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Non-Absorption of Ingested Lipophilic and Amphiphilic Allelochemicals by Generalist Grasshoppers: The Role of Extractive Ultrafiltration by the Peritrophic Envelope

Raymond V. Barbehenn

Department of Biology, University of Michigan, Ann Arbor, Michigan

The role of the peritrophic envelope in the non-absorption of three allelochemicals ingested by generalist grasshoppers was examined. This study tested the hypothesis that the association of lipophilic and amphiphilic allelochemicals with lipid aggregates (mixed micelles) reduces their permeability through the peritrophic envelope, a process similar to extractive ultrafiltration. Each of three allelochemicals (digitoxin, ouabain, and xanthotoxin) were solubilized in a lysolecithin suspension and injected separately into the midgut lumens of adult *Melanoplus sanguinipes* (Orthoptera: Acrididae). The low permeability of digitoxin through the peritrophic envelope was consistent with the extractive ultrafiltration of this compound. By comparison, ouabain and xanthotoxin permeability coefficients were 7- and 12-fold higher, respectively, than those of digitoxin. The results of extractive ultrafiltration assays confirmed that digitoxin is effectively extracted in lysolecithin micelles, but that neither ouabain nor xanthotoxin aggregates efficiently with these micelles. Arch. Insect Biochem. Physiol. 42:130–137, 1999. **© 1999 Wiley-Liss, Inc.**

Key words: *Melanoplus sanguinipes*; Orthoptera; Acrididae; peritrophic membrane.

INTRODUCTION

Previous work has shown that generalist grasshoppers can tolerate certain ingested plant allelochemicals by non-absorption. In particular, *Melanoplus sanguinipes* absorbs little or no digitoxin (Smirle and Isman, 1992), sesquiterpene lactones (Isman, 1985), α-terthienyl or xanthotoxin (Berenbaum and Isman, 1989). A study of another generalist grasshopper, *Schistocerca gregaria*, has also shown that it is protected from absorbing digitoxin and ouabain (Scudder and Meredith, 1982). Non-absorption of allelochemicals might result from the impermeability of the peritrophic envelope, the midgut epithelium, or both structures. However, none of the previous studies has distinguished between these possibilities.

The peritrophic envelope is a thin secreted tubular structure, composed of one or more peritrophic membranes, that surrounds all food in the midguts of insects. The peritrophic envelope has often been likened to an ultrafilter (e.g.,

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*Correspondence to: Raymond V. Barbehenn, University of Michigan, Department of Biology, Ann Arbor, MI 48109-1048. E-mail: rvb@umich.edu

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Zhuzhikov, 1970; Terra and Ferreira, 1981; Peters and Wiese, 1986; Miller and Lehane, 1990). Factors limiting the permeability of allelochemicals through this structure include size and possibly charge (Miller and Lehane, 1993; Barbehenn and Martin, 1995, 1997, 1998). Factors limiting the absorption of allelochemicals through the midgut epithelium include properties of the chemicals that influence their solubility in phospholipid membranes (polarity, size, and charge) (Shaw and Guthrie, 1970; Duffey, 1980), the efficiency of the detoxification systems in the midgut epithelium (Lindroth, 1991), and the rate of passage from the midgut epithelium to the hemocoel.

One potential means by which the peritrophic envelope could contribute to the process of allelochemical non-absorption is by the retention of allelochemicals sequestered in lipid aggregates in the gut fluid of herbivorous insects. Lipophilic and amphiphilic allelochemicals would be expected to partition into the lipid phase of gut fluid in inverse proportion to their water solubility (Florence, 1977; Kuksis, 1987). If these aggregates are sufficiently large, then allelochemical absorption could be impeded by a process similar to extractive ultrafiltration. In this separation process lipophilic and amphiphilic compounds are extracted into a non-aqueous phase that does not permeate an ultrafilter (Watters et al., 1989). The non-aqueous phase of the midgut fluid in herbivorous insects is composed primarily of micelles (Martin and Martin, 1984; Turunen and Chippendale, 1989; Small, 1986). Micelles form during the digestive process from the aggregation of lysophospholipids (surfactants), galactosyl glycerides, long chain fatty acids, and other amphiphilic and lipophilic compounds (Hawke, 1973; Turunen and Chippendale, 1989). Although pure surfactant micelles range in diameter from 3 to 12 nm, mixed micelles may be hundreds of nanometers in diameter (Vander et al., 1980; Mimms et al., 1981; Furth et al., 1984; Jones et al., 1987). Emulsified lipid droplets are also relatively large (≥1,000 nm) (Masoro, 1968; Vander et al., 1980), but these are a minor component of the midgut fluid of foliage-feeding insects (Hawke, 1973; Turunen and Chippendale, 1989). Maximum particle sizes that permeate the matrix of the peritrophic membranes of herbivorous insects have been estimated to range from 7.0–8.0 nm, based on digestive enzyme permeability (Santos and Terra, 1986; Ferreira et al., 1994), to 21–36 nm, based on FITC-dextran permeability (Barbehenn and Martin, 1995). Therefore, depending on the size limitation of pores in the matrix and the sizes of lipid-allelochemical aggregates, the association of allelochemicals with lipid aggregates might reduce allelochemical permeation, either by size exclusion or reduced rates of diffusion.

The allelochemicals examined in this study have a wide range of water solubilities: ouabain is a polar cardiac glycoside with a maximum solubility of 23 mM; digitoxin is a nonpolar cardiac glycoside with a maximum solubility of 0.013 mM; and xanthotoxin is a furanocoumarin that is "practically insoluble" (Merck, 1996). In this study the degree of association between each of these allelochemicals and lysolecithin micelles was determined using extractive ultrafiltration. To examine the potential of the peritrophic envelope to retain allelochemicals in mixed micelles, each of the three allelochemicals was solubilized in a lysolecithin suspension and injected separately into the midgut lumens of adult *M. sanguinipes*. The rates of permeation of these allelochemicals from two types of midgut preparations were compared. One preparation contained an intact peritrophic envelope surrounded by a midgut wall. A small hole was cut to expose the peritrophic envelope, thereby allowing the relative permeability of the peritrophic envelope to be measured for allelochemicals that do not permeate the midgut wall. A second type of midgut preparation, in which the wall remained intact but the peritrophic envelope was removed, was made to measure the permeability of the midgut wall. The results of these studies were considered consistent with extractive ultrafiltration if the permeability of the allelochemical through the peritrophic envelope is low and if the allelochemical is efficiently retained along with lysolecithin micelles in an extractive ultrafiltration assay.

MATERIALS AND METHODS

Chemicals

Digitoxin, xanthotoxin, and lysolecithin (soy and palmitoyl) were purchased from Sigma Chemical Co. (St. Louis, MO). Soy lysolecithin (lysophosphatidylcholine) is a mixture of fatty acid

esters, including linoleoyl (48.5%), palmitoyl (25%), stearoyl (8%), and linolenoyl (4.5%). Ouabain was purchased from Fluka Chemical Co. (Ronkonkoma, NY). All chemicals were weighed to the nearest 0.01 mg on a Cahn Electrobalance.

Insects

Eggs of *M. sanguinipes* (Orthoptera: Acrididae) were obtained from the USDA (Bozeman, MT). A colony was maintained in a screen cage (1 m × 0.5 m × 0.5 m) with a 150 W light bulb placed against the screen to provide a heat gradient (16 h L:8 h D). Nighttime temperature was ambient (ca. 22°C). Insects were reared from egg-hatch on romaine lettuce (*Lactuca sativa longifolia*) and wheat bran. Adult insects were used for all experiments.

Peritrophic Envelope and Midgut Wall Permeability

A separate experiment was performed for each allelochemical and midgut preparation type. Allelochemical solutions or suspensions were prepared by sonicating the allelochemical (20 min, 22°C) in an aqueous suspension of palmitoyl lysolecithin (41–71 mM). Each insect was chilled (–20°C, 9 min) and its entire gut was dissected free from the body wall under magnification from a dissecting microscope. The gut was placed in a small dish of incubating solution containing 5 mM Tris(hydroxymethyl)aminomethane, 45 mM Tris-(hydroxymethyl)aminomethane hydrochloride, 20 mM $CaCl_2$, 3 mM NaCl, 227 mM KCl, 41 mM $MgSO_4$, 6 mM ascorbic acid, and 440 mM fructose (pH 7.0). The foregut and anterior midgut (including the caeca) were removed, and the hindgut was ligated adjacent to the midgut with a silk suture (size 6-0). Allelochemicals were injected into the midgut lumen to allow large doses to be introduced rapidly. A Hamilton microsyringe (10 ml) containing an allelochemical solution or water (control) was mounted in a micromanipulator, and the gut preparation was tied onto the needle tip while remaining in the incubating solution. A 1.5 µl aliquot of a test solution was introduced into the closed endoperitrophic space. The gut preparation was pulled from the needle tip with tension on the suture ends to prevent the introduced solution from leaking. In gut preparations containing an intact peritrophic envelope, a small hole was cut in the mid-midgut wall to expose the peritrophic envelope. Damage to the peritrophic envelope was monitored by carefully observing any outflow of gut fluid into the incubating solution during dissection, and by examing the data for statistical outliers (Barbehenn and Martin, 1995; Barbehenn et al., 1996). Areas (mm^2) of holes made in experiments on the permeability of digitoxin, ouabain, and xanthotoxin averaged 1.8 (±0.3 SE), 1.1 (±0.2), and 0.9 (±0.1), respectively. After rinsing each gut preparation for approximately 10 sec in each of three beakers of incubating solution, they were incubated for 1.5 h at 22°C (±1°) in 1.5 ml of incubating solution containing palmitoyl lysolecithin (2.0 mM; pH 7.0). Following the incubation period, the entire volume of incubating solution was placed in a test tube, frozen in an ethanol-dry ice bath, and lyophilized. To determine the amount of allelochemical remaining in the midgut following the incubation period, the midgut contents were extracted in methanol (2 × 1.0 ml, 1 hr). The percentage of each allelochemical recovered at the end of the incubation period ([µg remaining in lumen + µg present in incubating solution]/µg injected) was 102.2% (digitoxin), 77.8% (ouabain), and 81.8% (xanthotoxin).

To determine the role of the midgut wall in limiting the permeation of test chemicals from gut preparations, the above procedures were repeated with the following exceptions: the peritrophic envelope and midgut contents were removed and the midgut wall remained intact. The efficiency of recovery of allelochemicals introduced into the midgut lumen was assumed to be similar to that measured from the entire midgut.

HPLC Analysis

Allelochemical concentrations in solutions and suspensions injected into the midgut lumen were measured in replicate aliquots (2, 4, or 5 µl) dissolved in 0.5 or 1.0 ml of methanol. Lyophilized incubating solutions were resolubilized sequentially in 200 µl of double-distilled water and 600 µl of methanol, and filtered (0.45 µm, Gelman GHP Acrodisc). Aliquots (10–30 µl) of samples and standards were injected onto a Vydac C-18 column (250x4.6 mm, 5 µ) and guard column using a Shimadzu autoinjector. Digitoxin and xanthotoxin were eluted at 1.0 ml/min using the fol-

lowing gradient: 0–15 min: 28% to 90% acetonitrile; 15–25 min: 90% to 28% acetonitrile; and 30–45 min: 28% acetonitrile. Ouabain was measured using the following gradient elution profile (1.0 ml/min): 0–15 min: 18% to 70% acetonitrile; 15–20 min: 70% to 18% acetonitrile; and 20–40 min: 18% acetonitrile. All compounds were measured with a Shimadzu SPD-6AV u.v.-visible detector (0.002 AUFS). Ouabain was detected at 220 nm, xanthotoxin at 249 nm, digitoxin at 240 nm, and soy lysolecithin at each wavelength. Peak areas were integrated with a Shimadzu C-R4A Chromatopac integrator, and converted to μg injected with standard curves for each compound. Average peak areas of interfering substances in control samples, when present, were subtracted from the peak areas in treatment samples.

Extractive Ultrafiltration

Suspensions of digitoxin, ouabain, and xanthotoxin were sonicated (20 min) in soy lysolecithin (1 mg/ml) in double-distilled water. Soy lysolecithin was substituted for palmitoyl lysolecithin to allow measurement with a u.v.-visible detector. Micellar suspensions were centrifuged (13,600g, 10 min, 21°C) and filtered (0.45 μm). Replicate aliquots (350 or 400 μl) were placed in ultrafiltration devices (Centrifree MPS-1; Amicon) containing 30,000 molecular weight cutoff (MWCO) ultrafilter membranes. Ultrafiltration devices were centrifuged (Sorvall RC-5B, 500g, 8 or 10 min), and the volumes of the ultrafiltrate (<30 kDa MWCO) and the retentate were measured. Concentrations of allelochemicals and lysolecithin in these fractions and in the unfiltered solutions were measured with HPLC, as described above.

Xanthotoxin Solubility

The putative insolubility of xanthotoxin in water was tested by sonicating 0.47 mg of xanthotoxin in double-distilled water (4.73 ml, 20 min), and centrifuging the suspension (13,600g, 4 min, 21°C). The supernatant was prefiltered (0.45 μm), and an aliquot (500 μl) was filtered through a 3000 MWCO ultrafilter (Microcon microconcentrator; Amicon) by centrifugation (13,600g, 15 min, 21°C). The concentrations of xanthotoxin in the supernatant suspension and in the ultrafiltrate were measured with HPLC, as described above.

Micelle Size

Micelle size was measured with a Coulter N4 Plus submicron particle size analyzer. Particle size measurements were made with the size distribution program at 62.6 or 90.0° light-scatter angles. Soy lysolecithin (10.0 mg/ml) or palmitoyl lysolecithin (l1.4 mg/ml) were solubilized in 10 mM HEPES buffer (pH 7.4) containing 0.145 M NaCl. The effect of digitoxin on micelle size was measured by examining micelle size in a soy lysolecithin solution (17.2 mg/ml) in HEPES buffer and remeasuring micelle size after solubilizing digitoxin with sonication (1.1 mM final concentration). Xanthotoxin particle size was measured in double-distilled water, prepared as described above (0.096 mM final concentration). All solutions were centrifuged (13,600g, 5 min, 21°C) and filtered (0.45 μm) to remove insoluble particles. Three to eleven replicates of each preparation were measured.

Statististical Analyses and Calculations

Pairwise comparisons of means were made using Mann-Whitney U-tests (SYSTAT; Wilkinson, 1990). Correlations between hole diameter in midgut preparations and percent permeation of each allelochemical were examined (SYSTAT; Wilkinson, 1990). No significant correlations were found in individual experiments or in pooled data and, therefore, hole diameter was not used in the calculation of permeability coefficients. The permeability of a gut preparation (P) was calculated from the equation $P = p/Ct$, where p is the total number of mmoles of the test chemical that diffused into the incubating solution in time t (hr) and C is the concentration (μmoles/cm^3) of the test chemical initially present in the midgut. Using this formula, P has the units cm^3/h. The average volumes of the sausage-shaped gut preparations with and without peritrophic envelopes (n=5 each) was 6 μl. Volumes were measured by placing midgut preparations (with the sutures and underlying tissue removed) in a microcentrifuge tube containing 50 μl of water, and homogenizing the contents with a spatula tip. The total volume was measured with an adjustable pipet (Gilson). The midgut volume was calculated as the total volume minus the original volume. Assuming that the surface areas of similar shapes with similar volumes are essentially the same, the permeabil-

ity coefficients can be compared between gut preparations. Back-diffusion of allelochemicals into midgut preparations was calculated to be negligible.

RESULTS

Permeability coefficients from midgut preparations that contained an intact peritrophic envelope were reduced 27% for digitoxin, 64% for ouabain, and 36% for xanthotoxin, compared with preparations from which the peritrophic envelope had been removed (Table 1), although only the reduced permeability of ouabain was statistically significant (P=0.01). Midgut preparations, with or without an intact peritrophic envelope, were much more permeable to ouabain and xanthotoxin than to digitoxin (Table 1). The permeability coefficients for ouabain and xanthotoxin are 14.5 and 14.0 times higher than the permeability coefficient for digitoxin in gut preparations without a peritrophic envelope and 7.2 and 12.3 times higher in gut preparations with a peritrophic envelope.

The large percentage of ouabain and xanthotoxin that diffused from the midgut preparations is less than would be predicted at equilibrium. Had equilibrium been reached during the 1.5 h incubation period, over 99% of the allelochemicals would have been in the incubation solutions, since the average volume of the *M. sanguinipes* gut preparations was 6 µl and the volume of the incubation solutions was 1.5 ml.

Lysolecithin micelles do not pass through an ultrafiltration membrane with a MWCO of 30,000 (Table 2). The concentration of lysolecithin in ultrafiltrates was only 1–4% of its concentration in the original suspensions. When digitoxin was added to a lysolecithin suspension, it was also extracted by ultrafiltration. The concentration of digitoxin in the ultrafiltrate was only 14% of its concentration in the original suspension (Table 2). By contrast, ultrafiltration does not result in the concentration of ouabain in a lysolecithin suspension (Table 2). These results suggest that the two cardiac glycosides have markedly different abilities to form mixed micelles. Xanthotoxin in a lysolecithin suspension is moderately concentrated by ultrafiltration. The concentration of xanthotoxin in the ultrafiltrate was 63% of its concentration in the original solution, suggesting that a substantial fraction of the xanthotoxin was not associated with lysolecithin micelles (Table 2). This result is consistent with the observation that the concentration of xanthotoxin in double-distilled water was between 52–59% of its concentration in lysolecithin suspensions. Further ultrafiltration (3000 MWCO) had no effect on the concentration of xanthotoxin in double-distilled water. These suspensions contained 1.0±0.2 nm diameter particles. By contrast, soy and palmitoyl lysolecithin micelles had diameters of 8.3±0.3 and 8.0±0.06 nm, respectively. Soy lysolecithin micelles in suspensions with and without digitoxin measured 7.3±0.1 nm and 7.6±0.2 nm, respectively.

DISCUSSION

The results of this study are consistent with the extractive ultrafiltration of digitoxin by the peritrophic envelope, i.e., digitoxin aggregates in lysolecithin micelles and has very low permeability coefficients through the peritrophic envelope in *M. sanguinipes*. Therefore, non-absorption of digitoxin by *M. sanguinipes* (Smirle and Isman,

TABLE 1. Permeability of *Melanoplus sanguinipes* Midgut Preparations to Allelochemicals*

Compound (N)	Type of gut preparation[a]	Concentration in midgut (µmoles cm^{-3})	Amount permeating (10^{-3} µmoles)	Percent permeating	Permeability coefficient (10^{-3} cm^{-3} h^{-1})
Digitoxin (6)	PE present	1.11	0.26 ± 0.10	2.5 ± 1.0[1]	0.16 ± 0.06[1]
Digitoxin (4)	PE absent	0.45	0.14 ± 0.10	3.3 ± 2.4[1]	0.22 ± 0.16[1]
Ouabain (7)	PE present	3.62	6.2 ± 1.0	18.2 ± 3.4[1]	1.15 ± 0.21[1]
Ouabain (6)	PE absent	2.20	11.0 ± 2.0	51.5 ± 12.0[2]	3.19 ± 0.64[2]
Xanthotoxin (7)	PE present	0.83	2.5 ± 0.3	31.3 ± 3.9[1]	1.97 ± 0.24[1]
Xanthotoxin (5)	PE absent	0.41	1.6 ± 0.2	40.5 ± 5.5[1]	3.08 ± 0.58[1]

*Data reported as mean ± SE. Summary statistics within compounds followed by different numbers are significantly different (P = 0.01).

[a]Midgut preparations with a peritrophic envelope present were also surrounded by a fenestrated gut wall. Midgut preparations without a peritrophic envelope had an intact gut wall.

TABLE 2. Extractive Ultrafiltration (30,000 MWCO) of Digitoxin, Ouabain, and Xanthotoxin in Lysolecithin Suspensions*

Compound[a]	Concentration (μM)		Permeability coefficient
	Original solution	Ultrafiltrate solution	(10^{-3} cm^{-3} h^{-1})
Lysolecithin	1193.4	25.2 ± 2.2	0.044 ± 0.02
Digitoxin	54.6	7.5 ± 0.3	0.15 ± 0.005
Ouabain	24.5	29.5 ± 0.2	1.60 ± 0.10
Xanthotoxin	190.3	120.4 ± 6.9	1.00 ± 0.07

*Data reported as mean ± SE.
[a]Suspension of digitoxin, ouabain and xanthotoxin were prepared by sonicating each chemical in a suspension of soy lysolecithin (1 mg/ml), followed by centrifugation (13,600g) and filtration (0.45 μm).

1992), and possibly also *S. gregaria* (Scudder and Meredith, 1982), appears to be the result of the combined low permeability of the peritrophic envelope and the midgut wall.

The results of previous work on *S. gregaria* showing that ouabain is not absorbed from the midgut (Scudder and Meredith, 1982) are in marked contrast to the results on *M. sanguinipes* in this study. Although the permeability of ouabain through *M. sanguinipes* gut preparations is significantly reduced by the presence of the peritrophic envelope, the permeability coefficient for ouabain remains relatively high. Indeed, the percent of ouabain permeating midgut preparations (18%) is similar to that for highly permeable compounds such as gallic acid (23%) (Barbehenn and Martin, 1992). Non-absorption of ouabain is most likely the result of an efficient detoxification system in the midgut epithelium, which is not necessarily fully functional in midgut preparations during the 1.5 h incubation period. It is also possible that detoxification systems are overwhelmed by the high concentration of ouabain injected into the midgut lumen in this study.

Xanthotoxin permeated complete midgut preparations more rapidly than did the amphiphilic cardiac glycosides, digitoxin and ouabain ($P<0.02$). This was the opposite of the result expected if xanthotoxin in micellar suspensions is sequestered in the lipophilic cores of lysolecithin micelles (Kuksis, 1987). However, the results of ultrafiltration and particle size studies demonstrated that a large fraction of xanthotoxin does not aggregate with lysolecithin but instead forms small (1 nm) micelles. Such small micelles (stacked arrangements) are formed by many hydrophobic polycyclic compounds in aqueous solutions (Atwood, 1983). Results on xanthotoxin permeability presented in this study also differ from previous work, which demonstrated non-absorption of xanthotoxin by *M. sanguinipes* (Berenbaum and Isman, 1989). Extensive work has shown that adapted insects chemically transform xanthotoxin in the midgut epithelium (Ivie et al., 1983; Bull et al., 1984; Nitao, 1989), whereas xanthotoxin permeates the peritrophic envelope and midgut wall in non-adapted insect species (Ivie et al., 1983). In vivo results on midgut wall permeability may differ from the results in this study if midgut detoxification systems in this study were not fully functional or were overwhelmed by a large dose of xanthotoxin. Further work is needed to examine whether lipophilic allelochemicals in general have such high peritrophic envelope permeabilities as does xanthotoxin, or whether this is an attribute of molecules that form small micelles.

The extent to which the molecular weights of the allelochemicals tested might explain their relative permeabilities was examined by estimating their diffusion rates. A regression equation was calculated for protein diffusion coefficients (D) and molecular weights (13.4–524 kDa) (Lehninger, 1975), and for FITC-dextran flux from midgut preparations and molecular weights (20–756 kDa) (Barbehenn and Martin, 1995). These equations ($D = -2.56 \times \ln MW + 35.40$ and $Flux = -270 \times \ln MW + 3{,}620$, respectively) provide surprisingly similar predictions for the relative diffusion rates and fluxes of the allelochemicals tested. The diffusion coefficient of free digitoxin (MW 765) is 96% of that of ouabain (MW 585), the same as predicted for their relative fluxes through the peritrophic envelope. By contrast, digitoxin aggregated in lysolecithin micelles (MW 92,000) would have a diffusion coefficient or flux

that is 29–34% of that of free digitoxin or ouabain. In this study the permeability coefficient for digitoxin from gut preparations containing a peritrophic envelope was only 14% of that of ouabain. These results suggest that digitoxin is aggregated in mixed micelles in the gut lumen in *M. sanguinipes*, and that these lipid aggregates are largely retained in the endoperitrophic space as a result of their low diffusion rates and extractive ultrafiltration by the peritrophic envelope.

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