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Specificity of the fluorescein transport process in Malpighian tubules of the cricket *Acheta domesticus*

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Summary

We demonstrate the presence of an efficient, multispecific transport system for excretion of organic anions in the Malpighian tubules of the cricket *Acheta domesticus* using fluorescein (FL) as a model substrate. Malpighian tubules rapidly accumulated FL *via* a high affinity process (K_m=7.75 μmol l^{-1}); uptake was completely eliminated by the prototypical organic anion transport inhibitor probenecid (1 mmol l^{-1}), but not by *p*-aminohippuric acid (3 mmol l^{-1}). FL uptake was inhibited by monocarboxylic acids at a high concentration (3 mmol l^{-1}), and inhibition was more effective with an increase in the carbon chain of the monocarboxylic acid (37% inhibition by 5-carbon valeric acid, and 89% inhibition by 7-carbon caprylic acid). Likewise, tests using a series of aliphatic glutathione conjugates indicated that only the compound with the longest side-chain (decyl-glutathione) significantly inhibited FL uptake (81% inhibition). FL uptake was inhibited by a number of xenobiotics, including a plant alkaloid (quinine), herbicides (2,4-dichlorophenoxyacetic acid and 4-(2,4-dichlorophenoxy)-butyric acid), and the insecticide metabolites malathion monocarboxylic acid (MMA) and 3-phenoxybenzoic acid (PBA), suggesting that this transport system plays an active role in excretion of xenobiotics from *Acheta* by Malpighian tubules. HPLC quantification of MMA and PBA accumulation into Malpighian tubules verified that MMA accumulation was *via* a mediated transport process, but suggested that PBA accumulation was by nonspecific binding. The presence of a transport system in Malpighian tubules that handles at least one pesticide metabolite (MMA) suggests that transport processes could be a mechanism conferring resistance to xenobiotic exposure in insects.

Key words: Malpighian tubules, fluorescein, organic anion transport, *Acheta domesticus*, pesticide.

Introduction

Excretory systems of animals rely on a suite of specific solute transporters to selectively secrete unwanted compounds (either exogenous substances, or endogenous waste products), or reabsorb nutrients. Within well-studied mammalian models, there are a set of transport systems that have broad, and sometimes overlapping, specificities based on the size and charge characteristics of a molecule. One of the first transport systems identified was that for organic anions, including a specific process identified on the basolateral surface of proximal kidney tubules, for which many aspects have subsequently been well characterized in vertebrates (see review by Burckhardt and Burckhardt, 2003). This basolateral step of the transport process consists of a family of related isoforms that handle compounds with an appropriately sized hydrophobic core and with a full or partial negative charge at physiological pH (Ullrich, 1997). In its 'classic' form, this uptake of organic anions is *via* exchange with α-ketoglutarate, which is maintained at a high concentration in the cell by a Na^+/monocarboxylate cotransporter. In turn, intracellular Na^+ is maintained at a low concentration through the operation of the Na^+/K^+ ATPase. This organic anion transporter (OAT) therefore operates as a tertiary exchanger (see reviews by Dantzler, 2002; Burckhardt and Burckhardt, 2003). From a functional standpoint, specificity studies in vertebrates have implicated the overall organic anion transport process (including both the basolateral and apical steps) as having a role in ridding the body of exogenous substances, including naturally occurring or synthetic environmental toxins (Dantzler, 2002). There are fewer studies on organic anion transport in invertebrates, despite the fact that invertebrates often live in environments where xenobiotic exposure is high.

The Malpighian tubule of insects performs a role analogous to that of vertebrate kidneys, and is recognized as a model transport epithelia with impressive capacities for both solute and water transport (Maddrell, 1991). Studies of transport function in this tissue have largely focused on elucidating mechanisms for ion transport, and have demonstrated the critical physiological role of this organ in surviving the wide range of conditions inhabited by insects (Pannabecker, 1995). For instance, the fast response and high transport rate in

Malpighian tubules of blood-sucking insects are recognized as crucial adaptations for a feeding strategy that relies on rapidly processing large, dilute blood meals (Maddrell, 1991). A number of studies have pointed to a similar adaptive role for the transport of organic substances by Malpighian tubules, including a role in the secretion of xenobiotics (e.g. Hanson et al., 1980; Meredith et al., 1984). Furthermore, evidence from several studies suggests that the upregulation of xenobiotic transport may confer resistance to pesticides in insects (Lanning et al., 1996), or other invertebrates (Cornwall et al., 1995). A role for transport in acquired xenobiotic resistance would not be surprising, given that transport-mediated drug resistance in vertebrate tissues is implicated in the ability of cells to tolerate xenobiotic exposures (Ambudkar et al., 1999).

Given that the organic anion transport process appears to be present in Malpighian tubules (Bresler et al., 1990; Linton and O'Donnell, 2000), and that this transport process handles environmentally relevant compounds in other species (e.g. Dawson and Renfro, 1993), we hypothesized that organic anion transport may play a key role in insect survival during exposure to toxins. We report here on the specificity of this process in the house cricket *Acheta domesticus*, and implicate the process in the handling of at least one insecticide metabolite – a metabolic product of malathion.

Materials and methods

Animals and solutions

Acheta domesticus L. were purchased from local pet stores and held without food for at least 1 day prior to experiments. All dissections and experiments were performed in Ringer's solution (Spring and Hazelton, 1987) consisting of (in mmol l^{-1}): NaCl (100), K_2SO_4 (10), $MgSO_4$ (10), $CaCl_2$ (3.5), Glucose (10), Hepes (25), Glycine (10), Proline (10), Glutamine (2), Histidine (2), Lysine (4), Leucine (2). Ringer's was adjusted to pH 7.4 with 3 mol l^{-1} NaOH. Pesticides and pesticide metabolites were purchased from ChemService (West Chester, PA, USA). Solvents and buffer solutions for HPLC analysis were made from HPLC-grade reagents (Sigma-Aldrich). After dissection, Malpighian tubules were held for at least 15 min in Ringer's solution prior to experimentation.

Quantification of fluorescein uptake by fluorescence microscopy

Fluorescein (FL) accumulation in mid-tubule sections of individual Malpighian tubules was assessed by the technique of Welborn et al. (1998). Tubules were stuck on coverslips coated with Cell-Tak (BD Biosciences; Bedford, MA, USA), which reduced movement of the tubules during experiments, and then covered with a Coverwell Perfusion chamber (0.5 mm deep, 4 mm × 32 mm; Electron Microscope Sciences, Washington, PA, USA). Ringer's solutions were pumped through the chamber at a rate of 4 ml min^{-1}, providing for complete change-out of chamber solution in approximately 30 s. FL accumulation was observed using a Nikon Microphot microscope equipped with a 20× lens (0.17 N.A., Nikon). Excitation at 490 nm was provided by a mercury lamp (Osram HBO, Munich Germany), coupled with a 420–490 nm bandpass excitation filter, a 510 nm dichoric mirror, and a 515–545 nm bandpass emission filter (Omega Optical, Brattleboro, VT, USA). Light was collected at 1 s intervals using a photomultiplier tube (HC120, Hamamatsu, Bridgewater, NJ, USA) and processed using a photon counting unit (C3866, Hamamatsu) connected to a computer.

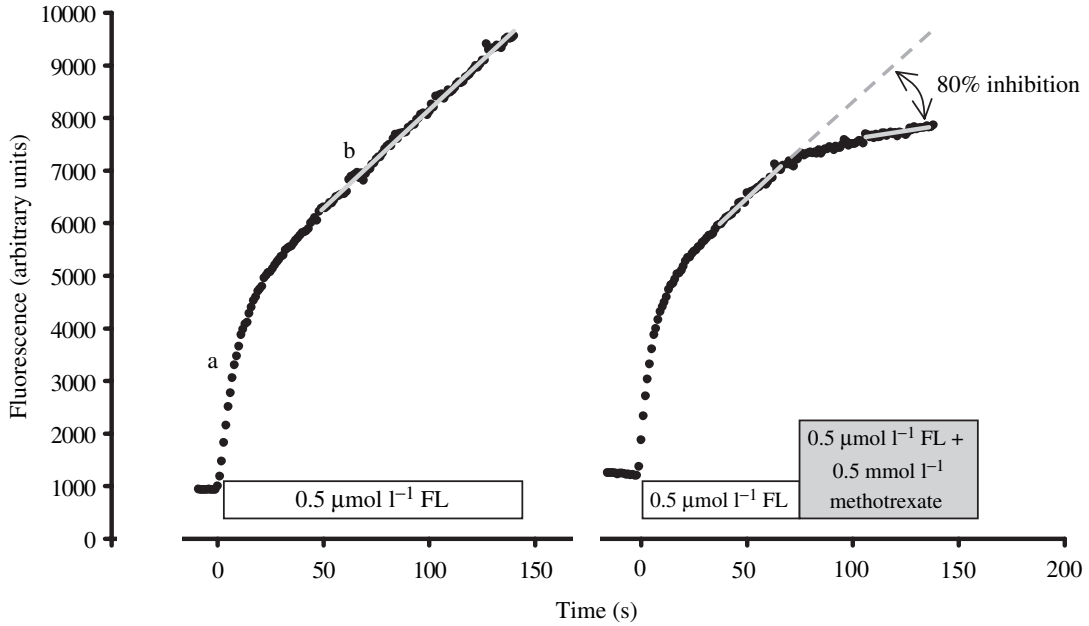

Fig. 1. Example fluorescence microscopy traces of fluorescein uptake in the presence and absence of a representative OAT inhibitor, methotrexate. During an uptake trial, the signal trace shows an initial rapid increase in fluorescence due to the change-out of chamber with fluid containing FL (a), followed by a slower increase representing accumulation in the tubule (b).

Standard testing procedure was to measure uptake in two consecutive periods (Fig. 1). During the first measurement period, we exposed tubules to Ringer's solution containing 0.5 μmol l^{-1} FL. We allowed chamber change-out for approximately 40 s, and then measured the rate of accumulation over the following 30–40 s. For the second measurement period, solution was switched to either a control solution (an identical solution, Ringer's + 0.5 μmol l^{-1} FL), or to a test solution (a solution containing 0.5 μmol l^{-1} FL + the test compound). As with the first measurement period, we measured uptake rates for 30–40 s after a 40 s chamber change-out period. After an uptake trial, the solution was switched back to plain Ringer's and tubules were allowed to recover (with FL washout) for at least 10 min. Multiple uptake tests could normally be performed for 2–3 h in this manner before there was a substantial decrease in FL accumulation; in no case was there evidence of obvious toxicity of the test compounds (i.e. lack of FL accumulation after the recovery period that followed a test exposure). All FL concentrations were 0.5 μmol l^{-1}, except for the trials to determine kinetics. In the kinetics trials, we calculated K_m values for four tubules that were each exposed to FL concentrations of 0.5, 2, 3.5, 7 and 14 μmol l^{-1}.

Quantification of fluorescein accumulation in whole tubules by fluorescence photometry

In selected cases, FL accumulation was also assessed for whole Malpighian tubules using fluorescence photometry. Individual Malpighian tubules were digitally photographed under a dissecting microscope (for length measurements), and then incubated in Ringer's containing 0.5 μmol l^{-1} FL in the presence or absence of a test compound. Tubules were rinsed three times for 30 s each in Ringer's alone, and then digested in 200 μl of 0.1 mol l^{-1} NaOH. Fluorescence intensity of tubule extracts and standard solutions were then measured using a luminescence spectrometer (LS-55, Perkin-Elmer) with excitation/emission wavelengths of 492 nm/520 nm, respectively. Uptake rates were standardized to the measured length of the tubule.

HPLC measurement of pesticide metabolites in Malpighian tubules

Accumulations of 3-phenoxybenzoic acid (PBA) and malathion monocarboxylic acid (MMA) in Malpighian tubules were measured directly by HPLC. Rather than using single Malpighian tubules, we pooled groups of Malpighian tubules from multiple crickets in order to increase the tissue mass to the point that we could detect the presence of these compounds. For each experiment, we dissected the complete complex of Malpighian tubules (representing approximately 100 tubules; Spring and Kim, 1993) from five crickets. When cut at the ureter, near its connection with the colon, each complex of Malpighian tubules stayed together as a single unit, connected *via* the ampula.

Each complex of Malpighian tubules (from a single cricket) was cut approximately in half; one half was used as a control (for exposure to the metabolite alone), and the other half was used in test solution (for exposure to metabolite plus inhibitor). For control trials, each one-half of a Malpighian tubule complex was exposed to 1 mmol l^{-1} of the metabolite for a 1 h period. The complex was then rinsed three times for 30 s each in Ringer's solution, and then extracted in 1 ml of HPLC grade water for at least 1 h. Each half-complex from the five crickets was combined into the same 1 ml extraction volume; each sample therefore represents the pooled tissue from five separate tubule complexes. Extracts were vortexed and then centrifuged prior to further processing. Test conditions followed the general procedure described above for controls, and took two forms. First, we tested the effect of organic anion transport inhibitors on metabolite accumulation. FL was used to test for inhibition of PBA accumulation. Because the FL retention time overlapped with the MMA metabolite peaks under the HPLC conditions used, we used PBD as an inhibitor of MMA accumulation. The second type of test condition exposed tubules for 1 min to HPLC-grade water, prior to exposure to the metabolite. Exposure to water alone for a short period stopped FL transport (data not shown), presumably by causing cellular rupture, but maintained some structural integrity of the tubule due to the basal lamina surrounding the cells of the Malpighian tubules. Metabolite accumulation after exposure to water is indicative of non-specific binding rather than mediated transport.

After extraction, samples were cleaned by solid-phase extraction (strong anion exchange cartridge; Accubond II SAX, Agilent Technologies, Palo Alto, CA, USA) based on the procedure of Abu-Qare and Abou-Donia (2001). Prior to sample loading, the SAX cartridge was prepared with 2 ml of retention buffer (HPLC-grade water adjusted to pH 7 with acetic acid and NaOH). The cartridge was loaded with 0.75 ml of centrifuged sample, rinsed with 2 ml of retention buffer, and then extracted with 2 ml of extraction buffer (50 mmol l^{-1} citric acid, pH 2). Extraction by SAX cartridges of standard solutions indicated a recovery of 89% for PBA, and 82% and 93% (for the first and second isomer peaks, respectively) for MMA. All samples were then run through a 0.45 μm filter (Millex-LH, Millipore, Billerica, MA, USA) prior to HPLC injection. We used a gradient from 55:45 acetonitrile:water (adjusted to pH 3.5 with acetic acid) to 100% ACN over a 20 min period on a 5 μm C18 column (Zorbax Eclipse XDB-C18, 4.6 mm × 250 mm). Compounds were detected by UV absorbance at 210 nm: a single peak for PBA was detected at approximately 7.9 min, and a double peak for MMA isomers eluted at 5.7 and 6.2 min.

Statistics

All uptake trials using fluorescence microscopy are reported as the rate of uptake during the second measurement period relative to the rate of uptake during the first measurement period. The effect of the presence of test compounds on FL uptake was analyzed with a paired *t*-test by comparing this change in uptake rate (between first and second measurement periods) in the presence *vs* absence of test compound. All reported values are means ± S.E.M.

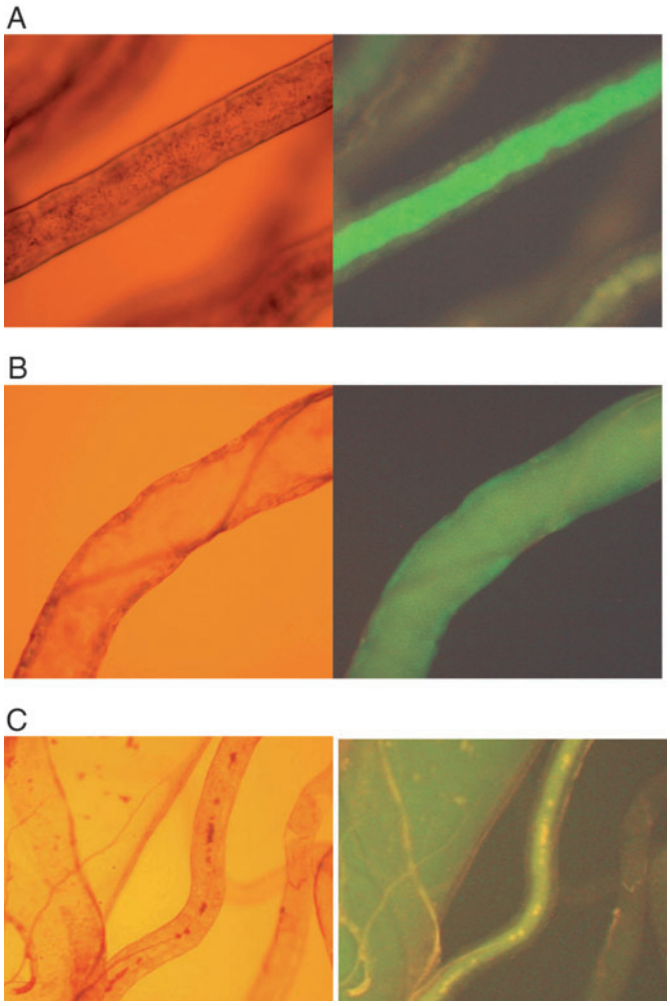

Fig. 2. Brightfield (left) and fluorescence (right) micrographs showing FL accumulation in *Acheta* Malpighian tubules. In some cases, fluorescence was stronger in the lumen (A), while in others it was more generally dispersed through lumen and cell (B). In (C) FL is present in both the Malpighian tubule (right) and the larger ureter (left) that connects Malpighian tubules to intestine.

Results

Fluorescein uptake by Malpighian tubules

Acheta Malpighian tubules show rapid accumulation of FL (Figs 1 and 2) into the mid-tubule section (but not the distal section) sections, indicating the presence of a vigorous organic anion transport (organic anion transport) process. FL rates measured at a range of concentrations indicated a high-affinity process with a K_m of 7.75±1.42 µmol l^{-1} (N=4). At an exposure concentration of 0.5 µmol l^{-1} FL, initial uptake rates of whole tubules measured by flourometry were 99.6±14.5 fmol min^{-1} mm^{-1} tubule length (N=8). The spatial pattern of accumulation varied; in some cases FL was obviously concentrated in lumen (Fig. 2A), whereas in others FL was either more concentrated in the cells or appeared equally distributed between lumen and cells (Fig. 2B). We noted no correlation of this variation with uptake rates or specificity; i.e. uptake occurred and was reduced by inhibitors regardless of where in the tubule FL was concentrated, suggesting most effects were due to inhibition on the basolateral surface. In some cases uptake was not linear; we collected data only from tubules in which uptake was generally linear over the ~2 min control FL exposure (e.g. Fig. 1A). As expected, FL was also present in the ureter (Fig. 2C), which drains urine from the Malpighian tubules, indicating that organic anion transport substrates would be excreted from the excretory organ.

Inhibition of fluorescein accumulation

Inhibitors of organic solute transport

We tested for inhibition of FL uptake by a range of compounds that represent known inhibitors of various organic solute transport processes (Table 1). Although the classic organic anion transport inhibitor *p*-aminohippuric acid (PAH) did not show inhibition at concentrations up to 3 mmol l^{-1}, other known blockers of organic anion transport (probenecid (PBD), bromosulfophthalein, methotrexate, salicyclic acid and penicillin) did block uptake. As expected, the classic organic cation transport blocker tetraethylammonium (TEA) showed no inhibition of FL uptake. Verapamil, a blocker of *p*-glycoprotein (*p*-gp; multi-drug resistance transporter) transport, was effective at blocking FL uptake.

Carboxylic acids

Since the uptake by organic anion transport is typically inhibited by aliphatic mono- and dicarboxylic acids in a defined size range (Ullrich et al., 1987), we tested for inhibition of FL uptake by carboxylic acids with a range of carbon backbone lengths. Inhibition by monocarboxylic acids increased with length of carbon chains (Fig. 3), although this inhibition was evident only at the higher (3 mmol l^{-1}) inhibitor concentrations. Evidence for inhibition by dicarboxylic acids was weak, with only the longest chain compound (sebacic acid with a 10 carbon backbone) showing any evidence of inhibition.

Glutathione conjugates

Organic anion transport in mammals has been implicated in export of xenobiotics *via* glutathione conjugation (Ullrich et al., 1989). We therefore tested for inhibition of FL accumulation by glutathione and several of its aliphatic conjugates (Fig. 4). At 0.2 mmol l^{-1}, inhibition occurred only with the largest conjugate (a 10 carbon chain), suggesting that glutathione conjugates are handled by organic anion transport in *Acheta* if they are sufficiently large.

Xenobiotics

We tested for FL inhibition by a range of compounds that represent foreign compounds (xenobiotics), and that in many cases are known toxins to insects (Table 2). Four insecticides were tested. The parent compounds did not inhibit FL uptake, with the exception of carbaryl and permethrin. The oxon metabolites of malathion and chlorpyrifos did not inhibit FL

Table 1. *Inhibition of fluorescein accumulation (at 0.5 μmol l^{-1}) by compounds known to inhibit organic solute transport in other systems*

Compound	Concentration (mmol l^{-1})	Uptake rate		*N*
		Control[a]	Treatment[b]	
p-Aminohippuric acid	3	93.6±4.7	75.8±16.3	5
Probenecid	0.1	96.9±3.6	92.5±8.1	5
Probenecid	1	90.7±3.7	–19.1±11.0*	6
Bromosulfophthalein	0.1	–	12.8±8.4	4
Methotrexate	0.5	89.7±2.6	10.8±8.6*	6
Verapamil	0.1	95.8±4.5	52.6±12.3*	5
Verapamil	1	94.6±5.3	–15.7±8.8*	6
Tetraethylammonium	1	98.2±3.5	95.4±6.8	6
Salicylic Acid	1	96.0±4.1	57.4±4.8*	6
Penicillin	1	91.3±2.7	63.8±5.3*	6

All values are means ± S.E.M.; *N* = number of tubules per sample. Negative values represent trials in which the introduction of test compound resulted in a net FL efflux from the tubule (a decrease in fluorescence).

[a]Control uptake rate (in absence of inhibitor) is the rate during the second measurement period (no inhibitor) expressed as percent of uptake measured in the first measurement period. See text for details.

[b]Treatment uptake rate (in presence of inhibitor) during second measurement period (inhibitor present) expressed as percent of uptake measured in the first measurement period.

*Change in uptake rate in presence of inhibitor significantly different from change in uptake rate in absence of inhibitor; $P<0.05$, paired *t*-test.

–, Missing control trials

uptake, but the carboxylic acid metabolites of malathion (malathion monocarboxylic acid; MMA) and permethrin (3-phenoxybenzoic acid; PBA) did show significant inhibition. Inhibition of FL uptake (at 1 μmol l^{-1}) by PBA was verified in whole tubules; FL uptake over a 2 min incubation period was 89.3±14.3 fmol mm^{-1} tubule length in the absence of PBA

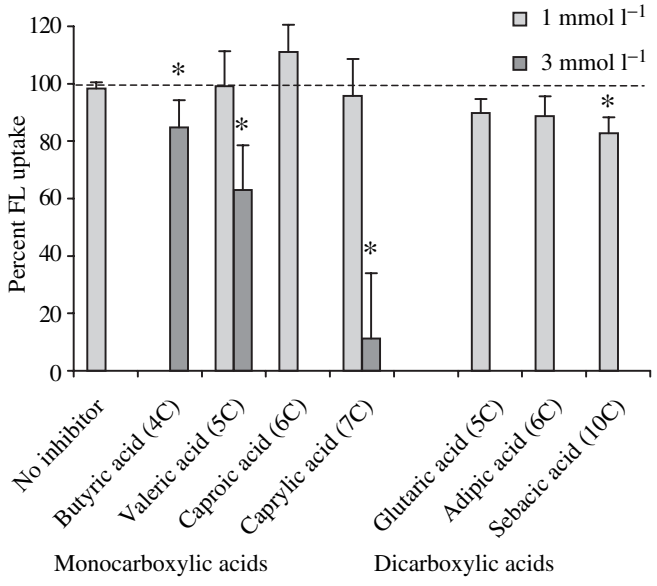

Fig. 3. Inhibition of FL accumulation by mono- and dicarboxylic acids representing a range of sizes (as indicated by the number of carbons in the backbone). Sample sizes range from 3 to 7 tubules, except for 'no inhibitor', which represents all control trials (N=27). *Change in uptake rate (second measurement period relative to first measurement period) in the presence of inhibitor significantly different from uptake rate in absence of inhibitor; $P<0.05$, paired *t*-test.

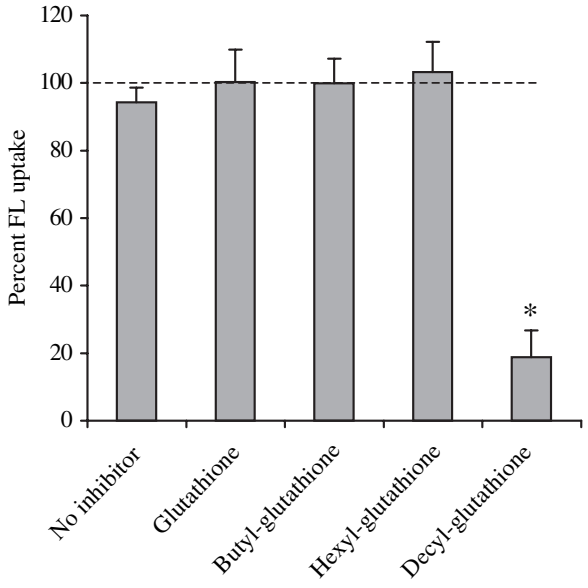

Fig. 4. Inhibition of FL accumulation by glutathione and a size range of its conjugates. All inhibitor concentrations are 0.2 mmol l^{-1}; sample sizes are 6 tubules, except for 'no inhibitor, which represents all control trials (N=23). *Change in uptake rate (second measurement period relative to first measurement period) in presence of inhibitor significantly different from uptake rate in absence of inhibitor; $P<0.05$, paired *t*-test.

Table 2. *Inhibition of fluorescein accumulation by various xenobiotic compounds*

Compound	Concentration (mmol l^{-1})	Uptake rate		*N*
		Control[a]	Treatment[b]	
Insecticides				
Malathion	0.5	94.2±3.4	85.0±10.6	6
Permethrin	0.5	89.1±1.8	76.9±5.1*	6
Chlorpyrifos	0.1	95.2±4.7	90.8±9.0	7
Chlorpyrifos	0.5	108.3±4.6	97.4±13.5	4
Carbaryl	0.25	95.2±3.1	75.4±5.1*	6
Metabolites				
Malathion oxon	0.5	86.3±1.4	88.9±5.4	6
Malathion monocarboxylic acid (isomer mixture) (MMA)	0.5	91.3±2.7	74.5±3.2*	6
MMA	1	93.6±4.8	38.9±7.5*	6
3-Phenoxybenzoic acid (PBA)	0.5	93.6±3.3	30.2±12.3*	9
Chlorpyrifos oxon	0.1	103.1±5.8	121.5±12.1	5
Chlorpyrifos oxon	0.5	99.9±7.2	72.6±16.6	4
Herbicides				
2,4-Dichlorophenoxyacetic acid (2,4-D)	1	94.0±5.1	50.4±9.6*	6
4-(2,4-Dichlorophenoxy)-butyric acid (2,4-DB)	1	95.0±4.3	–19.4±8.3*	7
Plant alkaloids				
Nicotine	0.5	100.0±12.4	77.6±9.3	3
Atropine	1	98.4±4.3	100.4±10.8	6
Quinine	1	96.0±4.9	45.0±12.6*	6

All values are means ±S.E.M. *N* = number of tubules per sample. Negative values represent trials in which the introduction of test compound resulted in a net FL efflux from the tubule (a decrease in fluorescence).

[a]Control uptake rate (in absence of inhibitor) is the rate during the second measurement period (no inhibitor) expressed as percent of uptake measured in the first measurement period. See text for details.

[b]Treatment uptake rate (in presence of inhibitor) during second measurement period (inhibitor present) expressed as percent of uptake measured in the first measurement period.

*Change in uptake rate in presence of inhibitor significantly different from change in uptake rate in absence of inhibitor; $P<0.05$, paired *t*-test.

and 54.2±6.6 fmol mm^{-1} tubule length in its presence (0.5 mmol l^{-1}; $P<0.05$). As expected (given their sizes and the presence of carboxyl side groups), the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-(2,4-dichlorophenoxy)-butyric acid (2,4-DB) were potent inhibitors of FL uptake. Finally, the plant alkaloid quinine also showed some inhibition of FL uptake. The specificity of organic anion transport in *Acheta* is therefore consistent with a role in the excretion of xenobiotic compounds.

Inhibition of FL accumulation in Drosophila

FL accumulation was also evident in Malpighian tubules of *Drosophila*; we therefore tested several compounds for FL inhibition in *Drosophila* (Fig. 5). As with *Acheta*, FL uptake was notably decreased by PBD and PBA. However, unlike in *Acheta*, the classic organic anion transport inhibitor PAH was effective at blocking FL uptake in *Drosophila*.

Uptake of PBA and MMA in Malpighian tubule bundles

Both PBA and MMA could be detected in Malpighian tubule bundles after a 1 h incubation in Ringer's containing one of these metabolites. After the 1 h exposure to PBA, samples (each containing bundle halves from five crickets) contained 2.23±0.53 nmol PBA (*N*=4). Neither prior exposure to distilled water (which irreversibly stops FL transport) nor concurrent exposure to 2 mmol l^{-1} FL resulted in inhibition of PBA accumulation (Fig. 6). Therefore, the observed PBA accumulation appeared to result from nonspecific binding. MMA was detected in living tissue in the absence of inhibitor (the sum of the two isomers in samples was 20.6±7.4 nmol, *N*=4, after 1 h exposure), but not in tissue after prior exposure to distilled water (Fig. 6). Accumulation of both isomers of MMA was decreased by concurrent exposure to 10 mmol l^{-1} PBD, suggesting that MMA accumulation is mediated, at least in part, by organic anion transport.

Discussion

Insects inhabit a diversity of habitats and rely on a variety of food sources, thus exposing them to a corresponding diversity of xenobiotic compounds. The presence of vigorous mechanisms for detoxification, including cellular transport processes, is therefore expected. This study demonstrates that Malpighian tubules from *Acheta* do indeed show a robust ability to secrete organic anions such as FL. The transport process had a high affinity for FL (K_m=7.75 μmol l^{-1}), and was

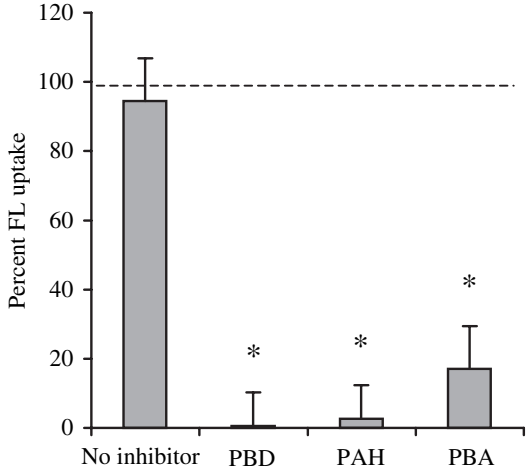

Fig. 5. FL accumulation in Malpighian tubules of *Drosophila*, and its inhibition by several potential substrates. *N*=3 tubules for each. PBD, 3-phenobenzoic acid (1 mmol l^{-1}); PAH, *p*-aminohippuyric acid (3 mmol l^{-1}); PBA, probenecid (0.5 mmol l^{-1}). *Change in uptake rate (second measurement period relative to first measurement period) in presence of inhibitor significantly different from uptake rate in absence of inhibitor; $P<0.05$, paired *t*-test.

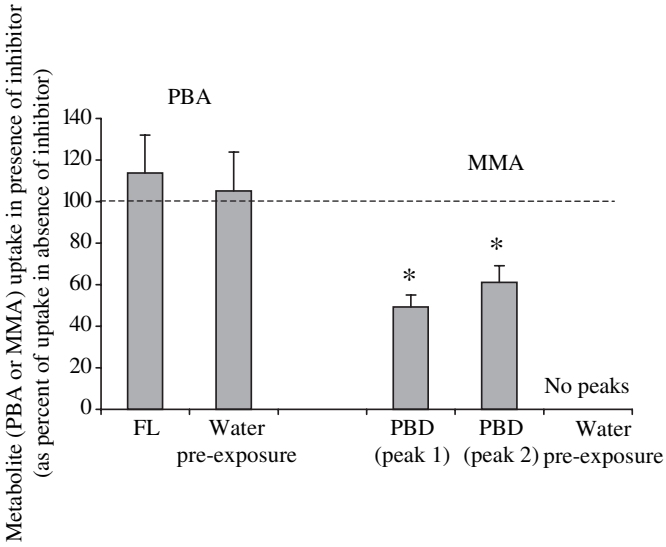

Fig. 6. Uptake of insecticide metabolites 3-phenoxybenzoic acid (PBA) and malathion monocarboxylic acid (MMA) in Malpighian tubule bundles, as detected by HPLC. Uptake in presence of inhibitor conditions (FL, 2 mmol l^{-1}; PBD, 10 mmol l^{-1}; or water pre-exposure) is plotted relative to control uptake (uptake with metabolite alone in Ringer's). *N*=4 samples for each. *Uptake of metabolite in presence of inhibitor condition significantly different from uptake in absence of inhibitor condition.

inhibited by compounds such as plant alkaloids, glutathione conjugates and pesticide metabolites, suggesting that the organic anion transport process may in fact serve as a conduit for the excretion of unwanted compounds for insects.

The demonstration of the transport of organic anions in *Acheta* is consistent with other studies that report organic anion transport in Malpighian tubules of insects (Bresler et al., 1990; Linton and O'Donnell, 2000; Maddrell and Gardiner, 1975). The specificity of the transport process in *Acheta* and other insects shows similarities with specific organic anion transporters in mammals. For instance, inhibition of organic anion transport in insects by PBD (present study; Linton and O'Donnell, 2000), a prototypical inhibitor of organic anion transport, mirrors results found in mammalian systems (Burckhardt and Burckhardt, 2003). Likewise, bromosulfophthalein, salicylic acid, penicillin, methotrexate, and the larger dicarboxylic acids all effectively inhibited FL accumulation in *Acheta,* and are known substrates for one or multiple isoforms of the OAT transporter in mammals (Burckhardt and Burckhardt, 2003).

In other respects, specificity of FL transport in *Acheta* does not match the 'classic' model for organic anion transport. A number of compounds that commonly inhibit organic anion transport in other animals did not affect FL accumulation in *Acheta*. FL accumulation was not affected by PAH, a prototypical organic anion transport inhibitor in mammals that also inhibits organic anion transport in *Drosophila* (Linton and O'Donnell, 2000) and several orthopteran species (Bresler et al., 1990). The monocarboxylate glutarate likewise had no effect on FL accumulation. This is consistent with the finding of Linton and O'Donnell (2000) in *Drosophila* who found that PAH transport was insensitive to preloading by α-ketoglutarate. Monocarboxylic acids such as α-ketoglutarate or glutarate act as a counterion in the classic model of organic anion transport. However, recent evidence indicates that not all isoforms of organic anion transporters in mammals have high affinity for PAH (Zhang et al., 2004), and that some isoforms do not act as exchangers with monocarboxylic acids (Burckhardt and Burckhardt, 2003). Thus FL transport in *Acheta* is more consistent with a basolateral transport step that does not follow the 'classic' pattern of specificity. Finally, organic anion transport in *Acheta* seemed to prefer larger compounds than organic anion transport in mammals, based on tests of related molecules of differing sizes. For instance, whereas dicarboxylic acids with shorter (5 carbon) backbones are effective at blocking FL in rat (Ullrich et al., 1987), dicarboxylic acids only inhibited FL in *Acheta* when of a larger size, and at a higher concentration.

FL transport in *Acheta* was inhibited by several compounds normally not associated with organic anion transport (OAT). Both verapamil and quinine, typically considered substrates of the multidrug resistant transporter *p*-gp (Ambudkar et al., 1999), decreased FL accumulation in *Acheta*. However, the decrease in FL accumulation seen in the presence of these, or other, test compounds could have been due to effects on either the basolateral or apical surfaces of the Malpighian tubule. In other systems, FL translocation across the basolateral surface occurs *via* an OAT transporter, but its translocation across the apical surface has been studied less. Thus, inhibition of FL transport by verapamil and quinine in *Acheta* may not indicate an OAT with unusual charge requirements.

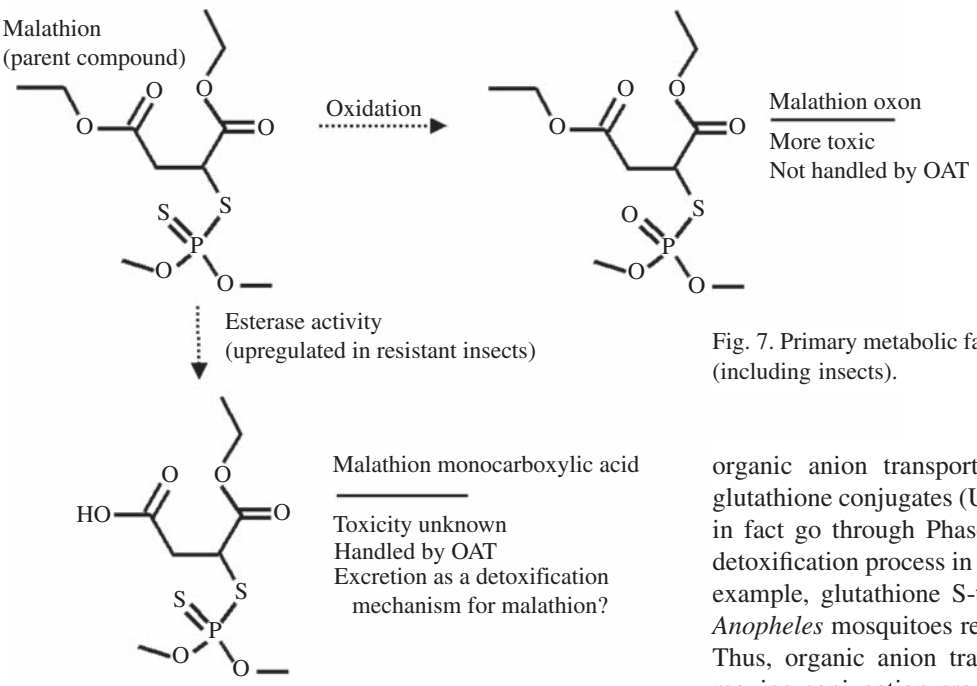

Fig. 7. Primary metabolic fates known for malathion in animal tissues (including insects).

In insects, studies in the handling of toxins have focused on the role of enzymatic biotransformations (e.g. Haubruge et al., 2002; Hemingway et al., 2004; Pasteur et al., 2001). To date, a smaller number of studies have investigated the possibility that excretion of toxins may be an important survival mechanism in insects (e.g. Bresler et al., 1990; Lanning et al., 1996). These have focused most extensively on the presence of the two 'multidrug resistance' transporters MRP (recently implicated in *Acheta*; Karnaky et al., 2001) and *p*-gp (Lanning et al., 1996). For instance, Lanning et al. (1996) found that *p*-gp expression was higher in a xenobiotic resistant strain in *Heliothis*, and that blocking *p*-gp increased toxicity of the insecticide thiodicarb. The specificity of the robust organic anion transport process in *Acheta* suggests that the organic anion transport process may also play a role in xenobiotic handling in insects. We identified FL inhibition by specific compounds from three classes of compounds – plant alkaloids, glutathione conjugates, and pesticides or pesticide metabolites – that would be candidates for excretion from the insect body.

Inhibition of FL transport by the plant alkaloid quinine suggest that organic anion transport may play a role in the excretion of naturally occurring toxins associated with plant diets. This is consistent with a number of other studies, suggesting that xenobiotic excretion allows insects to utilize plants that contain toxic alkaloids (Gaertner et al., 1998; Lanning et al., 1996). Inhibition of FL transport by decyl-glutathione in *Acheta* suggests that glutathione conjugates may represent a second class of xenobiotic compounds that could be handled by the organic anion transport pathway. In 'Phase II' (conjugative) biotransformation that uses glutathione, potentially toxic compounds are attached to the sulfhydryl group (on cysteine) of glutathione, making them substrates for excretion. The process is well studied in mammals, where organic anion transport is known to handle a variety of glutathione conjugates (Ullrich et al., 1989). Many xenobiotics in fact go through Phase II biotransformation as part of the detoxification process in insects (Hemingway et al., 2004). For example, glutathione S-transferase activity is upregulated in *Anopheles* mosquitoes resistant to DDT (Ranson et al., 2001). Thus, organic anion transport could also be a pathway for moving conjugation products through the Malpighian tubules for excretion.

Herbicides, insecticides and insecticide metabolites represent a third class of compounds that are potential substrates for transport *via* organic anion transport. One of the most common herbicides in use, 2,4-D, has a moderate size and a carboxyl group, making it a likely substrate for organic anion transport, as it is in other species (e.g. Dawson and Renfro, 1993). Indeed, both 2,4-D and 2,4-DB (a related herbicide with a longer carbon chain on its carboxyl side group) were potent inhibitors of FL transport in *Acheta*. Furthermore, 2,4-DB (the more effective inhibitor) stimulated a net loss of FL from the tubule (Table 2), perhaps representing *trans*-stimulation of efflux (a FL efflux that is stimulated by the increased influx of its counterion). A similar *trans*-stimulation of organic anion transport was reported for a number of other chlorophenoxy acid herbicides in flounder proximal tubules (Dawson and Renfro, 1993),

We tested a number of insecticides and their metabolites for FL transport. Although several parent compounds (permethrin and carbaryl) showed modest inhibition, a more marked effect was noted for the Phase I biotransformation products of malathion and permethrin (malathion monocarboxylic acid, MMA, and 3-phenoxybenzoic acid, PBA, respectively). In fact, the moderate size and carboxylic acid side groups of both compounds make them candidates for organic anion transport. Since inhibition of FL transport does not necessarily imply that the test compound is translocated, we quantified MMA and PBA accumulation more directly with HPLC. Both MMA and PBA clearly accumulated in Malpighian tubules, but only MMA showed evidence of mediated transport – MMA accumulation was partially inhibited both by PBA, an organic anion transport inhibitor, and by pretreatment with water (which killed tubules). PBA accumulation, on the other hand, was unaffected by

either FL or water pretreatment, suggesting that accumulation of this metabolite reflected nonspecific binding.

Acquired resistance to pesticides such as malathion clearly involves an increase in enzymatic biotransformation (e.g. Haubruge et al., 2002; Pasteur et al., 2001). During acquisition of malathion resistance, carboxyesterases are commonly upregulated, increasing MMA production and diverting malathion away from the more toxic malathion oxon derivative (Fig. 7), which is not an organic anion transport substrate (Table 2). In mosquitoes, carboxyesterases are particularly common in the alimentary canal, where they would be positioned to transform malathion into its carboxylic acid derivative as the toxin is taken up into the insect body (Pasteur et al., 2001). Thus, upregulation of MMA excretion by the Malpighian tubules of *Acheta* may complement an upregulation of malathion biotransformation to MMA.

It has become clear that molecular mechanisms of resistance acquisition can differ between strains of insects, even for the same insecticide (e.g. Pasteur et al., 2001). Likewise, different species have differing sensitivities to pesticides (e.g. Karunaratne and Hemingway, 2001). We suggest that, just as enzyme activity underlies some differences in pesticide susceptibility between some species and strains, so might differences in transport activity underlie differences in pesticide susceptibility. Thus, the difference in the ability of organic anion transport to use MMA and PBA as substrates could in part explain the observation that insects sometimes do not show cross-resistance between malathion and permethrin (Bisset et al., 1991): increased excretion of the malathion metabolite would not imply an increased excretion of the permethrin metabolite. In addition, competitive interactions at the site of the transporter could underlie the effectiveness of some pesticide adjuvants. Inclusion of a transport inhibitor in a pesticide mixture could effectively decrease the transport detoxification mechanism for the insect, just as the use of inhibition of transport can modulate chemical nephrotoxicity in mammals (Berndt, 1998).

In summary, we have demonstrated a robust process for the transport of organic anions in Malpighian tubules of *Acheta*. Plant alkaloids, glutathione conjugates and pesticide metabolites all interacted with the transporter, and at least one pesticide metabolite (MMA) was accumulated by Malpighian tubules *via* mediated transport. A similar handling of pesticide metabolites by organic anion transport could occur in humans and other mammals, given that MMA is rapidly produced and excreted from humans (Tuomianen et al., 2002). To our knowledge, there are no reports of whether MMA interacts with organic anion transport in mammalian systems. From a functional standpoint, the organic anion transport process in insects is thus poised to act as a key component in acquired resistance to xenobiotics.

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