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Effects of chronic exposure to dietary salicylate on elimination and renal excretion of salicylate by *Drosophila melanogaster* larvae

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Summary

The effects of chronic exposure to dietary salicylate on elimination and renal excretion of salicylate by *D. melanogaster* larvae were evaluated using salicylate-selective microelectrodes. Larvae chronically exposed to dietary salicylate showed 25% less salicylate in the haemolymph compared to the control group after feeding on a salicylate-enriched diet. By 1 h after transfer to a salicylate-free diet the levels of salicylate in the haemolymph of larvae raised on dietary salicylate were 46% lower than in the control group. Salicylate flux increased dramatically across Malpighian tubules but not across midgut or hindgut isolated from larvae chronically exposed to dietary salicylate, relative to the control group. Malpighian tubules isolated from experimental larvae showed a 4.7-fold increase in K_t and a nearly 5-fold increase in J_{max} relative to the control. These changes in salicylate transport were accompanied by a 3.2-fold increase in fluid secretion rate. Moreover, the high rates of fluid secretion by the Malpighian tubules isolated from experimental larvae were stimulated 2.1-fold and 2.8-fold when tubules were challenged with 1 mmol l^{-1} cAMP and 10 μmol l^{-1} leucokinin I, respectively. Taken together, these results indicate that chronic exposure of *D. melanogaster* larvae to dietary salicylate alters elimination of such toxins from the haemolymph and increases the basal rate of fluid secretion and excretion of salicylate by the Malpighian tubules.

Key words: salicylate, insect renal system, toxin elimination.

Introduction

Salicylate is a naturally occurring plant secondary metabolite that inhibits the growth of insect species such as the moth larvae [*Operophtera brumata* (Ruuhola et al., 2001)] and the corn earworm [*Helicoverpa zea* (Li et al., 2002)]. Various mechanisms have been developed by insects to cope with potentially harmful compounds. In this context, the activation of detoxification enzyme systems (cytochrome P450 monooxygenases, esterases and glutathione S-transferases) has been the most widely studied mechanism (Li et al., 2002; Li et al., 2007; Wilson, 2001), and less attention has been paid to the activation of the renal system as a possible mechanism of toxin elimination from the haemolymph.

Insect Malpighian (renal) tubules can excrete a wide range of plant secondary metabolites, such as nicotine (Gaertner et al., 1998; Maddrell and Gardiner, 1976), ouabain (Rafaeli-Bernstein and Mordue, 1978; Torrie et al., 2004), and salicylate (O'Donnell and Rheault, 2005). It has been proposed, therefore, that the Malpighian tubules may play an important role in the detoxification process and adaptation to xenobiotics in insects (Gaertner et al., 1998; Neufeld et al., 2005). This proposal is supported by evidence that the insect renal system upregulates the elimination of metabolic wastes or toxins acquired from food sources in some species. For example, the transport of *p*-aminohippuric acid and uric acid by the Malpighian tubules of *Rhodnius prolixus* is increased several days after consumption of a protein-rich meal (Maddrell and Gardiner, 1975; O'Donnell et al., 1983). Similarly, tetraethylammonium (TEA) excretion rate is higher in Malpighian tubules isolated from *D. melanogaster* larvae acutely exposed (24 h) to a TEA-enriched diet compared to the control (Bijelic et al., 2005). There is also evidence that the Malpighian tubules isolated from the plant feeder *Zonocerus variegatus*, fed for more than 12 days on a diet containing ouabain, secreted this glycoside at higher rates that those of insects fed an ouabain-free diet (Rafaeli-Bernstein and Mordue, 1978). Taken together, these results suggest that the activation of renal transport mechanisms might be related to food consumption in insects, which in turn would represent a suitable line of defence against ingested toxins or the products of their metabolism.

In this study, salicylate-selective microelectrodes were used to evaluate the effects of chronic exposure to dietary salicylate on haemolymph levels of salicylate and its elimination from *D. melanogaster* larvae. A previous study showed haemolymph to lumen flux of salicylate across the Malpighian tubules, posterior midgut and hindgut (O'Donnell and Rheault, 2005).

We have therefore measured the effects of chronic exposure to dietary salicylate on salicylate flux by these tissues.

Materials and methods

Animals and diet preparation

Oregon R strain *Drosophila melanogaster* Meigen were raised on standard diet and kept at 20–23°C in laboratory culture vials filled with ~10 ml of medium. The diet was prepared as described by Roberts and Stander (Roberts and Stander, 1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 1 g KH_2PO_4, 8 g potassium sodium tartrate, 0.5 g NaCl, 0.5 g $MgCl_2$ and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tapwater and 50 g dry active yeast. The two solutions were autoclaved, then combined and stirred. After cooling to 60°C, 7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept; Sigma-Aldrich, Oakville, ON, Canada) dissolved in ethanol and 10 ml of an acid mix (11 parts tapwater, 10 parts propionic acid, and 1 part 85% *o*-phosphoric acid) were added to the mixture. Salicylate-enriched diets were prepared by the addition of appropriate volumes of stock solutions to produce diet concentrations of 1–100 mmol l^{-1} sodium salicylate. Corresponding control diets were prepared by substituting NaCl for sodium salicylate.

Dissection and renal tissue assays

The composition of the experimental salines is shown in Table 1.

For Ramsay secretion assays, Malpighian tubules were dissected under control saline and set up as described by O'Donnell and Rheault (O'Donnell and Rheault, 2005). Briefly, pairs of Malpighian tubules were arranged so that one tubule was in a 50 μl bathing droplet containing salicylate, while the other was wrapped around a steel pin positioned approximately 1.5 mm away from the bathing droplet under paraffin oil. The ureter was positioned just outside the bathing droplet, and secreted droplets forming at the ureter were removed every 30–60 min with a fine glass rod and placed on the bottom of the Petri dish. Bathing saline contained 25–500 μmol l^{-1} salicylate. Fluid secretion rates are unaltered by salicylate at these concentrations (O'Donnell and Rheault, 2005). Salicylate flux across the Malpighian tubules was also evaluated in Na^+-free bathing saline containing 50 μmol l^{-1} salicylate.

Table 1. *Composition of experimental salines*

	Control	30 mmol l^{-1} Cl^-	Na^+-free
NaCl	117.5	–	–
KCl	20	9	5.5
$MgCl_2$	8.5	8.5	8.5
$CaCl_2$	2	2	2
Glucose	20	20	20
$NaHCO_3$	10.2	10.2	–
NaH_2PO_4	4.3	4.3	–
Hepes	8.6	8.6	8.6
Glutamine	10	10	10
Na_2SO_4	–	58.63	–
K_2SO_4	–	5.5	–
$KHCO_3$	–	-	10.2
KH_2PO_4	–	-	4.3
NMDG*	–	-	132

All values are mmol l^{-1}. *NMDG: *N*-methyl-D-glucamine.
All solutions were adjusted to pH 7.

For analysis of salicylate flux across isolated gut tissue, salicylate-selective self-referencing (Sal-SeR) microelectrodes (see below) were used. Midgut and hindgut were isolated under 30 mmol l^{-1} Cl^- *D. melanogaster* saline solution and transferred to a 35 mm diameter Petri dishes filled with 4 ml of the same saline containing 100 μmol l^{-1} salicylate. This saline mimics the Cl^- levels in the haemolymph and permits measurement of lower salicylate concentrations by salicylate-selective microelectrodes (O'Donnell and Rheault, 2005). Dishes were pre-coated with 50 μl droplets of 125 μg ml^{-1} poly-L-lysine and air dried before filling with saline to facilitate adherence of the tissue to the bottom of the dish. Salicylate influxes across midgut and hindgut were measured at three sites separated by 480 and 200 μm, respectively.

Haemolymph sampling

Dissection and haemolymph sampling were performed as described by Bijelic et al. (Bijelic et al., 2005). Briefly, larvae removed from 35 mm Petri dishes containing the control diet or the salicylate-enriched diet were rinsed in distilled water, dried on filter paper, and subsequently transferred to another Petri dish containing paraffin oil. The abdominal cuticle was torn with forceps and a sample of haemolymph that exuded from the wound was collected with a pipette. Samples of haemolymph were then transferred to another Petri dish containing paraffin oil to measure the salicylate concentration.

Measurements of salicylate concentrations in haemolymph and in fluid secreted by the Malpighian tubules

Salicylate concentrations in the haemolymph and in the fluid secreted by the Malpighian tubules were measured with salicylate-selective microelectrodes, as described in detail by O'Donnell and Rheault (O'Donnell and Rheault, 2005). Briefly, micropipettes were pulled to tip diameters of 5 μm on a programmable puller (P-97 Flaming-Brown, Sutter Instrument Co., Novato, CA, USA), silanized by treatment with *N*, *N*-dimethyltrimethylsilylamine (200°C, 60 min), cooled and then stored. Prior to use, microelectrodes were backfilled with 150 mmol l^{-1} KCl and frontfilled with a short column length (100 μm) of the ion exchanger cocktail, which consisted of 9% (w/v) tridodecylmethylammonium chloride (TDMA Cl^-; Fluka, Buchs, Switzerland) in 2-nitrophenyl octyl ether. The tip of each microelectrode was coated with a thin (~1 μm) layer of poly vinyl chloride (PVC; Fluka) dissolved in tetrahydrofuran (Sigma-Aldrich) to prevent displacement of the ion exchanger cocktail by paraffin oil (O'Donnell and Rheault, 2005). The reference microelectrode had a tip diameter of 1 μm and was

filled with 500 mmol l^{-1} KCl. Both the salicylate-selective microelectrode and the reference microelectrode were connected through chlorided silver wires to a FD 223 amplifier (World Precision Instruments, Inc., Sarasota, FL, USA), which in turn was connected to a computerized data acquisition and analysis system, PowerLab 4/25 with Chart 5 (ADInstruments, Inc., Colorado Springs, CO, USA). The signal value was then converted into a salicylate concentration using a microelectrode calibration curve that related voltage output to salicylate concentration in saline. Corrections for the effects of other anions in the secreted fluid (typically less than 3% of the salicylate concentration) were made as described (O'Donnell and Rheault, 2005). Calibration solutions were made up in 30 $mmol^{-1}$ Cl^- or in control *D. melanogaster* saline for measurements of salicylate concentration in the haemolymph and secreted fluid, respectively. The slopes for a change from 0.5 to 0.05 mmol l^{-1} salicylate were on average 60 mV in 30 mmol l^{-1} Cl^- and 35 mV in control *D. melanogaster* saline. The slope for a change from 0.5 to 5 mmol l^{-1} salicylate was on average 60 mV for both calibration solutions. Preliminary measurements showed that the levels of salicylate measured in the haemolymph correspond only to the parent compound. The main product of salicylate metabolism in insects, salicin (Ruuhola et al., 2001), is not detected by salicylate-selective microelectrodes (unpublished observations). An earlier study indicated that salicylate is secreted unchanged into the lumen of the Malpighian tubules (Ruiz-Sanchez and O'Donnell, 2007).

Measurements of salicylate flux across isolated guts

Influx of salicylate into the midgut and hindgut (ileum and rectum) was measured using salicylate-selective microelectrodes and the scanning ion electrode technique [SIET; also known as the self-referencing ion selective electrode technique (O'Donnell and Rheault, 2005)]. Salicylate-selective microelectrodes were constructed as described above. The reference electrode, however, consisted of a 10 cm long, 1.5 mm diameter glass capillary tube (TW150-4, World Precision Instruments, Inc.) filled with a mixture of 3 mmol l^{-1} KCl and 3% agar inserted into a microelectrode holder Ag–AgCl half-cell filled with 3 mol l^{-1} KCl. Both electrodes were connected through chlorided silver wires to an IPA-2 ion/polarographic amplifier and computerized data acquisition and analysis system (Applicable Electronics, Forestdale, MA, USA). At each measurement site, the Sal-SeR microelectrode, controlled through automated scanning electrode technique software (ASET, Science Wares, Inc., East Falmouth, MA, USA), was moved perpendicular to the tissue surface between two positions separated by 100 μm. The microelectrode remained stationary during the 9 s waiting period and microelectrode voltage was recorded and averaged for 0.5 s during the sample period. The voltage difference across the excursion distance was converted into a salicylate concentration difference (μmol cm^{-3}) using slopes from calibration solutions containing 0.05, 0.5 and 5 mmol l^{-1} salicylate in 30 mmol l^{-1} Cl^- *D. melanogaster* saline. Salicylate flux (pmol s^{-1} cm^{-2}) was then calculated from Fick's equation:

$$J_{sal} = D(\Delta C/\Delta x) ,$$

where J_{sal} is the flux, D is the diffusion coefficient for salicylate (0.959×10^{-5} cm^{-2} s^{-1}) (Lide, 2002), ΔC is the salicylate concentration difference (μmol cm^{-3}), and Δx is the microelectrode excursion distance (0.01 cm).

Kinetics, accumulation and elimination of salicylate from the haemolymph

The time-course of salicylate accumulation in the haemolymph was measured in third instar larvae fed for 3, 6, 9, 12 or 24 h on a 20 mmol l^{-1} salicylate-enriched diet. Concentration–response curves were plotted for haemolymph collected from larvae fed for 24 h on 5, 10, 20, 50 or 100 mmol l^{-1} salicylate in the diet.

Levels of salicylate in the haemolymph of third instar larvae fed for 24 h on a salicylate-enriched diet were compared between experimental and control groups of larvae. The experimental group was chronically exposed (10 days), from egg through third instar, to a diet containing 10 mmol l^{-1} salicylate, transferred to a salicylate-free diet for 15 h, then transferred back to a diet containing 20 mmol l^{-1} salicylate for 24 h. The control group was raised on the salicylate-free diet and then exposed to a diet containing 20 mmol l^{-1} salicylate for 24 h.

The rate of elimination of salicylate from the haemolymph was determined by feeding control or experimental larvae on a diet containing 20 mmol l^{-1} salicylate, and then sampling haemolymph at 0, 1, 4 and 6 h after transfer to a salicylate-free diet.

Effects of leucokinin I and cAMP effects on fluid secretion by the Malpighian tubules

Malpighian tubules isolated from third instar experimental or control larvae were dissected and set up in the Ramsay assay using control saline. The first secreted droplet was collected at 30 min. Either 1 mmol l^{-1} cAMP or 10 μmol l^{-1} leucokinin I was added to the bath and the second secreted droplet from each tubule was collected after a further 30 min.

Statistical analysis

Data describing the time-course of salicylate accumulation in the haemolymph were fitted to a Michaelis–Menten equation using non-linear regression analysis. Kinetic parameters for time-course curve were calculated using Eqn 1:

$$A = A_{max}T / (A_t+T) , \quad (1)$$

where A is the concentration of salicylate in the haemolymph (mmol l^{-1}); A_{max}, the maximum concentration of salicylate (mmol l^{-1}); T, time of exposure to the salicylate-enriched diet (h); and A_t, the time required to reach one-half the maximum concentration of salicylate in the haemolymph (h).

For the concentration–response curve, the kinetic parameters were calculated using Eqn 2:

$$B = B_{max}C / (B_t+C) , \quad (2)$$

where B is the concentration of salicylate in the haemolymph

(mmol l^{-1}); B_{max}, the maximum concentration of salicylate in the haemolymph (mmol l^{-1}); C, the salicylate concentration in the diet (mmol l^{-1}); and B_t, the concentration of salicylate in the diet corresponding to one-half the maximum concentration of salicylate in the haemolymph (mmol l^{-1}).

Kinetic parameters for salicylate flux across the Malpighian tubules were calculated using Eqn 3:

$$J = J_{max}S / (K_t+S) , \quad (3)$$

where J represents the salicylate flux across the Malpighian tubule (pmol $tubule^{-1}$ min^{-1}); J_{max}, the maximum rate of salicylate flux (pmol $tubule^{-1}$ min^{-1}); S, the concentration of salicylate in the bath (mmol l^{-1}); and K_t, the half saturation concentration for the salicylate transport system (mmol l^{-1}).

Curves relating elimination of salicylate from the haemolymph were fitted to the Eqn 4, describing a one-compartment model:

$$Y = Y_0e^{-Kx} , \quad (4)$$

where Y is the concentration of salicylate (mmol l^{-1}) at time x; Y_0, the value of Y at time zero (mmol l^{-1}); K, the elimination rate constant; and x, the time after transfer to salicylate-free diet (h).

Data from all experiments were expressed as means ± s.e.m. for the indicated numbers of replicates (N). Significant differences (P<0.05) were determined using the two-sample t-test assuming either equal or unequal variance, according to the outcome of a two-sample F-test. Statistical analyses and curve fitting were performed using GraphPad InStat and Prim 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Salicylate accumulation in the haemolymph

Levels of salicylate in the haemolymph of larvae fed on a 20 mmol l^{-1} salicylate-enriched diet for different periods (1–24 h) followed first order kinetics. The maximum concentration (A_{max}) of salicylate in the haemolymph was 0.57 mmol l^{-1} and was reached after approximately 6 h of exposure (Fig. 1A). The concentration–response experiments showed that levels of salicylate in the haemolymph of larvae fed for 24 h on diets containing different concentrations of salicylate (1–100 mmol l^{-1}) reached their maximum concentration (B_{max}) of 1.06 mmol l^{-1} when larvae were fed on diets containing 30 mmol l^{-1}, or higher concentrations of salicylate (Fig. 1B).

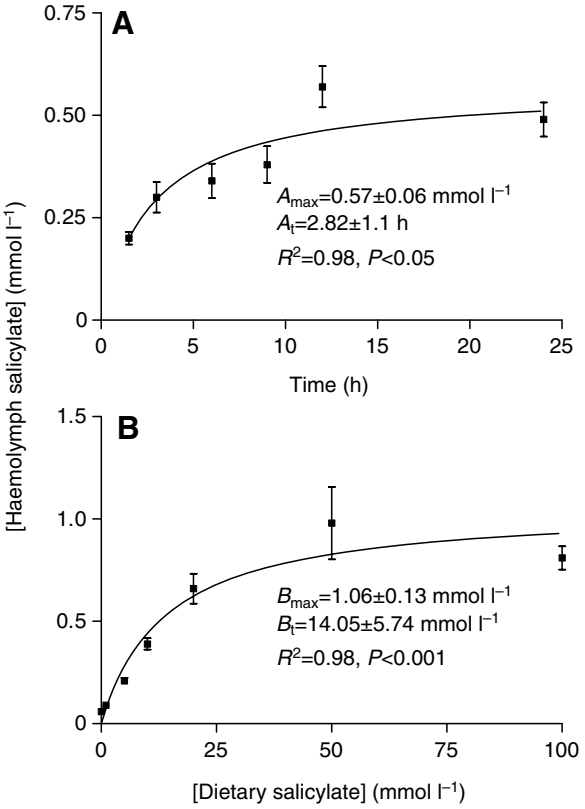

Fig. 1. Salicylate levels in the haemolymph of third instar larvae fed on a salicylate-enriched diet. (A) Levels of salicylate in the haemolymph of larvae fed on a 20 mmol l^{-1} salicylate-enriched diet for different periods. (B) Levels of salicylate in the haemolymph of larvae fed for 24 h on diets with different concentrations of salicylate. Each point represents the mean ± s.e.m. of 25–50 samples. The curve was fitted to the Michaelis–Menten equation by non-linear regression analysis.

Effects of chronic exposure to dietary salicylate on haemolymph levels of salicylate and its elimination

Salicylate concentration in the haemolymph after 24 h feeding on a diet containing 20 mmol l^{-1} salicylate was 25% lower (P<0.05; unpaired t-test; N=25–30) in larvae raised on dietary salicylate than in those of the control group (Fig. 2). By 1 h after transfer to the salicylate-free diet the levels of salicylate in the haemolymph of the experimental larvae were 46% lower than in the control group (P<0.005; unpaired t-test; N=21–26; Fig. 2). The lower values of haemolymph salicylate at both 0 and 1 h after transfer to the salicylate-free diet indicate more rapid elimination of salicylate from the larvae in the experimental group. Salicylate elimination from the haemolymph followed a one compartment, first order kinetic model (Fig. 2). The elimination rate constant (K) for salicylate was 0.024 min^{-1} for larvae chronically exposed to salicylate and 0.015 min^{-1} for the control group. Although the difference in K between the control and experimental groups of larvae was not statistically significant (P>0.05), this is in part a reflection of the very low levels of salicylate in the samples collected at 4 and 6 h.

Effects of chronic exposure to dietary salicylate on salicylate flux across isolated Malpighian tubules

Fluid secretion rate of Malpighian tubules isolated from larvae exposed chronically to salicylate was on average 3.2-fold higher (P<0.01; unpaired t-test; N=5) than from the control group (Fig. 3A). By contrast, salicylate concentration in the secreted fluid by the Malpighian tubules was similar in experimental and control groups (Fig. 3B).

Kinetic parameters for salicylate flux across the Malpighian

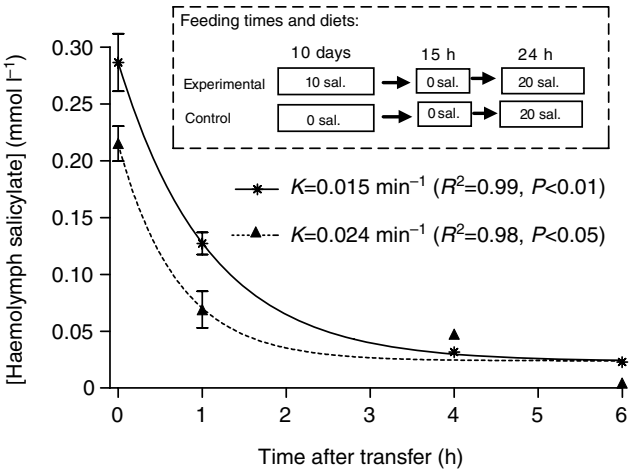

Fig. 2. Kinetics of salicylate elimination from the haemolymph of third instar *D. melanogaster* larvae. Haemolymph salicylate concentration was measured (0–6 h) after transfer of larvae that had been fed for 24 h on a 20 mmol l^{-1} salicylate-enriched diet to a salicylate-free diet. Solid and broken lines indicate the control and experimental group, respectively. Experimental larvae were raised for 10 days on a 10 mmol l^{-1} salicylate-enriched diet and subsequently transferred for 15 h to a salicylate-free diet, whereas control larvae were raised on a salicylate-free diet (inset). Each point represents the mean (± s.e.m.) of 25–50 samples. The curve was fitted to a one-compartment model by non-linear regression analysis.

tubules were dramatically affected by chronic exposure of larvae to dietary salicylate. The maximum rate of salicylate flux (J_{max}) was increased more than 4-fold, from 5.81± 0.46 pmol min^{-1} $tubule^{-1}$ in Malpighian tubules isolated from control larvae to 28.13±5.82 pmol min^{-1} $tubule^{-1}$ in tubules from larvae chronically exposed to dietary salicylate. The half saturation concentration (K_t) for the salicylate transport system increased 4.7-fold, from 0.09±0.02 mmol l^{-1} in tubules isolated from control larvae to 0.42±0.16 mmol l^{-1} for tubules isolated from larvae chronically exposed to dietary salicylate (Fig. 3C).

Na^+ dependence of salicylate flux in control and salicylate-exposed larvae

The increases in salicylate flux across Malpighian tubules isolated from larvae chronically exposed to dietary salicylate raised the possibility of expression of another transport system, in addition to the Na^+-dependent transport of salicylate described previously (Ruiz-Sanchez and O'Donnell, 2007). Thus, the effect of Na^+-free bathing saline on salicylate flux was evaluated. Our results showed that in Na^+-free bathing saline there was a decrease of 74% and 79% in salicylate flux across the Malpighian tubules isolated from larvae exposed to dietary salicylate and for those of the control group, respectively (Fig. 4B). Fluid secretion rate was not affected by Na^+-free bathing saline (Fig. 4A).

Effects of stimulants of fluid secretion in Malpighian tubules isolated from control and salicylate- exposed larvae

To determine whether the stimulation in fluid secretion rate

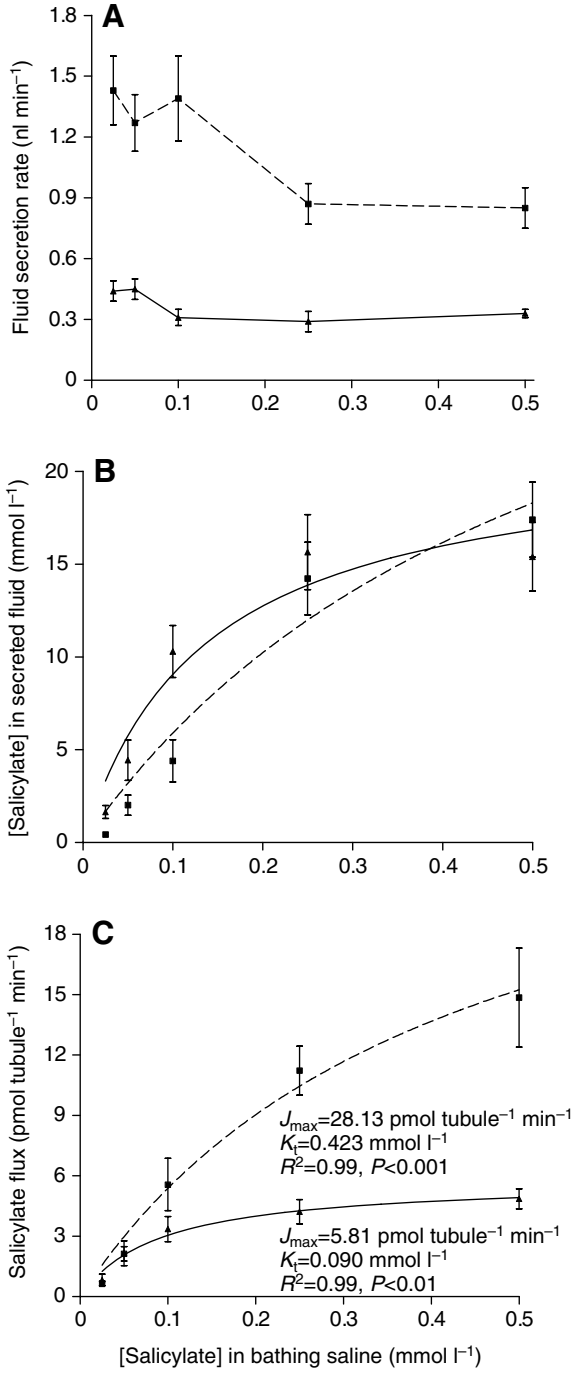

Fig. 3. Effects of chronic exposure of *D. melanogaster* larvae to dietary salicylate on (A) fluid secretion rate, (B) salicylate concentration in the secreted fluid and (C) transepithelial flux of salicylate across the main segment of isolated Malpighian tubules set up in the Ramsay assay. Each point 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.

by Malpighian tubules isolated from larvae chronically exposed to dietary salicylate was due to an increase in basal

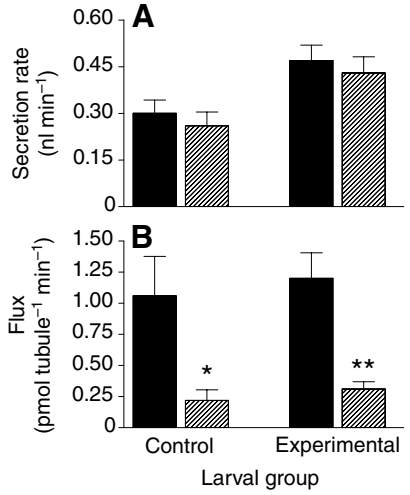

Fig. 4. Effects of Na^+-free bathing saline on (A) fluid secretion rate and (B) transepithelial flux of salicylate across the main segment of isolated Malpighian tubules set up in the Ramsay assay. Malpighian tubules were bathed in control saline (solid bars) or Na^+-free saline (hatched bars) containing 50 μmol l^{-1} salicylate. 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. Secreted fluid droplets were collected after 40 min. Values are means ± s.e.m. (*N*=6–9 tubules). Asterisks indicate significant differences between control and experimental groups (**P*<0.05, ***P*<0.01; *t*-test, *N*=6–8).

levels of intracellular second messengers, the effects of cAMP and leucokinin I were evaluated. Cyclic AMP increases fluid secretion through an increase in apical H^+-ATPase activity, whereas leucokinin I increases transepithelial Cl^- conductance through an increase in intracellular Ca^{2+}. Details on the mechanisms of action of these fluid secretion stimulants have been described previously (O'Donnell et al., 1996; Terhzaz et al., 1999). Our results showed that the stimulation of fluid secretion by 1 mmol l^{-1} cAMP was 2.1- and 2.5-fold in Malpighian tubules isolated from larvae chronically exposed to

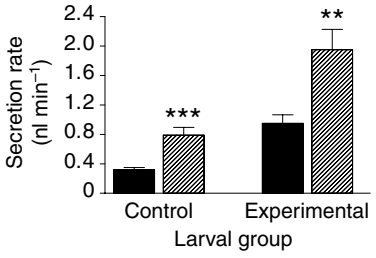

Fig. 5. Effects of 1 mmol l^{-1} cAMP on the rate of fluid secretion by the main segment of isolated Malpighian tubules set up in the Ramsay assay. The first secreted droplet was collected at 30 min (solid bars) and the second secreted droplet (hatched bars) was collected 30 min after adding cAMP. Experimental larvae were raised for 10 days on a 10 mmol l^{-1} salicylate-enriched diet. Control larvae were raised on a salicylate-free diet. Values are means ± s.e.m. (*N*=8–10 tubules). Asterisks indicate significant differences in fluid secretion rate before and after adding cAMP (***P*<0.01, ****P*<0.001; paired *t*-test, *N*=8–10).

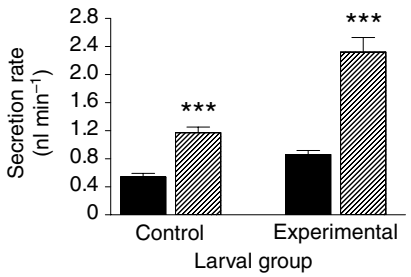

Fig. 6. Effects of 10 μmol l^{-1} leucokinin I on the rate of fluid secretion by the main segment of isolated Malpighian tubules set up in the Ramsay assay. The first secreted droplet was collected at 30 min (solid bars) and the second secreted droplet (hatched bars) was collected 30 min after adding leucokinin I. 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. Values are means ± s.e.m. (*N*=7–9 tubules). Asterisks indicate significant differences in fluid secretion rate before and after adding leucokinin are indicated (****P*<0.001; paired *t*-test, *N*=7–9).

dietary salicylate and in those from the control group, respectively (Fig. 5). Leukonin I, however, increased the fluid secretion rate 2.8-fold in Malpighian tubules isolated from larvae chronically exposed to dietary salicylate, whereas there was an increase of 2.2-fold in the control group (Fig. 6).

Salicylate influx across midgut and hindgut

The posterior midgut and hindgut (ileum and rectum) also transport salicylate from bath to lumen (O'Donnell and Rheault, 2005). Our results showed that there was no significant difference (*P*>0.05; unpaired *t*-test; *N*=4–5) in salicylate influx across posterior midgut, ileum and rectum isolated from larvae chronically exposed to dietary salicylate relative to those from the control group (Fig. 7).

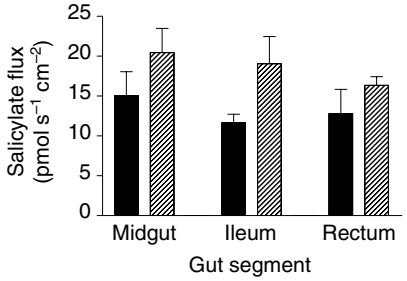

Fig. 7. Effects of chronic exposure of *D. melanogaster* larvae to dietary salicylate on salicylate influx across three segments of the isolated gut. Fluxes were measured using the scanning ion electrode technique as described in the Materials and methods. Gut segments were bathed in 30 mmol l^{-1} Cl^- saline containing 100 μmol l^{-1} salicylate. Flux for each segment of each larva was calculated as the mean value for three sites separated by 480 μm for the midgut and 200 μm for the ileum and rectum. Solid and hatched bars indicate mean ± s.e.m. for 4–5 larvae in 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. No significant differences between groups were observed (*P*>0.05).

Discussion

Chronic exposure to dietary salicylate increases both the rate of elimination of salicylate from the haemolymph and the rate of secretion of salicylate by isolated Malpighian tubules.

Larvae chronically exposed to dietary salicylate showed 25% less salicylate in the haemolymph compared to the control group after 24 h of feeding on a 20 mmol l^{-1} salicylate-enriched diet. Moreover, by 1 h after transfer to a salicylate-free diet the levels of salicylate in the haemolymph of the experimental larvae were 46% lower than in the control group (Fig. 2), consistent with more rapid elimination of salicylate from the haemolymph of the experimental larvae.

We have also found dramatic changes in salicylate flux across Malpighian tubules but not across midgut or hindgut isolated from larvae chronically exposed to dietary salicylate. The maximum rate of salicylate flux (J_{max}) across Malpighian tubules isolated from larvae chronically exposed to dietary salicylate was nearly 5-fold greater than that of the control larvae (Fig. 3C). Although salicylate flux across the Malpighian tubules isolated from salicylate-fed larvae clearly does not reach saturation within the range of salicylate concentration (25–500 μmol l^{-1}) used in the bathing saline (Fig. 3C), higher concentrations of salicylate could not be evaluated because it has been previously shown that fluid secretion rate by *D. melanogaster* Malpighian tubules significantly decreases when bathed in saline containing more than 500 μmol l^{-1} salicylate (O'Donnell and Rheault, 2005).

The posterior midgut and hindgut of *D. melanogaster* have been found to transport salicylate into the lumen (O'Donnell and Rheault, 2005). Our results show that salicylate influx across the posterior midgut, ileum and rectum was not altered in larvae chronically exposed to dietary salicylate (Fig. 7). It is worth noting that Taylor (Taylor, 1985) showed that Ca^{2+} absorption across the midgut of flies is not altered in response to variations in dietary Ca^{2+} levels. Instead, whole animal Ca^{2+} homeostasis is achieved by excretion of excess Ca^{2+} by the Malpighian tubules. Our results indicate that absorption by the gut is not downregulated in response to excess dietary salicylate, but that excretion is enhanced by upregulation of Malpighian tubule salicylate transport.

Previous work on accumulation and elimination of TEA in *D. melanogaster* larvae by Bijelic et al. (Bijelic et al., 2005) showed that this organic cation is excreted at slightly but significantly higher rates in Malpighian tubules isolated from larvae exposed for 24 h to dietary TEA than in those of the control group. This modest increase in TEA flux might have been due to the short-term exposure to dietary TEA.

Interestingly, salicylate transport across the Malpighian tubules isolated from larvae chronically exposed to dietary salicylate showed a half saturation concentration (K_t) approximately 4.7 times higher than that of the control group. Whereas an increase in J_{max} alone would be consistent with an increase in the number of transporters, the change in K_t suggests that chronic exposure to dietary salicylate results in expression of an additional transporter with a lower affinity for salicylate. This system, like that found in tubules of the control larvae, shows sodium dependence (Fig. 4). Previous studies have shown that the rate-limiting step for transepithelial salicylate transport is transport across the apical membrane. Uptake of salicylate across the basolateral membrane involves a Na^+/salicylate cotrasport system with high affinity (K_t=0.03 mmol l^{-1}) and high capacity (12.6 pmol min^{-1} $tubule^{-1}$) (Ruiz-Sanchez and O'Donnell, 2006).

The increase in salicylate flux across the Malpighian tubules of larvae chronically exposed to dietary salicylate was accompanied by an increase in the rate of fluid secretion (Fig. 3A,C). The current view of inorganic ion transport proposes that neuropetides, acting through cAMP and cGMP, enhance fluid secretion by increasing the activity of the electrogenic V-H^+-ATPase in the epical membrane (Broderick et al., 2003; O'Donnell et al., 1996). By contrast, an increase in intracellular Ca^{2+} in response to leucokinin or tyramine enhances the fluid secretion rate by increasing transepithelial Cl^- permeability (Blumenthal, 2003; O'Donnell et al., 1996; Terhzaz et al., 1999). The increase in the fluid secretion rate in tubules isolated from larvae chronically exposed to dietary salicylate does not simply represent an increase in the basal level of these intracellular second messengers. Although the fluid secretion rate of unstimulated tubules from salicylate-fed larvae is comparable to that of cAMP-stimulated control tubules, the addition of cAMP to the experimental tubules results in a dramatic 2.1-fold stimulation of fluid secretion rate (Fig. 5). Similarly, tubules isolated from salicylate-fed larvae increased the rate of fluid secretion 2.8-fold in response to leucokinin I (Fig. 6). Therefore, we suggest as a working hypothesis that Malpighian tubules isolated from experimental larvae are modulated through the insertion of more ion transporters into the cell membranes, rather than through an increase in the basal level of cAMP or Ca^{2+} in the cell. The most plausible candidate is the V-H^+-ATPase, which energizes not only the apical membrane but also the basolateral membrane of the Malpighian tubules (Beyenbach et al., 2000). An increase in the number of proton pumps in the apical membrane of the principal cells will therefore lead to an increase in secretion of both cations (Na^+ and K^+) and Cl^-, with a corresponding increase in the fluid secretion rate. This rate could be further increased through cyclic AMP or through leucokinin, as observed. It is worth nothing that Donini et al. (Donini et al., 2006) showed that changes in rearing salinity for cultures of *Aedes aegypti* and *Ochlerotatus taeniorhynchus* affect the intrinsic ion transport machinery of the Malpighian tubules. In both cases then, a change in ambient salinity or an increase in dietary toxins may result in a remodelling of the epithelium so that more and/or different transporters are expressed.

An important consequence of the increase in the rate of fluid secretion is that the concentration of salicylate in the tubule lumen is maintained at relatively low level and diffusive backflux of salicylate from the tubule lumen to the peritubular solution is thereby minimized. We have shown elsewhere that transepithelial salicylate transport increases with the fluid

secretion rate irrespective of whether fluid secretion is increased by intracellular second messengers or changes in bathing saline osmolality (Ruiz-Sanchez and O'Donnell, 2007). An additional advantage of this system is that elimination of any toxins to which the tubule wall is permeable will be enhanced by an increase in the rate of fluid secretion. Thus, elimination of small molecules, such as salicylate, and larger molecules, such as *p*-glycoprotein substrates and MRP2 substrates (O'Donnell and Leader, 2006) will increase if exposure to dietary toxins produces an increase in the basal rate of Malpighian tubule fluid secretion.

It is also important to note that secretion of salicylate by the Malpighian tubules can account for the decline in haemolymph salicylate concentration in both control larvae and those reared on 10 mmol l^{-1} salicylate. Using an estimated haemolymph volume of 2 μl (Carton et al., 2002) and initial haemolymph salicylate concentrations as in Fig. 2, secretion by the main segments of the four tubules in each larva can reduce the haemolymph salicylate concentration to one-half the initial value in ~6 min for larvae reared on salicylate-enriched diets and ~16 min for control larvae. Although backflux of salicylate across the lower tubule will reduce net secretion by approximately one third (O'Donnell and Rheault, 2005), with a corresponding increase in half-times for clearance, these estimated values are still well below the measured half times of 29 min and 45 min for experimental and control larvae, respectively. The predominant role of the tubules in clearance of the organic anion salicylate can be contrasted with clearance of the organic cation TEA. For organic cations, it appears that active transport across the tubules and posterior midgut can account for only about 10% of the observed rate of decline in haemolymph TEA concentration, and that passive loss across the gut is important when larvae are transferred to TEA-free diet (Bijelic et al., 2005).

In conclusion, our results show that larvae chronically exposed to dietary salicylate show lower levels of salicylate in the haemolymph and increased elimination of salicylate after feeding on salicylate-enriched diet. Exposure to dietary salicylate leads to an increase in salicylate excretion by isolated Malpighian tubules. This increase is accompanied by an increase in Malpighian tubule fluid secretion rate. We suggest that these changes provide the larvae with an effective means of defence against ingested toxins.

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