rates determined as above and the concentration of salicylate was measured by liquid scintillation counting in a LKB Wallac 1217 Rackbeta Liquid Scintillation Counter.

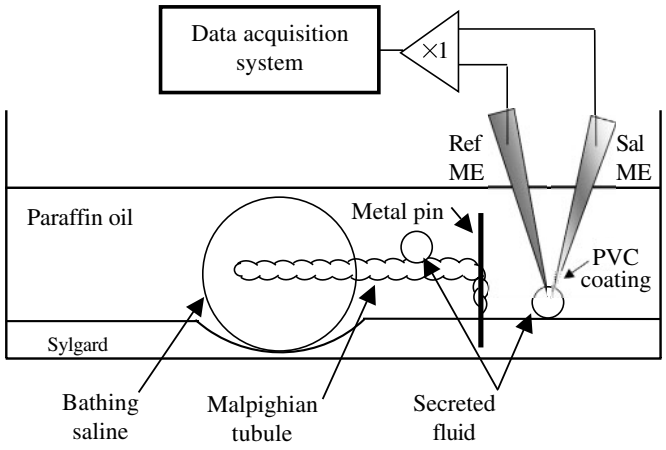

Fig. 2. Use of salicylate-selective microelectrodes for measurement of the concentration of salicylate in droplets of fluid secreted by isolated Malpighian tubules in the Ramsay assay. An isolated pair of Malpighian tubules is placed in a droplet of bathing saline under paraffin oil. One Malpighian tubule 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 collect at the ureter which is positioned just outside the bathing saline droplet. Secreted fluid droplets are collected on glass rods and placed on the bottom of the dish adjacent to calibration droplets containing known concentrations of salicylate in *Drosophila* saline. For each droplet, the potential difference between the salicylate-selective microelectrode (Sal ME) and the reference microelectrode is measured by a unity gain high impedance (>10^{15} Ω) operational amplifier. Voltages are digitized and recorded on a PC-based data acquisition system. Salicylate concentration in the secreted droplets is calculated from the voltage difference between the secreted droplet and the calibration droplets, as described in the methods.

Salicylate flux was calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid salicylate concentration (mmol l^{-1}) determined using either technique described above.

Injection of salicylate into the haemocoel

Third instar larvae were secured to the bottom of a Petri dish using double-sided tape. A 1 ml plastic tuberculin syringe pulled out to a fine tip over a low flame was used to transfer 0.1 μl of 100 mmol l^{-1} salicylate from a Gilson micropipette into the tip of a glass micropipette whose tip was broken back to a diameter of ~3 μm. The micropipette was mounted on a micromanipulator and the tip was introduced into the haemocoel of the larva observed under a dissecting microscope. Air pressure supplied by a 60 ml syringe connected to the back of the micropipette through plastic tubing was used to expel the salicylate solution into the haemocoel.

Measurement of chloride and salicylate concentrations in haemolymph samples

Chloride concentration in haemolymph samples was measured using Cl^--selective microelectrodes based on

chloride ionophore I, cocktail A (Fluka) and backfilled with 500 mmol l^{-1} KCl. The microelectrodes were front-filled with the cocktail and coated with PVC as described above. The reference microelectrode was backfilled with 1 mol l^{-1} Na^+ acetate near the tip and 1 mol l^{-1} KCl in the upper part of the barrel.

For Cl^- measurements, third instar larvae were removed from the culture tubes, washed for 5–10 s in 5 ml distilled water and dried on tissue paper. The larvae were transferred to a Petri dish filled with Paraffin oil and the cuticle was torn with forceps to permit the escape of haemolymph. Care was taken to avoid damaging the gut. Haemolymph samples were transferred by micropipette to another oil-filled dish containing calibration droplets (10 mmol l^{-1} and 100 mmol l^{-1} KCl).

For salicylate measurements, haemolymph samples were collected from larvae that had been injected with salicylate or fed salicylate-rich or control diets. Haemolymph samples were collected from larvae that had been washed and dried as above, then secured to a 35 mm Petri dish with double-sided tape. This permitted collection of multiple haemolymph samples from the same larvae by inserting a glass micropipette into the haemocoel under the control of a micromanipulator and applying suction using a syringe connected to the back of the micropipette through plastic tubing. Salicylate concentration was measured using the same procedures as for secreted fluid samples except that the calibration solutions of 5, 0.5 and 0.05 mmol l^{-1} salicylate were made up in 30 mmol l^{-1} KCl.

Calculations and statistics

Values are expressed as mean ±S.E.M. Two-sample F-tests were used to compare the variances of the data for the control and experimental groups. Depending on the outcome of each F-test, differences between experimental and control groups were compared using unpaired Student's *t*-tests appropriate for data with either equal or unequal variances. Fluid secretion rates of isolated tubules in salines containing different concentrations of salicylate were analysed by one-way ANOVA (analysis of variance) with Dunnett's *post hoc* test (GraphPad InStat version 3.05, GraphPad Software, San Diego CA). Differences were considered significant if $P<0.05$. The responses of the same group of tubules or guts before and after an experimental treatment were compared using a paired *t*-test. Dose-response curves relating salicylate flux or secreted fluid salicylate concentration to bathing saline salicylate concentration were fitted by non-linear regression (SigmaPlot 2000, SPSS Inc., Chicago).

Results

Characteristics of salicylate-selective microelectrodes

Microelectrodes based on the ion exchanger tridodecylmethylammonium chloride showed a high selectivity for salicylate relative to the other anions present in *Drosophila* saline, haemolymph or secreted fluid collected from isolated Malpighian tubules. For chloride, the predominant anion in *Drosophila* saline, the selectivity coefficient K_{SalCl} was $10^{3.3}$, indicating that the electrode was ~2000 times more selective for salicylate than Cl^-. Based on the assumption that transepithelial Cl^- flux is approximately equal to that of K^+ (400 pmol cm^{-2} s^{-1}; Rheault and O'Donnell, 2001), application of the Nicolsky-Eisenman equation (Ammann, 1986) indicates that the interference of Cl^- flux on salicylate-selective microelectrodes was less than <1 μV, which is less than 0.2% of the typical differential signal (>400 μV) recorded by the Sal-SeR microelectrode in the experiments described below. Selectivity coefficients (in brackets) for salicylate relative to other inorganic or organic anions were: HCO_3^- ($10^{4.2}$); $H_2PO_4^-$ ($10^{4.7}$); PAH ($10^{3.8}$); cAMP ($10^{4.5}$).

The high selectivity of TDMA-based electrodes for salicylate relative to other anions was also indicated by the electrode slopes for a 10-fold change in salicylate concentration for solutions made up in *Drosophila* saline, which contains 158.5 mmol l^{-1} Cl^-, 10.2 mmol l^{-1} HCO_3^- and 4.2 mmol l^{-1} $H_2PO_4^-$. Slopes were 59.3+0.4 mV (N=16) between 10 and 1 mmol l^{-1} salicylate, 59.2±0.4 (N=15) between 5 and 0.5 mmol l^{-1} salicylate and 54.7±0.2 between 2.5 and 0.25 mmol l^{-1} salicylate (N=7). The slope was 31.9±1.3 mV between 0.5 and 0.05 mmol l^{-1} salicylate (N=15) in *Drosophila* saline containing 158.5 mmol l^{-1} Cl^-. However, the slope between 0.5 and 0.05 mmol l^{-1} salicylate was 56.5±1.7 (N=5) in 30 mmol l^{-1} KCl. The latter solution was chosen to represent the upper limit of haemolymph Cl^- levels in the larvae (see below).

The relatively large tip diameter (~5 μm) of the microelectrodes used in this study and the low resistivity of ion exchanger-based microelectrodes (relative to neutral carrier-based ion-selective microelectrodes) contributed to rapid response times. The 90% response times for Sal-SeR microelectrodes used in this study were typically in the range of 0.3–0.7 s. Electrode noise based on the scatter of measurements with self-referencing electrodes in saline containing 0.5 mmol l^{-1} salicylate was typically of the order of ±30 μV in for individual measurements, but was reduced to ±20 μV for averages of three measurements and to ±10 μV for averages of five measurements (Fig. 3). At the lowest noise level, changes in salicylate concentration of less then 0.04% could be resolved. Salicylate flux was calculated after subtracting the noise at a reference position 1 mm or more from the preparation from the differential signal measured at the site of interest near the preparation. This subtraction corrects the signals measured near the preparation for any minute regional variations in salicylate concentration in the bathing dish. The typical signal to noise ratio was in the range of 10–50.

Measurement of salicylate flux using the Sal-SeR microelectrode

A representative recording of salicylate flux across the main segment of an isolated Malpighian tubule is shown in Fig. 4A. In these experiments, a positive differential signal denotes a decrease in the salicylate concentration of the unstirred layer near the basolateral surface of the tubule. This decrease reflects

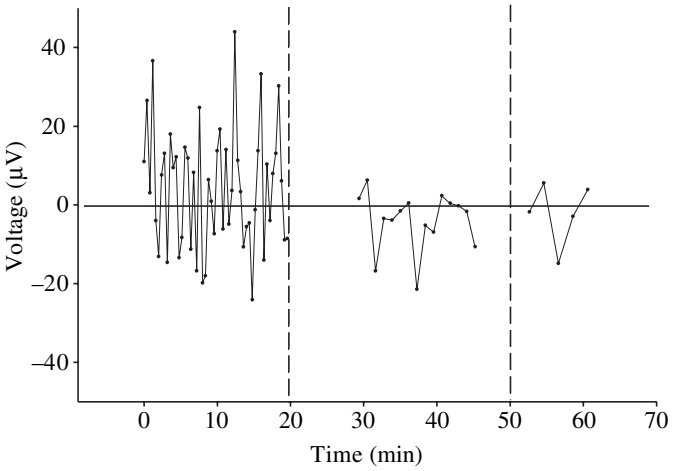

Fig. 3. Sal-SeR microelectrode voltage recorded over an excursion of 100 μm in *Drosophila* saline containing 0.5 mmol l^{-1} salicylate. Each point indicates the differential signal recorded between the excursion limits of 100 μm, using the 'move, wait, sample' protocol described in Materials and methods. In the absence of any gradients in salicylate concentration the voltage is indicative of the noise of the electrode and measurement system. Variation in the signal is reduced when individual measurements (0–20 min) are compared with averages of three (30–45 min) or five (52–60 min) measurements.

secretion of salicylate from the bath into the tubule (i.e. salicylate influx). In general, there was a slight increase in the magnitude of the differential signal at all sites over the 2 h recording period, but the data indicate substantial spatial and temporal heterogeneity. Although most of the sites in Fig. 4A showed relatively constant differential signals for periods of 40–60 min, signals at some sites varied substantially over time. Increases in differential signal were more dramatic in the three left-most sites in Fig. 4A. In Fig. 4B,C, the electrode signal has been plotted as a function of time for eight sites on two different tubules. Fig. 4B,C shows that substantial variations in differential signal occurred over distances which were only a few times larger than the diameter of the principal cells in the tubules (~30 um). The largest differential signal (site 1 in Fig. 4B) exceeded the smallest signal (Site 4) more than 7-fold. The two sites (1 and 2) with the highest initial signals in Fig. 4B also showed oscillations in the differential signal. There were peaks in the signals near 0, 30 and 80 min and troughs near 20 and 60 min. In Fig. 4C, there was a small but steady increase in differential signal at site 1, whereas there was a pronounced peak in signal between 30 and 70 min for sites 3 and 4. The significance and possible mechanisms of temporal and spatial variations in differential signal are discussed below.

Measurements of differential signal and calculated salicylate flux in different segments of the Malpighian tubule and gut are summarized in Fig. 5. There was a small but significant influx of salicylate in the distal segment of the anterior tubules, and the largest fluxes were observed in the secretory main segment of the tubule. There was evidence of an efflux of salicylate (from tubule lumen to bathing saline) in the lower Malpighian tubule.

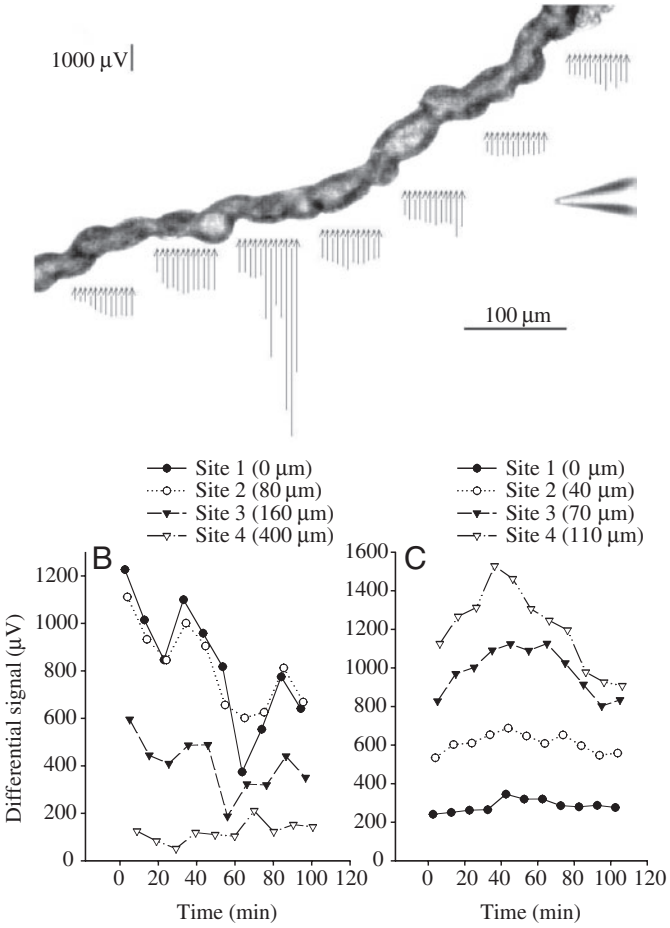

Fig. 4. (A) Representative scan showing spatial and temporal heterogeneity of salicylate flux at seven locations along the main segment of the *Drosophila* Malpighian tubule. At each location, the position of the Sal-SeR microelectrode at the epithelial surface is indicated by the arrowhead of the left-most arrow. The arrows corresponding to 11 additional scans made at intervals of 10 min at the same seven sites are indicated by the adjacent arrows, which have been offset to the right for clarity. ASET software calculated the salicylate-specific signal differences 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 mean differential signal based on three measurements at each time interval. (B,C) Plots of differential signal of the Sal-SeR microelectrode as a function of time at four sites in the main segments of two Malpighian tubules. The positions of sites 2–4 relative to site 1 are indicated in the legend. Each point is the mean of three measurements.

Preliminary measurements showed no differences in the fluxes of salicylate across the anterior *versus* the posterior midgut, and the data have been combined. Salicylate influxes in the midgut, ileum and rectum were 21%, 63% and 45%, respectively, of those in the main segment of the Malpighian tubule.

The dependence of salicylate flux on active transport was assessed by comparing flux before and after inhibition of metabolism with the metabolic inhibitor sodium cyanide. Based on measurements at 13 sites in four tubules, salicylate flux changed from 8.3±0.6 pmol cm^{-2} s^{-1} in control saline to

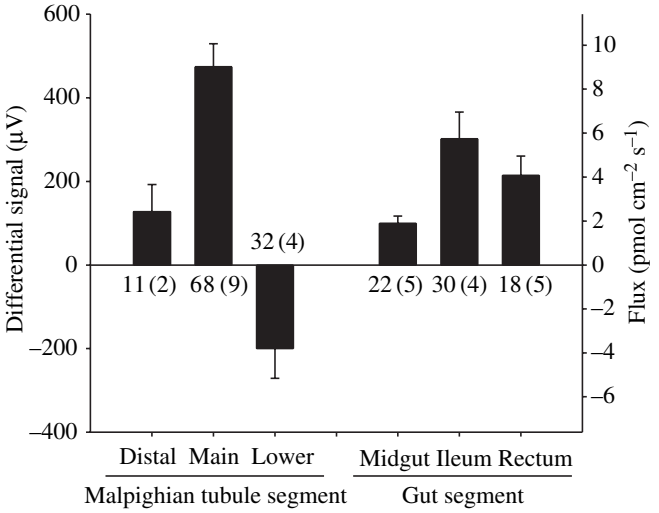

Fig. 5. Salicylate flux (right axis) and differential signal (left axis) in three segments of the Malpighian tubule, and in the midgut, the ileum and the rectum. The height of each bar is the mean ±S.E.M. for the number of sites indicated at the bottom of each bar. The number of different preparations is indicated in brackets. At each site, the salicylate flux was calculated as the mean of five replicate measurements.

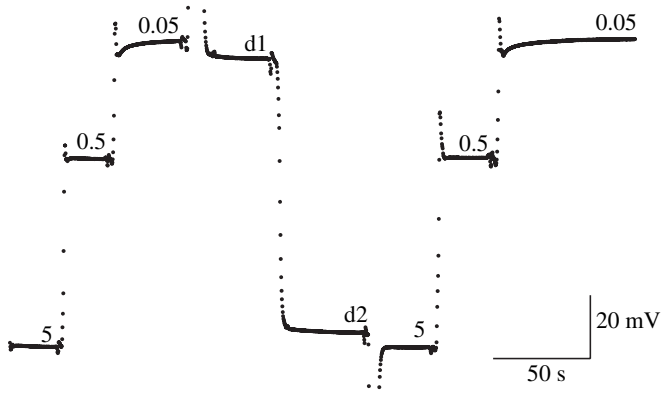

Fig. 6. Sample recording showing the change in electrical potential of a salicylate-selective microelectrode positioned in droplets of secreted fluid or calibration solutions. Microelectrode voltage was sampled at 3 Hz by the data acquisition system. The labels 5, 0.5 and 0.05 refer to calibration solutions containing 5, 0.5 or 0.05 mmol l^{-1} salicylate, respectively, in *Drosophila* saline. d1 indicates the voltage recorded in a droplet that was secreted over 30 min prior to the addition of salicylate to the bathing droplet and indicates the background voltage recorded due to endogenously secreted compounds. d2 indicates the voltage recorded in a droplet secreted 30 min after the addition of 0.05 mmol l^{-1} salicylate to the bathing saline.

–13.2±4.2 pmol cm^{-2} s^{-1} and 1.4±0.3 pmol cm^{-2} s^{-1} by 15 and 30 min, respectively, after addition of 2 mmol l^{-1} NaCN to saline containing 0.5 mmol l^{-1} salicylate. The negative values at 15 min indicate that the flux transiently shifted from an influx to an efflux in response to cyanide. The basis for this effect is discussed below on the basis of the high lumen concentrations of salicylate maintained in metabolically active Malpighian tubules.

The finding of salicylate flux from bath to midgut lumen raised the question of whether salicylate is normally absorbed from the gut into the haemolymph if the insects feed on diets containing this compound. We therefore measured salicylate fluxes across midguts dissected from 3rd instar larvae that had been fed for 24 h on *Drosophila* diet containing 100 mmol l^{-1} salicylate. In saline containing 0.5 mmol l^{-1} salicylate there was a flux of salicylate from lumen to bath of 7.6±2.4 pmol cm^{-2} s^{-1} (N=20 sites in N=5 guts).

Measurements of salicylate flux using the Ramsay assay

A sample recording of the voltage from a PVC-coated TDMA-based microelectrode is shown in Fig. 6. The 90% response time of the PVC-coated microelectrodes used to measure salicylate concentrations in droplets under paraffin oil was ~5 s. The measurements of secreted fluid droplets before and 30 min after addition of 0.05 mmol l^{-1} salicylate are bracketed by measurements of calibration droplets containing 0.05, 0.5 and 5 mmol l^{-1} salicylate in *Drosophila* saline. The voltage recorded in a droplet of fluid secreted by an isolated tubule in the absence of salicylate in the bathing saline (d1) was equivalent to that produced by 0.06 mmol l^{-1} salicylate in *Drosophila* saline. This background represents the interference of other anions in the secreted fluid on the salicylate microelectrode. This background must be subtracted from the signals measured after addition of salicylate to the bathing saline. The voltage recorded in a droplet of secreted fluid (d2) after the addition of 0.05 mmol l^{-1} salicylate to the bathing saline was equivalent to that produced by 4.90 mmol l^{-1} salicylate. The corrected concentration of 4.84 mmol l^{-1} salicylate in the secreted fluid was then calculated by subtracting the background concentration in d1 from d2.

Dose–response curves relating fluid secretion rate for tubules, secreted fluid salicylate concentration and salicylate flux to bathing saline salicylate concentration are shown in Fig. 7. Although salicylate at concentrations below 0.5 mmol l^{-1} had no significant effect on fluid secretion rate for tubules from adult flies (ANOVA, P>0.05), concentrations of 1.25 and 2.5 mmol l^{-1} were inhibitory (Fig. 7A). The maximum concentration of salicylate in the secreted fluid was ~7 mmol l^{-1} (Fig. 7B). At lower bathing saline concentrations (0.0025–0.125 mmol l^{-1}) the concentration of salicylate in the secreted fluid was elevated 50-fold to 100-fold (Fig. 7B, inset). Values of J_{max} (2.72 pmol min^{-1}) and K_t (0.046 mmol l^{-1}) were obtained by fitting the salicylate flux data to the Michaelis–Menten equation (Fig. 7C). It should be noted that these kinetic values represent the steady-state consequence of at least two transport steps operating in series in the tubule epithelium.

Salicylate was also secreted by isolated Malpighian tubules dissected from 3rd instar *Drosophila* larvae. Fluid secretion rates, secreted fluid salicylate concentration and salicylate flux for larval tubules did not differ significantly from the corresponding values for tubules from adult flies (Student's t-tests, P>0.05). Fluid secretion rates for tubules from larvae and adult flies bathed in saline containing 0.05 mmol l^{-1} salicylate

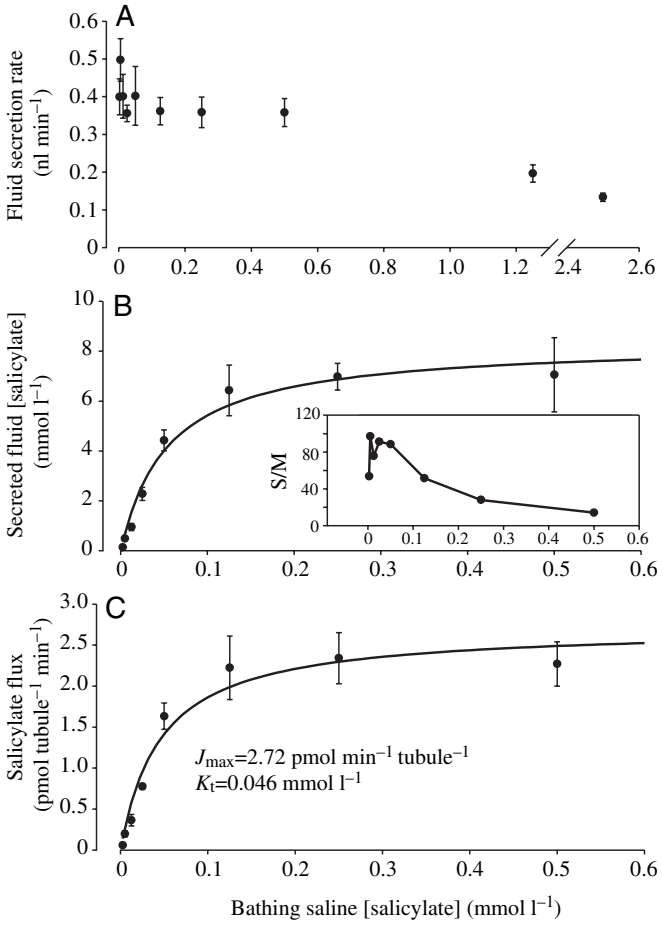

Fig. 7. The effects of bathing saline salicylate concentration on (A) fluid secretion rate, (B) secreted fluid salicylate concentration and (C) salicylate flux. Each point is the mean ±S.E.M. for 6–12 tubules. The inset in B shows the relationship between bathing saline salicylate concentration (mmol l^{-1}) and S/M, the ratio of salicylate concentration in the secreted fluid to that in the bathing medium. Curves in B and C were fitted to the Michaelis-Menten equation using nonlinear regression analysis. J_{max} refers top the maximum rate of transport and K_t to the concentration of salicylate required to produce 50% of the maximum flux.

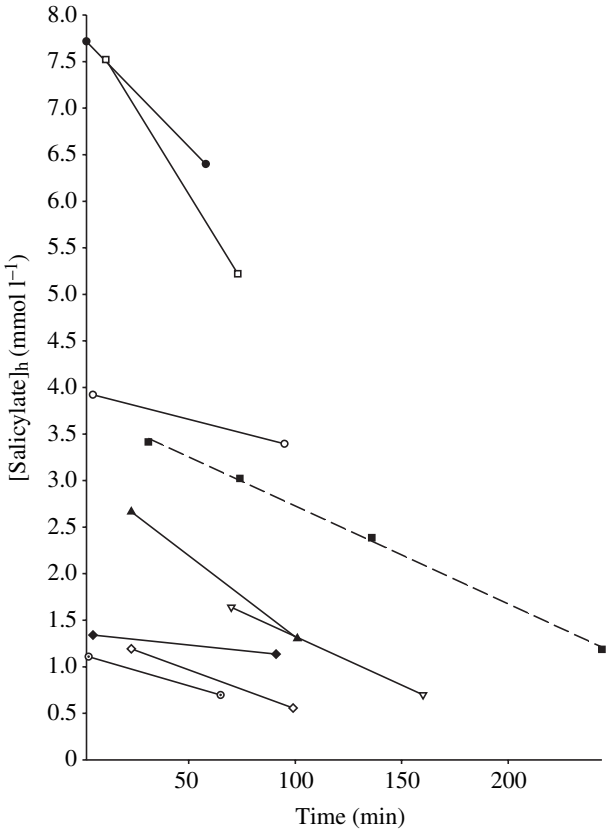

Fig. 8. Salicylate concentration in haemolymph samples collected at the times indicated after injection of 0.1 µl of 100 mmol l^{-1} salicylate into the haemocoel of 3rd instar larvae. Each line indicates a different larva. The dashed line was fit by linear regression (r^2=0.998) and describes the decline in concentration of salicylate in four samples collected from a single larva. The corresponding equation is: [Salicylate]$_h$=–0.0105t+3.78, where [Salicylate]$_h$ is haemolymph salicylate concentration in mmol l^{-1} and t is in minutes.

were 0.53±0.06 nl min^{-1} (N=8) and 0.51±0.06 nl min^{-1} (N=9), respectively. Fluid secreted by tubules of larval and adult flies contained 2.58±0.35 mmol l^{-1} salicylate and 3.13±0.24 mmol l^{-1} salicylate, respectively. Salicylate fluxes across tubules of larval and adult flies were 1.35±0.22 pmol min^{-1} and 1.57±0.16 pmol min^{-1}, respectively.

The Sal-SeR microelectrode experiments indicated that salicylate was transported from bath to lumen across the main segment of the Malpighian tubule, but that there was a loss of salicylate from the lumen to the bath across the lower Malpighian tubule of adult flies (Fig. 5). Transport of salicylate from lumen to bath would be expected to reduce the lumenal concentration of salicylate. This possibility was tested by comparing the concentration of salicylate in fluid secreted by whole tubules with that of the main segments of the same tubules. Whole pairs of tubules set up in a Ramsay assay and bathed in saline containing 0.5 mmol l^{-1} salicylate secreted fluid containing 5.82±0.49 mmol l^{-1} salicylate (N=8). When the tubules were repositioned so that only the main segment of one of the tubules remained in the bathing saline, the secreted fluid contained 7.78±0.97 mmol l^{-1} salicylate. The significant difference (P<0.02; paired t-test) in the concentration of salicylate is consistent with the loss of salicylate from lumen to bath as the fluid passes through the lower Malpighian tubule.

Flux of [^{14}C]-salicylate

The mean salicylate flux for isolated tubules bathed in saline containing 0.033 mmol l^{-1} ^{14}C-labelled salicylate was 0.95±0.10 pmol min^{-1} (N=6). This value is between the values of 0.78±0.04 pmol min^{-1} and 1.63±0.16 pmol min^{-1} based on measurements with salicylate-selective microelectrodes for tubules bathed in saline containing 0.025 and 0.05 mmol l^{-1} salicylate, respectively (Fig. 7). Based on the regression equation fit to the data in Fig. 7C, the flux predicted in a bathing saline concentration of 0.033 mmol l^{-1} salicylate was 1.14 pmol min^{-1}.

Salicylate levels in haemolymph of Drosophila *larvae fed salicylate-rich diets or injected with salicylate*

We also measured salicylate in haemolymph samples collected from 3rd instar larvae fed either the control diet or diet containing 100 mmol l^{-1} salicylate. There was no evidence for increased mortality or effects on pupation or adult emergence in the experimental group relative to the controls. The levels of salicylate in the haemolymph after feeding for 3–6 or 24 h on the salicylate diet were 0.50±0.25 mmol l^{-1} (N=8) and 0.40±0.09 mmol l^{-1} (N=15), respectively. The level of salicylate in the haemolymph increased to 0.93±0.13 mmol l^{-1} (N=7) when larvae were fed on the salicylate diet for 3–6 h and then chilled to 4°C for 30–70 min to reduce metabolic rate. The electrode signal in haemolymph collected from animals on the control diet without salicylate was equivalent to a salicylate concentration of 0.006±0.002 mmol l^{-1} (N=7). This level of interference is less than that of ~0.06 mmol l^{-1} reported above for droplets of fluid secreted by Malpighian tubules (Fig. 6), presumably due to the lower concentration of chloride in the haemolymph (22.3±3.1 mmol l^{-1}; N=9) relative to that in secreted fluid.

The rate of change of salicylate concentration in the haemolymph was measured after larvae were injected with ~0.1 μl of 100 mmol l^{-1} salicylate and two or more samples of haemolymph of ~10 nl each were collected at intervals of 60–100 min (Fig. 8). The mean concentration of salicylate in the first sample of haemolymph was 3.4±0.9 mmol l^{-1} (N=9). The mean rate of decline in haemolymph salicylate concentration, determined from the concentration difference between successive samples, was 0.0136±0.0036 mmol l^{-1} min^{-1} (N=9 larvae). Fig. 8 also shows that haemolymph salicylate concentration declined linearly (r^2=0.998) from 3.4 to 1.2 mmol l^{-1} in four samples collected from a single larva.

Discussion

This paper describes novel microelectrode techniques for analysis of salicylate transport and the application of these techniques to the study of organic anion transport in insects. The techniques are of use *in vivo*, for measurement of salicylate concentration in samples of haemolymph, and *in vitro*, for measurement of salicylate flux across isolated tissues. The results are significant for two reasons. First, the techniques described may be applicable to the study of salicylate transport by isolated tissues of other organisms and to measurement of salicylate concentration in small (nl) volumes of biological fluids. Second, this is the first report of salicylate transport by the Malpighian tubules and gut in an insect. The sections below describe these aspects in more detail.

Salicylate-selective microelectrodes

Microelectrodes based on the anion exchanger tridodecylmethylammonium chloride provide rapid, sensitive and low-cost methods for measurement of salicylate concentration in biological fluids. Salicylate flux can be calculated as the product of fluid secretion rate and secreted fluid salicylate concentration in the Ramsay assay, or it can be measured directly by the Sal-SeR microelectrode technique. Salicylate-selective microelectrodes can also be used to measure salicylate concentrations in the haemolymph after feeding animals salicylate-enriched diets or injecting salicylate into the circulatory system. Such measurements permit estimates of the rates of clearance of salicylate from the haemolymph.

For Malpighian tubules set up in the Ramsay assay the results show good agreement between salicylate flux determined using salicylate-selective microelectrodes and that determined using liquid scintillation counting of radiolabelled salicylate. Dose-response curves relating salicylate flux across isolated Malpighian tubules to bathing saline salicylate concentration can be determined using the Ramsay assay and either radiolabelled salicylate or salicylate-selective microelectrodes. The latter technique is preferable for reasons of cost, time, safety and accuracy. The tip diameter of the PVC coated microelectrodes used in this study (~5 μm) would permit the use of droplets as small as 2–3 times this diameter, corresponding to volume of much less than one nanoliter. In practice, we have readily measured salicylate concentration in 0.1 nl droplets. The maximum specific activity of commercially available radiolabelled salicylate does not permit ready measurement of its concentration in such small volumes. For example, the highest commercially available specific activity of ^{14}C-labelled salicylate is 56 mCi $mmol^{-1}$, equivalent to ~25 cpm for a 1 nl droplet containing 0.2 mmol l^{-1} salicylate. This level of radioactivity is approximately equal to the typical background level (~30 cpm) of radioactivity encountered in liquid scintillation counting of ^{14}C-labelled compounds. By contrast, the salicylate-selective microelectrode maintains a near-Nernstian response down to concentrations of 0.2 mmol l^{-1} salicylate in fluids containing ~150 mmol l^{-1} Cl^-, and can be used in droplets that are ten times smaller (0.1 nl).

Salicylate flux measured using the Sal-SeR microelectrode technique can also be compared with the flux calculated from the Ramsay assay. The two techniques are complementary, as both measure steady-state rates of salicylate transport. Comparison of main segment salicylate flux for the Ramsay technique with the corresponding flux measured with the Sal-SeR microelectrode requires division by the tubule surface area ×60 to produce the units measured by the self-referencing microelectrode (pmol cm^{-2} s^{-1}). For tubules bathed in saline containing 0.5 mmol l^{-1} salicylate, the flux in the Ramsay assay was 2.27 pmol min^{-1} $tubule^{-1}$ (Fig. 7C) and the flux measured by the Sal-SeR microelectrode was 9.03 pmol cm^{-2} s^{-1} (Fig. 5). Assuming that the tubule can be represented as a cylinder, a nominal surface area of the basolateral aspect of the tubule can be estimated from πDL, where D is diameter and L is length. The outside diameter of tubules measured with an eyepiece micrometer is ~46 μm and the length of the tubule in the Ramsay assay is ~2.18 mm, giving a surface area of 0.0032 cm^2 (Rheault and O'Donnell, 2004). The salicylate flux measured using the Ramsay assay is

therefore equivalent to 12.0 pmol cm^{-2} s^{-1}, which is 1.3 times greater than the flux measured directly by the Sal-SeR microelectrode technique. This ratio is undoubtedly smaller, and the agreement between the two techniques closer, because our estimation of tubule surface area is based on the assumption that the tubule is a cylinder. The functional surface area of a tubule will be larger than our estimation due to the extensive infoldings of the basolateral membrane (O'Donnell et al., 1985). The agreement between the flux of salicylate measured in the Ramsay assay with that measured using the Sal-SER microelectrode technique indicates that the flux measured by the latter corresponds primarily to transepithelial salicylate transport, as opposed to transport across the basolateral membrane followed by sequestration in intracellular organelles. However, it is important to note that if tubule surface area is greatly underestimated, then the flux measured by the Sal-SeR microelectrode may be larger than that measured in the Ramsay assay. The difference could be accounted for by sequestration of salicylate within the cells.

The self-referencing microelectrode technique is of particular advantage for tissues, such as the gut in small insects like as *Drosophila*, which may be difficult to perfuse or set up as flat sheets or everted sac preparations, as has been done successfully for analysis of epithelial transport in larger species, such as locusts and lepidopteran larvae (e.g. Phillips, 1964, Moffett, 1980). Self-referencing microelectrodes have previously been used in studies of H^+ and Cl^- transport by the gut of the mosquito *Aedes aegypti* (Boudko et al., 2001). The technique is also of use for comparison of associated gut regions and the Malpighian tubules in a single preparation. The tubules can be removed or left intact to assess the effects of high lumen concentrations on transport across regions of the gut such as the rectum and ileum that are downstream of the point at which the tubules join the gut.

The Sal-SeR microelectrode technique may be of use in future studies of salicylate transport in other systems. Salicylate is known to activate heat shock proteins in other animals (Ishihara et al., 2003), induce apoptosis (Lee et al., 2003) and alter sensory cell functioning (Cazals, 2000), as well as act as a substrate or competitive inhibitor of organic anion transporters (Russel et al., 2002).

The major limitation of the salicylate-selective microelectrode techniques described in this paper is that other organic anions may interfere with the response of the electrode to salicylate. The results indicate that the organic anions PAH and cAMP are poorly detected by the electrode and it is therefore feasible to use appropriate concentrations of these compounds and assess their impact on salicylate transport *in vitro* or *in vivo*. However, detailed studies of competitive and non-competitive inhibitors of salicylate transport that do interfere with the electrodes will require the use of alternative approaches, such as measurement of radiolabelled salicylate fluxes.

Salicylate transport by Drosophila

Our results show that the organic anion salicylate is transported at high rates by the isolated Malpighian tubules and gut of *Drosophila*. This study also documents the extraordinary capacity of insects to clear the haemolymph of organic anions. Salicylate-selective microelectrodes can be used to monitor the concentrations of salicylate in the haemolymph in response to dietary loading or direct injection into the haemocoel. Measurements in animals fed salicylate-enriched diet revealed that the haemolymph salicylate concentration is far below that in the food. Slowing metabolism by chilling the animals resulted in an increase in haemolymph salicylate concentration, suggesting that metabolism maintains salicylate levels in the haemolymph below those in passive electrochemical equilibrium with the gut contents.

The contribution of the Malpighian tubules to clearance of salicylate from the haemolymph can be assessed by comparison of the rates of decline of haemolymph salicylate concentration *in vivo* with the rates of transport of salicylate by isolated Malpighian tubules *in vitro*. Repeated measurements of salicylate concentration in the haemolymph of 3rd instar larvae that had previously been injected with salicylate permit indicated that salicylate concentration declines at the rate of 0.0136 mmol l^{-1} min^{-1} (Fig. 8). For the initial mean concentration of 3.4 mmol l^{-1} in the haemolymph after injection of salicylate, the concentration of salicylate in the haemolymph would decline by 50% in ~125 min. This half-time for clearance can be compared with estimates based on the salicylate transport rates of isolated Malpighian tubules. The maximum flux across a single isolated tubule is 2.72 pmol min^{-1} $tubule^{-1}$. Given that the haemolymph volume of a 3rd instar larvae is ~1 µl (Carton et al., 2002), then four tubules transporting at the maximal rate will reduce the salicylate concentration from 3.4 mmol l^{-1} to 1.7 mmol l^{-1} in ~156 min. This value will increase if there is reabsorption of salicylate across the lower tubule, but it is nonetheless of the same order of magnitude as the half-time estimated from the rate of decline in haemolymph salicylate concentration. These calculations suggest that the Malpighian tubules may play an important role in the elimination of salicylate.

Salicylate excretion will be further aided by transport across the gut, which will constrain movement of salicylate from gut lumen to haemolymph when the fly's diet contains salicylate. When very high concentrations of salicylate are present, the capacity for haemolymph to lumen secretion of salicylate may be exceeded, so that a net flux from lumen to haemolymph will occur. In support of this view is our finding that fluxes measured with Sal-SeR microelectrodes indicated transport of salicylate from lumen to bath for guts isolated from animals fed salicylate-rich diet. Estimates of the gut's contribution to salicylate excretion are complicated by difficulties in estimation of gut surface area. Such estimates are required to convert the Sal-SeR microelectrode flux data (pmol cm^{-2} s^{-1}) into flux across the entire gut. It is also worth noting that our data on the decline of salicylate concentrations in the haemolymph do not reveal whether salicylate has been excreted, sequestered within tissues or metabolized to neutral compounds such as salicin and catechol (Ruuhola et al., 2001) that are not detected by the electrode.

As noted above, the concentration of salicylate in the lumen is as much as 50 to 100-fold higher than that of the bathing saline. This finding indicates active transport of salicylate, since the lumen-positive transepithelial potential of 45 mV (O'Donnell et al., 1996) could account for an elevation of only 6-fold above that in the bath under conditions of passive electrochemical equilibrium.

High lumenal concentrations of salicylate provide an explanation for the transient reversal of salicylate flux in response to metabolic inhibition of isolated Malpighian tubules. The Ramsay assays indicate that the concentration of salicylate in the tubule lumen is ~7 mmol l^{-1} when the bathing saline contains 0.5 mmol l^{-1} salicylate. During the first 15 min after addition of NaCN the flux of salicylate reversed direction, so that net transport from lumen to bath was observed. We suggest that this reversal reflects passive leakage of salicylate down its concentration gradient, from the ~14-fold higher concentration in the lumen to the lower concentration in the bath. As this concentration gradient dissipates, the efflux progressively declines.

Our results also revealed pronounced spatial and temporal heterogeneity in the transport of salicylate across the main segment of the Malpighian tubule. Previous studies have shown that transport of tetraethylammonium is nearly constant over the length of the main segment and shows little variation over time (Rheault and O'Donnell, 2004). However, studies with self-referencing K^+ microelectrodes have indicated that not all morphologically similar cells participate equally in K^+ transport, nor do they respond equally to stimulation of K^+ transport with cAMP (Rheault and O'Donnell, 2001). Similarly, 'hot-spots' in extracellular Cl^--dependent current density are associated with stellate rather than principal cells and current density can vary dramatically between stellate cells in a single tubule (O'Donnell et al., 1998). Salicylate influx across the MT main segment also shows pronounced spatial variation (Fig. 4). One possible explanation for this finding is that local variations in transepithelial or apical membrane potential may affect either active salicylate transport or the extent of passive backflux from lumen to bath, thereby altering the net flux. It is worth noting in this context that the space constant of the MTs of another dipteran, the mosquito *Aedes aegypti*, is ~300 μm (Veenstra et al., 1997), so variations in transepithelial potential along the length of the MT are feasible.

Temporal variations in transepithelial ion flux have also been observed in earlier studies. The rate of K^+ reabsorption across the lower Malpighian tubule oscillates with a period of 0.8 min (Rheault and O'Donnell, 2001). In addition, the transepithelial potential across the main segment of *Drosophila* tubules oscillates with a period of 0.6 min, and it appears that these oscillations reflect Ca^{2+}-dependent changes in transepithelial Cl^- permeability (Blumenthal, 2001). Oscillations in salicylate transport across the main segment were much slower than either of the above phenomena, with a period of ~40 min. One possible explanation is that high intracellular or lumenal concentrations of salicylate affect other cellular processes, as evidenced by the decline in fluid secretion rate when the salicylate concentration in the bathing saline exceeds 0.5 mmol l^{-1}.

Further studies are required to describe the mechanisms of transepithelial salicylate transport by the Malpighian tubules and hindgut. It is worth noting that salicylate has been shown in other studies to be a substrate or a competitive inhibitor of several membrane transporters. These include the organic anion transporter OAT1 (Apiwattanakul et al., 1999; Russel et al., 2002), the voltage-dependent organic anion transporter OAT_V1 (Jutabha et al., 2003), a proton-linked monocarboxylate transporter (Emoto et al., 2002) and the sodium phosphate transporter NPT1 (Uchino et al., 2000).

In summary, we have developed ion-selective microelectrodes suitable for measurement of haemolymph levels of salicylate and for analysis of salicylate transport across isolated tissues. We have used these techniques to demonstrate transport of salicylate by the Malpighian tubules and gut of the fruit fly. Further studies will address modulation of salicylate transport by peptides and intracellular second messengers, or in insects fed on different diets.

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