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REASSESSMENT OF THE ROLES OF THE PERITROPHIC ENVELOPE AND HYDROLYSIS IN PROTECTING POLYPHAGOUS GRASSHOPPERS FROM INGESTED HYDROLYZABLE TANNINS

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Abstract—We examined several of the mechanisms that have been reported to enable polyphagous grasshoppers (Orthoptera: Acrididae) to tolerate ingested hydrolyzable tannins: hydrolysis, adsorption on the peritrophic envelope, and peritrophic envelope impermeability. None of these mechanisms explain the tolerance of *Melanoplus sanguinipes* to ingested tannic acid. In this species, tannin hydrolysis was 12–47% complete, adsorption accounted for less than 1% of the tannic acid contained in the midgut, and the peritrophic envelope was permeated by several gallotannins. The foregut is the main site for the chemical transformation of tannic acid in this species. In *Phoetaliotes nebrascensis*, hydrolysis was more extensive (82% complete), but the peritrophic envelope was readily permeated by two gallotannins. Oxidizing redox conditions were found in the guts of both species, and ingested tannins were oxidized in *M. sanguinipes*. We hypothesize that the tolerance of some polyphagous grasshoppers to ingested hydrolyzable tannins may be the consequence of their ability to tolerate the reactive oxygen species generated by polyphenol oxidation, whereas others may rely on rapid and extensive hydrolysis.

Key Words—Orthoptera, Acrididae, grasshopper, *Melanoplus sanguinipes*, *Phoetaliotes nebrascensis*, peritrophic envelope, peritrophic membrane, hydrolyzable tannin, tannic acid, oxidation, hydrolysis.

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INTRODUCTION

Grasshoppers (Orthoptera: Acrididae) vary greatly in their sensitivity to ingested hydrolyzable tannins. Polyphagous species are remarkably tolerant, being able to survive on diets containing 20% tannic acid without inhibition of feeding, reduction in the efficiency of utilization of nutrients, formation of lesions in the midgut epithelium, or any other adverse effect commonly associated with tannin sensitivity (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980). Graminivorous species are less tolerant of tannic acid than polyphagous species. For several graminivorous grasshoppers, tannic acid has been shown to reduce feeding and cause lesions in the midgut, which may prove lethal if the feeding period is sufficiently long (Bernays, 1978; Bernays et al., 1980; Raubenheimer, 1992). Two mechanisms have been proposed to contribute to the tolerance of polyphagous grasshoppers to tannic acid: rapid hydrolysis (Bernays, 1978; Bernays and Chamberlain, 1980) and containment within the peritrophic envelope (Bernays et al., 1980; Bernays and Chamberlain, 1980).

Since the publication of the seminal papers of Bernays and others (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980), there have been a number of advances in our understanding of the effects of tannins on insects and of the permeability properties of the peritrophic envelope, advances suggesting that the explanation proposed for tannin tolerance in generalist grasshoppers is still not complete. For example, our recent studies of tolerance in Lepidoptera (Barbehenn and Martin, 1992, 1994), which demonstrated that the containment of tannic acid within the endoperitrophic space is not sufficient to confer tannin tolerance, requires a re-examination of the putative protective role of the peritrophic envelope in grasshoppers. In addition, the recognition that the agents responsible for the adverse effects of ingested polyphenols are often not the polyphenols themselves, but rather oxidation products, such as quinones, or by-products of oxidation, including reactive oxygen species such as hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals (Appel, 1993; Ahmad, 1995; Cadenas, 1995; Felton, 1995; Summers and Felton, 1994), requires a re-examination of the chemical fate of ingested polyphenols in grasshoppers in order to determine whether or not they are oxidized.

The purpose of the present study is to re-examine the basis for tannin tolerance in polyphagous grasshoppers. We have examined several potential mechanisms in two grasshopper species in the subfamily Melanoplinae, *Melanoplus sanguinipes* and *Phoetaliotes nebrascensis*. *M. sanguinipes* is a highly polyphagous species with a diet varying from 50–78% forbs over its range in the midwestern United States (Gangwere et al., 1976; Joern, 1983). Although *P. nebrascensis* consumes more grasses and fewer forbs than *M. sanguinipes* (Joern, 1983), it has mixed-feeder mandibles (Isely, 1944) indicative of a polyphagous species. *M. sanguinipes* has a high tolerance to ingested tannins (Ber-

nays et al., 1980). We are unaware of any studies to determine the sensitivity of *P. nebrascensis* to ingested tannins. However, tannic acid is not an effective feeding deterrent for either species (Bernays et al., 1980; Mole and Joern, 1994).

In our experiments we used commercial tannic acid from oak galls so that we could compare our results with those of earlier workers. However, since the simple gallotannins comprising tannic acid differ from the more complex gallotannins found in the common host plants of polyphagous grasshoppers, we have also included in our experiments a tannin isolated from fireweed (*Epilobium angustifolium*), oenothein B. This tannin is representative of a widely occurring class of oligomeric hydrolyzable tannins that consist of core carbohydrates linked by oxidatively coupled galloyl groups (Okuda et al., 1993).

In this study, we have conducted experiments that address the following questions: (1) How extensively are hydrolyzable tannins hydrolyzed while passing through the gut? (2) In what region of the gut does most of the hydrolysis occur? (3) Do redox conditions in the gut favor polyphenol oxidation? (4) Are ingested tannins oxidized in the gut? (5) How does the fate of a complex hydrolyzable tannin (fireweed tannin) differ from the fate of the simple gallotannins found in tannic acid? (6) How much tannic acid is adsorbed by the peritrophic envelope? (7) Is the peritrophic envelope permeable to tannic acid?

METHODS AND MATERIALS

Insects and Plants. Eggs of *M. sanguinipes* 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 (16h L:8h D). Nighttime temperature was ambient (ca. 22°C). Insects were reared from egg-hatch on romaine lettuce (*Lactuca sativa longifolia*) and wheat bran. *P. nebrascensis* were collected as third- and fourth-instar nymphs in western Nebraska. A colony was maintained on rye (*Secale cereale*) seedlings and bran under the same conditions described for *M. sanguinipes*. Adult insects were used for all experiments.

Polyphenols. Tannic acid from *Quercus infectoria* (Fagaceae) galls was purchased from Sigma Chemical Company (St. Louis, MO; lot 64F-0049). Fireweed tannin was extracted from *E. angustifolium* (Onagraceae) leaves (Zhao, 1995). Gallic acid was purchased from Sigma Chemical Co.

Polyphenol Analysis. The gallotannins present in tannic acid were identified by comparing chromatograms (normal- and reverse-phase HPLC) with chromatogams of authentic samples of galloyl glucose esters isolated from tannic acid on a Sephadex LH-20 column (Hagerman et al., 1992). Calibration curves (plotting log retention time as a function of the number of galloyl groups) were constructed using data obtained with authentic standards in either normal- or

reverse-phase HPLC. The regression equations are log retention time = (0.085 × no. galloyl groups) + 0.415 (r^2 = 0.999) for normal-phase, using the linear portion of the curve up to pentagalloyl glucose (Figure 1), and log retention time = 0.160 × no. galloyl groups) + 0.098 (r^2 = 0.998) for reverse-phase, using the linear portion of the curve between tri- and pentagalloyl glucose. Purified fireweed tannin is composed primarily of oenothein B (Okuda et al., 1993), as determined by NMR and mass spectrometry (Zhao, 1995).

Quantitative analyses of polyphenols in sample extracts were conducted with reverse-phase HPLC using a Vydac C-18 column (4.5 mm × 250 mm; 5 μ) and Supelco C-18 guard column (10 μ). The mobile phase was 23% (v/v) acetonitrile in double-distilled water containing 1% acetic acid, unless otherwise noted. The flow rate of the mobile phase was 1.0 ml/min. Sample aliquots (20–30 μl) were injected with a Shimadzu autoinjector. Peaks were detected at 280 nm (Shimadzu UV-visible detector, 0.002 AUFS) and quantified with a Shimadzu C-R4A integrator. In all cases, peak areas were corrected for the presence of interfering substances found in the control samples. Standard curves were calculated for tannic acid, fireweed tannin and gallic acid to convert peak areas to μg polyphenol.

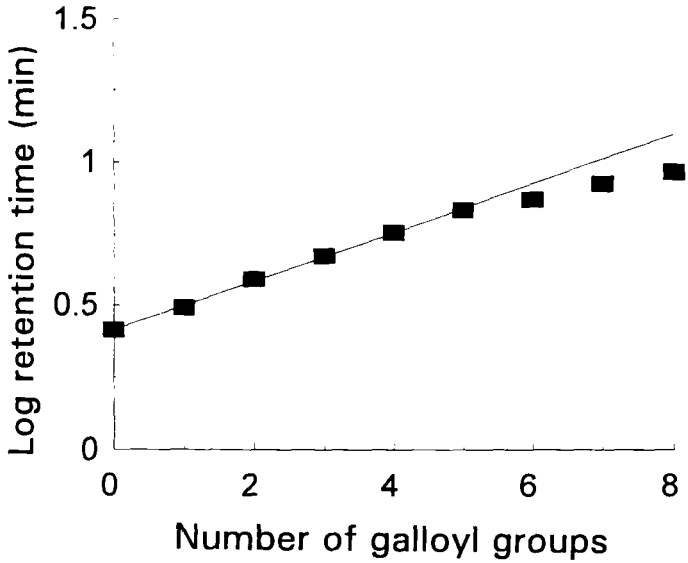

FIG. 1. Normal-phase HPLC calibration curve for gallic acid and galloyl glucose esters. The linear portion of the curve fits the regression equation log retention time = (0.085 × no. galloyl groups) + 0.415 (r^2 = 0.999).

Polyphenol Budgets. Dry-mass polyphenol budgets were determined using insects fed either artificial diet or foliage. Polyphenol-containing artificial diet was prepared by mixing the polyphenol (2.6% DW tannic acid, 2.6% DW fireweed tannin, or 2.2% gallic acid) with the dry ingredients of the Douglas fir tussock moth diet (Bioserve; Frenchtown, NJ), and hydrating as directed by the manufacturer. Polyphenol-coated lettuce (2.5% DW tannic acid or 4.6% DW gallic acid) and rye grass (4.0% DW tannic acid or 4.2% DW gallic acid) were prepared by allowing 15 μl or 13 μl of a 70% acetone solution of the polyphenol to evaporate on 22 mm diameter lettuce discs or 8 cm long rye grass leaves. Control lettuce discs and grass leaves were treated with 15 μl and 13 μl of 70% acetone, respectively.

Insects were placed individually in ventilated 470 ml plastic containers in an incubator (30°C; 16:8 light:dark) and randomly assigned to treated or control food. Initial fresh weights of food offered to each insect, and fresh weights of representative samples of each food, were measured to the nearest 0.1 mg. After feeding for one day they were fed a polyphenol-free chaser diet (foliage for insects previously fed artificial diet, and artificial diet for insects previously fed foliage). Frass was collected once approximately 24 hr after the initiation of feeding on the treated or control diets, and again after chaser diet appeared in the frass. All frass pellets were examined under a dissecting microscope, so that pellets containing egested polyphenols (i.e., pellets containing the treated diet) could be separated from pellets consisting entirely of the chaser diet. To confirm that all egested polyphenols were contained in the frass from the treated food, samples of frass from the chaser diets in three experiments were also analyzed for polyphenols. No tannic acid was found in frass from the chaser diets, but 1–4% of the total gallic acid egested was contained in this frass. Frass samples were stored at −20°C until all samples were collected, and lyophilized. Food samples for determining %DW, and uneaten food remains, were dried at 60°C (2 d). The amount of food consumed (DW) was calculated as the difference between food offered and food remaining.

Polyphenols were quantified in ground lyophilized frass and food samples. Entire samples or 5 mg aliquots were extracted three times in 1.0 ml of 70% acetone containing 0.001 M ascorbic acid at 22°C (±1°C). Extracts were centrifuged (4 min, 13,600 g), and the supernatant solutions were pooled, concentrated under a stream of nitrogen, and lyophilized. Lyophilized extracts were resolubilized in 500 μl of mobile phase (20 or 23% acetonitrile in double-distilled water containing 1% acetic acid), filtered (0.45 μ, Gelman GHP), and analyzed as described above. In most experiments using artificial diet, the gallotannins that eluted in the range of 4–8 min (Figure 2) were quantified. The gallotannins that eluted in the range of 5–13 min (Figure 2) were quantified for budgets using lettuce or rye grass. The water content of each polyphenol used was measured after drying at 60°C and subtracted from the weights of standards

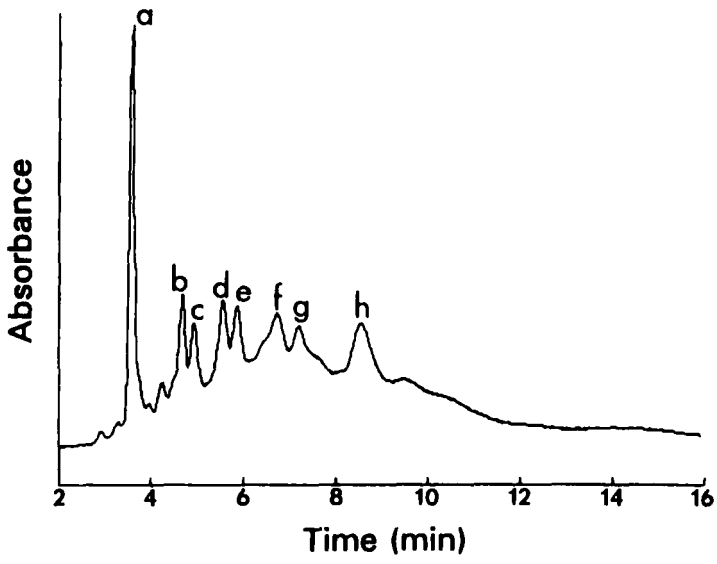

FIG. 2. Tannic acid separated on a Vydac reverse phase (C-18) column using 23% aqueous acetonitrile (with 1% acetic acid) as the mobile phase. Peak a is gallic acid, b and c are isomers of trigalloyl glucose, d and e are isomers of tetragalloyl glucose, f and g are isomers of pentagalloyl glucose, and h is hexagalloyl glucose.

and amounts of polyphenols added to food. Tannic acid is stable in the artificial diet that we used, and can be completely recovered using our extraction and HPLC methods (Barbehenn and Martin, 1992).

Tannin Hydrolysis. The extent of tannin hydrolysis was calculated from measurements of the amounts of gallic acid (GA) ingested and egested by insects used to measure tannin budgets. The extent of hydrolysis of galloyl esters in the ingested tannin was calculated from the expression 100 × (μg GA in frass derived from hydrolysis)/(theoretical μg GA yielded by complete hydrolysis of ingested tannin). The amount of gallic acid in frass derived from hydrolysis was calculated as (μg GA egested) − [(μg free GA ingested) × (% recovery in frass of ingested GA)]. The theoretical amount of gallic acid yielded by complete hydrolysis of ingested tannin was calculated from the expression (μg tannin ingested) × (μg GA produced per μg tannin hydrolyzed) × (% recovery in frass of ingested GA). The weights of gallic acid and tannin ingested were calculated from the expressions (mg food consumed) × (% GA in food) and (mg food consumed) × (% tannin in food), respectively. Tannic acid was found to yield 70% of its weight in gallic acid upon complete hydrolysis (Barbehenn and Martin, 1992). Based upon this determination, we calculated that tannic

acid would yield 69% gallic acid if the free gallic acid was removed from the tannic acid before hydrolysis. We used the calculated yield of 69% gallic acid in this study. Using the structural formula for fireweed tannin (Okuda et al., 1993), it can be calculated that this tannin would yield 22% of its weight in gallic acid upon complete hydrolysis. The percent recovery in frass of ingested gallic acid was found to be 81.5% in artificial diet and 79.8% on lettuce for *M. sanguinipes*, while 100% of ingested gallic acid was recovered in the frass of *P. nebrascensis*.

Changes in Tannic Acid and Gallic Acid Concentrations Along the Gut. *M. sanguinipes* were fed an artificial diet containing 2.6% tannic acid. Insects were dissected, as described above, and samples (2–20 mg fresh weight) of the foregut, anterior midgut, posterior midgut, and hindgut contents were quickly removed and weighed to the nearest 0.01 mg on a Cahn microbalance. Samples were immediately placed in test tubes containing 1.0 ml of 70% acetone containing 0.001 M ascorbic acid, macerated, extracted, and stored covered with parafilm at −20°C (4 d). Extracts were centrifuged, and supernatant solutions were evaporated under a stream of nitrogen and lyophilized. Lyophilized extracts were resolubilized in 500 μl of mobile phase and analyzed using HPLC as described above (30 μl aliquots). Polyphenols that eluted between 4.5–10 min (Figure 2) were quantified in standards and extracts. Concentrations of polyphenols were expressed on a dry weight basis by determining the average percent dry weight (60°C, 2d) of the gut contents in each of the regions of the gut from three insects.

The concentrations of tannic acid and gallic acid that would have been present had there been no assimilation of nutrients from the gut were calculated from the actual concentrations measured: 100 × (μg polyphenol)/[(μg sample) × (1 − assimilation efficiency)]. The assimilation efficiency (55.9% ±4.3 SE) was measured in *M. sanguinipes* using food and other conditions identical to those used in this experiment. The assumption underlying this calculation is that an assimilation efficiency of 55.9% approximates the actual assimilation efficiency throughout the midgut (Dadd, 1970; House, 1974). No correction of the polyphenol concentrations measured in the foregut was necessary since absorption from the foregut is negligible in grasshoppers (Maddrell and Gardiner, 1980).

Gut pH and Redox Conditions. Insects that had fed continuously on their rearing plants were chilled and dissected as described above. Microneedle pH (MI-408P) and silver-silver chloride reference (MI-401) microelectrodes (Microelectrodes, Londonderry, NH) were inserted through pairs of small holes cut in the gut wall. Redox potentials were measured with 0.02 in. platinum redox (MI-800) and silver-silver chloride reference (MI-401) microelectrodes using the same holes immediately following the pH measurements. Measurements in *M. sanguinipes* were made with a Metrohm/Brinkman (model 103)

millivolt and pH meter, and those in *P. nebrascensis* were made with a Corning (model 340) millivolt and pH meter. Observed redox potentials were converted to standard redox potentials (E_h) by adding 200 mV, and pe was calculated as $E_h/59.2$ (Appel and Martin, 1990). Overall redox conditions were summarized by the "redox parameter" (pH + pe), which gives equal weight to proton and electron availability in determining the thermodynamic favorability of oxidation in a complex system (Appel and Martin, 1990).

Polyphenol Oxidation. The amounts of soluble brown pigments in the food and frass of control and polyphenol-fed grasshoppers were measured as an indicator of the oxidation of ingested polyphenols (Barbehenn and Martin, 1994). Aliquots (5 mg) were extracted three times in 1.0 ml of 70% acetone containing 0.001 M ascorbic acid at 22°C (±1°C). Extracts were centrifuged (4 min, 13,600 g). The supernatant solutions were pooled, and the absorbance (420 nm) of each solution was measured with a Zeiss spectrophotometer. The net amount of brown pigment formed per mg frass was calculated by subtracting the amount of brown pigment present per mg in the food. Brown pigment formation in the frass of insects fed food with or without added polyphenols was compared with Mann-Whitney U-tests using SYSTAT (Wilkinson, 1990).

Adsorption of Tannic Acid on the Peritrophic Envelope. Tannic acid adsorption *in vivo* was assayed by feeding *M. sanguinipes* tannic acid-coated lettuce leaf discs (3.3% tannic acid). Leaf discs were coated with 15 μl of a solution of tannic acid (27 mg/ml) in 70% acetone (treatment) or 70% acetone (control). After the acetone had evaporated, two leaf discs were fed to each insect. Freshly treated leaf discs were offered after 12 hr. The insects were kept in ventilated plastic containers in an incubator at 30°C (16 hr L: 8hr D). After approximately 24 hr, chilled (−20°C, 12 min) insects were dissected, and the peritrophic envelope surrounding the food in the midgut and anterior hindgut, but not the caeca, was removed as one piece from the gut. Although the peritrophic envelope present in the caeca was not extracted, the peritrophic envelope in the main region of the midgut includes peritrophic envelope that had previously been in the caeca. The peritrophic envelope was cut longitudinally, separated from the food contents, and rinsed for 15–30 sec in three 100 μl drops of a buffer (pH 7.0) 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. The peritrophic envelope was drained for 10 sec on filter paper and weighed to the nearest 0.01 mg. Polyphenols adsorbed on the peritrophic envelope were extracted twice at 22°C in 500 μl of 70% acetone containing 0.001 M ascorbic acid. Extracts were centrifuged (4 min, 13,600g) and the pooled supernatant solutions were concentrated under a stream of nitrogen after brief storage (12 h, −20°C). Lyophilized extracts were resolubilized in 250 μl of mobile phase, filtered, and 30 μl aliquots were analyzed with HPLC as described above. Fresh

weights and dry weights (60°C, 2 d) of the peritrophic envelope of six control insects were measured to the nearest 0.01 mg in order to express the amount of polyphenols adsorbed on the peritrophic envelope on a dry weight basis. The dry weight/wet weight ratio of the peritrophic envelopes was 0.19 ± 0.09. The gut contents of the dissected insects were also dried and weighed to calculate the maximum amount of tannic acid that was present in the endoperitrophic space.

To confirm that tannic acid was completely removed from the peritrophic envelopes by extraction in 70% acetone, each peritrophic envelope was hydrolyzed in 500 μl of 2 M HCl at 100°C for 20 min. Hydrolysates were neutralized with 500 μl of 2 M NaOH and lyophilized. The lyophilized hydrolysates were resolubilized in mobile phase (300 μl), filtered, and 30 μl aliquots were analyzed with HPLC, as described above. No gallic acid was observed in the chromatograms of the hydrolysates, demonstrating that tannic acid had been completely extracted from the peritrophic envelopes in 70% acetone.

Permeability of the Peritrophic Envelope to Polyphenols. M. sanguinipes that had fed on lettuce were chilled (−20°C, 9 min) and their guts were excised. The foregut and anterior midgut (including the caeca) were removed. The hindgut was ligated with a silk suture (size 6-0). Tannic acid (40 mg) was dissolved in 1.0 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.01 M ascorbic acid, and the solution was purged with nitrogen to prevent oxidation before being introduced into the insect. Using a Hamilton microsyringe (10 μl) held with a micromanipulator, 2.0 μl of phosphate buffer (N = 3) or tannic acid solution (N = 10) was injected through the open (anterior) end of the midgut into the endoperitrophic space. A suture was used to clamp the anterior end of the midgut around the needle of the syringe to prevent leakage of the injected polyphenol solution. The anterior midgut was then ligated, and a small hole (0.9 ± 0.1 mm^2) was cut through the midgut wall to expose the peritrophic envelope. After rinsing the gut preparation for approximately 10 sec in each of three beakers of Tris buffer (described above), each gut preparation was placed in 1.5 ml of Tris buffer in the well of a tissue culture plate and incubated for 1.5-2 hr. Following incubation, the incubating solutions (1.5 ml) were transferred to test tubes, frozen (−20°C) and lyophilized. The contents of the midgut of each insect was extracted twice in 1.0 ml of 70% acetone (containing 0.001 M ascorbic acid) for 30 min at 22°C. Extracts were combined and centrifuged (4 min, 13,600g), and the supernatant solutions were concentrated under a stream of nitrogen. Concentrated extracts were lyophilized and stored at −20°C in a dessicator until they were analyzed with HPLC. Lyophilized samples were resolubilized in 0.5 ml (for incubating solutions) or 1.0 ml (for gut contents) of 20% acetonitrile containing 1% acetic acid, and filtered. Aliquots of samples (30 μl for incubating solutions, 20 μl for gut contents) and standards (25 μl) were analyzed with HPLC as described above. The mobile phase was 20% acetonitrile containing

1% acetic acid for incubating solutions, and 25% acetonitrile containing 1% acetic acid for gut contents.

This experiment was repeated using *P. nebrascensis*, with the following modifications: all insects were fed rye grass seedlings, distilled water (2 μl) was injected into the midguts of four insects (controls), tannic acid was injected into the midguts of six insects, gallic acid (2 μl, 40 mg/ml) was injected into the midguts of four insects, holes cut through the midgut wall averaged 2.2 ± 0.5 mm^2 and the mobile phase was 20% acetonitrile containing 1% acetic acid for all samples and standards.

Damage to the peritrophic envelopes during the dissection process was determined by observing whether a plume of dark gut fluid leaked from the exposed area. Any damage that was not detected during dissection was determined by examining the tannin permeation data for statistical outliers (Wilkinson, 1990). No outliers were observed in the data sets. Data from both experiments are presented as total μg polyphenols permeating the peritrophic envelope, since the amounts of polyphenols permeating the peritrophic envelope were not significantly correlated with either incubation time or hole diameter.

RESULTS

Composition of Tannins. Using either normal- or reverse-phase HPLC, the components of tannic acid elute in order of increasing number of galloyl esters (Figures 1 and 2; Hagerman et al., 1992). The conditions chosen for reverse-phase HPLC did not resolve the components of the tannic acid completely, but allowed for the higher molecular weight components to be measured (e.g., peak h; Figure 2). Isomers of several polygalloyl glucose esters were resolved as split peaks (Figure 2). Tannic acid from *Q. infectoria* (Sigma) contained an average of 10.2% trigalloyl glucose (peaks b and c), 21.1% tetragalloyl glucose (peaks d and e), 24.6% pentagalloyl glucose (peaks f and g), and 20.2% hexagalloyl glucose (peak h). The tannic acid used in this study also contained an average of 4.4% gallic acid (peak a). Mono- and digalloyl glucose and galloyl glucose esters larger than hexagalloyl glucose, which collectively account for the remaining 19.5% of the tannic acid, could not be quantified accurately with reverse-phase HPLC. Fireweed tannin contained oenothein B (>80%), gallic acid (<1%), and some lower molecular weight compounds that were not identified.

Tannin Budgets. Only a small fraction of the tannins ingested by either *M. sanguinipes* (5–25%) or *P. nebrascensis* (22%) could be recovered from their frass (Table 1). The tannins that were recovered from the frass of *P. nebrascensis* consisted primarily of tetragalloyl glucose, whereas low concentrations of several gallotannins were present in the frass of *M. sanguinipes*, but these were poorly resolved using HPLC.

Tannin Hydrolysis. In *M. sanguinipes* 12.1-46.9%, and in *P. nebrascensis* 82.0%, of the galloyl ester groups of the ingested tannins were hydrolyzed (Table 2). In *M. sanguinipes* tannic acid was more extensively hydrolyzed when ingested in an artificial diet than when ingested on lettuce leaves, and tannic acid was more susceptible to hydrolysis than fireweed tannin.

Changes in Tannic Acid and Gallic Acid Concentrations Along the Gut. The tannic acid content of the foregut was substantially less than that of the diet, identifying the foregut as a major site of transformation of tannic acid (Figure 3). In contrast, the gallic acid content of the foregut was 3.6 times higher than in the food, demonstrating that hydrolysis accounts for the loss of some tannic acid in the foregut. However, we calculate that hydrolysis can

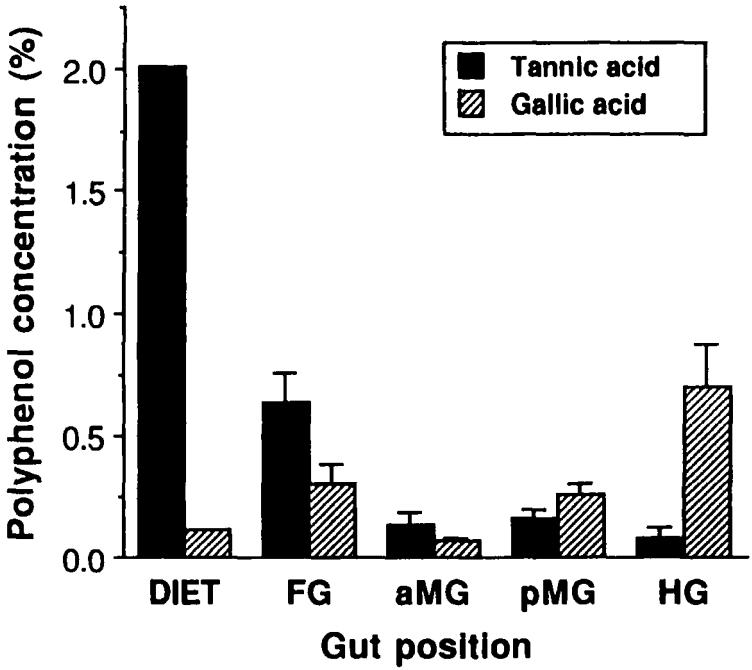

FIG. 3. Change in the percentage (DW) of tannic acid and gallic acid in artificial diet passing through the guts of *Melanoplus sanguinipes*. Gallic acid in the diet is an impurity in tannic acid. The percentage of polyphenols in the absorptive regions of the gut (midgut and hindgut) were transformed to the percentage of polyphenols in an equivalent mass of food, using the expression: (μg polyphenol)/[(μg sample/(1 − 0.559)], where 0.559 is the average approximate digestibility of the food. FG = foregut, aMG = anterior midgut, pMG = posterior midgut, and HG = hindgut.

TABLE 1. DRY MASS BUDGETS FOR TANNIC ACID (TA) AND FIREWEED TANNIN (FWT) INGESTED BY *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Diet (N)	Tannin ingested	Tannin egested	Percent tannin recovered
Melanoplus sanguinipes			
TA in artificial diet[b] (12)	666.3 ± 74.9	153.1 ± 23.6	22.5 ± 2.4
TA in artificial diet[c] (9)	34.4 ± 4.7	7.4 ± 0.7	24.7 ± 3.6
FWT in artificial diet (11)	1402.7 ± 91.2	166.4 ± 21.7	12.3 ± 1.6
TA on lettuce leaves[d] (12)	398.5 ± 25.1	21.6 ± 4.2	5.3 ± 1.0
TA on lettuce leaves[d] (10)	478.9 ± 49.4	22.7 ± 7.4	5.4 ± 1.6
Phoetaliotes nebrascensis			
TA on rye leaves[d] (9)	420.8 ± 8.7	94.3 ± 21.4	22.5 ± 5.2

[a] Data expressed as mean μg/insect ± SE.
[b] TA components eluting between 4–8 min (Figure 2) from a Vydac C-18 column were quantified.
[c] TA ingested was estimated from the weight of frass produced and an average approximate digestibility of 55.9%. TA egested was measured in frass pellets dissected from the hindgut. TA components eluting between 4.5–10 min (Figure 2) from a Vydac C-18 column were quantified.
[d] TA components eluting between 5–13 min (Figure 2) from a Vydac C-18 column were quantified.

TABLE 2. HYDROLYSIS OF TANNIC ACID (TA) AND FIREWEED TANNIN (FWT) DURING PASSAGE THROUGH THE GUTS OF *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Diet (N)	Total tannin ingested	Net GA egested[b]	Percent galloyl esters hydrolyzed[c]
Melanoplus sanguinipes			
TA in artificial diet (9)	40.1 ± 5.4[d]	8.3 ± 2.0	46.9 ± 12.3
FWT in artificial diet (11)	1402.7 ± 91.2[e]	52.7 ± 5.3	19.7 ± 1.6
TA on lettuce leaves (12)	526.8 ± 33.2[e]	34.6 ± 19.3	12.1 ± 6.6
TA on lettuce leaves (10)	694.3 ± 66.5[e]	32.1 ± 17.8	13.0 ± 7.6
Phoetaliotes nebrascensis			
TA on rye leaves (9)	438.3 ± 8.7[f]	250.3 ± 28.7	82.0 ± 8.2

[a] Data expressed as mean μg/insect ± SE.
[b] Calculated from the expression (μg GA egested) − [(μg GA ingested) × (%recovery of ingested GA)].
[c] Calculated from the expression (net μg GA produced)/[(μg tannin ingested) × (μg GA produced per μg tannin hydrolyzed) × (%recovery of ingested GA)]. TA yields 69% GA and FWT yields 22% GA upon hydrolysis. The percent recovery of GA ingested in artificial diet by *M. sanguinipes* was 81.5, and 79.8 when fed on lettuce. In *P. nebrascensis* all GA was recovered.
[d] TA ingested was calculated from the weight of frass produced and an average approximate digestibility of 55.9%.
[e] Tannin ingested was calculated from the %DW of tannin added to the food and the amount of food consumed.

account for no more than 45% (±7.6 SE) of the tannic acid lost from the diet in the foregut. There was a further drop in the concentration of tannic acid in the anterior midgut, identifying this region as an additional site of transformation of tannic acid. The correct concentration of tannic acid and gallic acid in the anterior midgut depends upon the extent of nutrient assimilation in this region, and may be less if an assimilation efficiency of 55.9%, which we used to correct the food mass, is excessive. By contrast, if no nutrient assimilation had occurred in the anterior midgut, then the percent tannic acid and gallic acid in the anterior midgut would have been 0.88 ± 0.15% and 0.33 ± 0.04%, respectively. The large increase in the concentration of gallic acid in the hindgut was presumably due to the excretion of assimilated gallic acid by the Malpighian tubules.

Gut pH and Redox Conditions. The pH throughout the guts of *M. sanguinipes* and *P. nebrascensis* is mildly acidic to neutral (Table 3). The redox conditions measured throughout the guts of both species are oxidizing (positive Eh, pH + pe > 9) (Table 3).

Polyphenol Oxidation. Polyphenol oxidation generates brown pigments (Hathway and Seakins, 1957; Thomson, 1962; Leatham et al., 1980; Igarashi and Yasui, 1985; Cilliers and Singleton, 1989). There was a significant increase ($P < 0.01$) in the amounts of brown pigments in the frass of insects that consumed tannic acid or fireweed tannin in an artificial diet (Table 4). There was no significant increase in brown pigment formation in *M. sanguinipes* that ingested gallic acid in artificial diet, consistent with the high recovery of ingested gallic acid in the frass (81.6%). Because of the high background absorbance in plant extracts, this method could not be used as an indicator of polyphenol oxidation in insects fed treated foliage.

Adsorption of Tannic Acid on the Peritrophic Envelope. When *M. sanguinipes* was fed tannic acid-coated lettuce, less than 1% of the total tannic acid

TABLE 3. pH AND REDOX CONDITIONS IN THE GUTS OF *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Gut position (N)	pH	Eh (mV)	pe	pH + pe
M. sanguinipes				
Foregut (14)	5.52 ± 0.07	270 ± 20	4.55 ± 0.33	10.07 ± 0.33
Midgut (14)	6.75 ± 0.14	168 ± 18	2.84 ± 0.31	9.58 ± 0.30
Hindgut (8)	6.80 ± 0.08	238 ± 16	4.01 ± 0.28	10.81 ± 0.28
P. nebrascensis				
Foregut (7)	6.03 ± 0.08	187 ± 7	3.17 ± 0.12	9.19 ± 0.12
Midgut (7)	7.12 ± 0.17	118 ± 8	1.99 ± 0.14	9.11 ± 0.22
Hindgut (7)	6.11 ± 0.14	213 ± 9	3.60 ± 0.16	9.71 ± 0.09

[a] Data are presented as mean ± SE.

TABLE 4. BROWN PIGMENTS (A_{420}) PRODUCED IN *Melanoplus sanguinipes* FED TANNIC ACID, FIREWEED TANNIN, AND GALLIC ACID IN ARTIFICIAL DIET[a]

Polyphenol	Control (N) (A_{420}/mg)	Treatment (N) (A_{420}/mg)
Tannic acid (2.6%)	0.008 ± 0.001 (6)	0.028 ± 0.009 (12)**
Fireweed tannin (2.6%)	0.029 ± 0.003 (10)	0.048 ± 0.004 (11)**
Gallic acid (2.2%)	0.016 ± 0.003 (2)	0.013 ± 0.002 (7)

[a]Data are presented as mean ± SE. **indicates $P < 0.01$ (Mann-Whitney U-test).

present in its midgut was adsorbed on the peritrophic envelope (Table 5). The tannic acid that was adsorbed on the peritrophic envelope of *M. sanguinipes* consisted of roughly equal amounts of tetragalloyl and hexagalloyl glucose.

Permeability of the Peritrophic Envelope to Polyphenols. Several gallotannins were found to permeate the peritrophic envelope of *M. sanguinipes* (Table 6). For example, the peritrophic envelope was permeated by 10.8% of the tetragalloyl glucose present in the tannic acid that was injected into the midgut, or 15.0% of that which was still detectable in the midgut and incubating solution at the end of the incubation period. In *P. nebrascensis*, the peritrophic envelope was permeated by 19.8% of the tetragalloyl glucose that was initially injected into the midgut, or 26.9% of that which was still detectable in the midgut and incubating solution at the end of the incubation period. The peritrophic envelopes of both species were freely permeable to gallic acid (Table 6). When pure gallic acid (80 μg) was introduced into the midgut of *P. nebrascensis*, an average of 28.7 μg (35.9 ± 5.0%) diffused through the peritrophic envelope.

DISCUSSION

Most of the tannin ingested by *M. sanguinipes* (75–95%) and *P. nebrascensis* (78%) is removed during passage through the gut. In *M. sanguinipes*,

TABLE 5. ADSORPTION OF TANNIC ACID (TA) ON THE PERITROPHIC ENVELOPE (PE) OF *Melanoplus sanguinipes in vivo*[a]

TA in Midgut	PE Dry weight[b]	TA Adsorbed on PE
107.3 ± 10.3	97.8 ± 16.8	0.95 ± 0.24

[a]Data expressed as mean μg ± SE. N = 10.
[b]Does not include peritrophic envelope in the caeca.

TABLE 6. POLYPHENOLS IN TANNIC ACID RETAINED WITHIN OR PERMEATING THE PERITROPHIC ENVELOPES OF *Melanoplus sanguinipes* AND *Phoetaliotes nebrascensis*[a]

Polyphenol	μg Introduced into midgut	μg Retained within PE	μg Recovered in incubating solution
M. sanguinipes (10)			
Gallic acid	3.5	10.1 ± 1.9	6.6 ± 1.2
Tannin components	52.8	18.3 ± 1.3	2.3 ± 0.5
Tetragalloyl glucose	16.9	11.5 ± 1.2	1.8 ± 0.4
Pentagalloyl glucose	19.7	3.4 ± 0.6	0.01 ± 0.006
Hexagalloyl glucose	16.2	3.4 ± 0.8	0.4 ± 0.2
P. nebrascensis (6)			
Gallic acid	3.5	1.3 ± 0.2	2.9 ± 0.8
Tannin components	52.8	11.4 ± 2.4	4.0 ± 1.3
Tetragalloyl glucose	16.9	7.3 ± 0.6	3.3 ± 0.9
Pentagalloyl glucose	19.7	2.1 ± 0.8	0.6 ± 0.4
Hexagalloyl glucose	16.2	2.0 ± 1.1	0

[a]Tannic acid was introduced into the midguts of insects by injecting 2 μl of a 4% solution of tannic acid in pH 7 phosphate buffer. Gut preparations were incubated for 1.5–2 hr. Data are presented as mean ± SE.

the primary sites of tannin removal are the foregut and the anterior midgut. We can envision *a priori* three processes that would result in the removal of tannins from an insect's gut: hydrolysis, adsorption on the peritrophic envelope, and oxidation. The first two are potential detoxification mechanisms. By contrast, the third is a process that potentially contributes to tannin toxicity.

In agreement with earlier studies (Bernays, 1978; Bernays and Chamberlain, 1980), we found that gallotannins are hydrolyzed in the guts of polyphagous grasshoppers. The extent of hydrolysis was much less in *M. sanguinipes* than in *P. nebrascensis*, and varied widely depending upon the type of tannin and nature of the diet in which it was ingested. The limited extent of tannin hydrolysis in *M. sanguinipes* argues strongly against the idea that this transformation plays a significant role in the detoxification of tannins in this species. For hydrolysis to result in detoxification, it must be extensive and it must result in the formation of nontoxic products. Partial hydrolysis, in which one gallotannin is converted into another with fewer galloyl groups, will be an effective detoxification mechanism only if the lower molecular weight gallotannin is significantly less toxic than the higher molecular weight analogue. The failure of partial hydrolysis to protect an insect from tannins is illustrated by the tannin-sensitivity of *Locusta migratoria*, a grasshopper in which 43% of the galloyl groups of ingested tannic acid are hydrolyzed (Bernays, 1978). We conclude, therefore, that hydrolysis is not a general mechanism that is sufficient to explain the widespread tolerance among polyphagous grasshoppers of hydrolyzable tannins.

Adsorption on the peritrophic envelope is a second mechanism by which tannins might be removed from the gut milieu, thereby protecting grasshoppers from the potential toxic effects of ingested tannins (Bernays and Chamberlain, 1980). We have not confirmed the earlier report that significant quantities of tannic acid are adsorbed by the peritrophic envelopes of polyphagous grasshoppers. Whereas Bernays and Chamberlain (1980) reported that the peritrophic envelope of *Schistocerca gregaria* adsorbed 100% of its weight in tannic acid *in vivo*, we found that the peritrophic envelope of *M. sanguinipes* adsorbed less than 1% of its weight in tannic acid. The different results obtained in the two studies may reflect the selection of different species or the use of different analytical methods. In the earlier study, adsorbed tannic acid was estimated by measuring the A_{270} of the HCl-hydrolyzate of the peritrophic envelopes of insects fed tannic acid-treated foliage. However, it was not established whether the A_{270} was due to gallic acid or to other compounds solubilized during hydrolysis. The method used in the present study (HPLC) entailed both the identification and quantification of specific gallotannins adsorbed by the peritrophic envelope. Whether the different results are due to our use of different species or different techniques, the conclusion is the same: the removal of tannic acid from the gut lumen by adsorption on the peritrophic envelope is not a general mechanism explaining the widespread tolerance among polyphagous grasshoppers of hydrolyzable tannins.

In further contrast to the earlier report that the peritrophic envelopes of tannin-tolerant grasshoppers are impermeable to tannic acid (Bernays et al., 1980), we have found that some of the gallotannins in tannic acid readily move across the peritrophic envelopes of *M. sanguinipes* and *P. nebrascensis*. In previous studies, the impermeability of the peritrophic envelope to tannins was inferred from the absence of lesions in histological sections (Bernays et al., 1980; Steinly and Berenbaum, 1985). Alternatively, the permeability of the peritrophic envelope to tannins was inferred from the presence of midgut lesions, or from the accumulation of polyphenols in damaged midgut tissues. The validity of this indirect approach is questionable; it is possible that tannins could cross the peritrophic envelope but not damage, or accumulate in, midgut tissues. In this study, we evaluated the permeability of the peritrophic envelope to tannins by direct measurement of the amounts of specific gallotannins that diffused from the endo- to the ectoperitrophic space. From our observation that the peritrophic envelopes of *M. sanguinipes* and *P. nebrascensis* are permeable to some gallotannins, we conclude that the tannin-tolerance of generalist grasshoppers does not result simply from the containment of tannic acid within the endoperitrophic space.

In our studies of *Malacosoma disstria* and *Orgyia leucostigma* (Lepidoptera), we found that tannic acid was oxidized in the midgut of *M. disstria*, a tannin-sensitive species, but not in the midgut of *O. leucostigma*, a tannin-

tolerant species (Barbehenn and Martin, 1992, 1994). These findings lead us to expect that tannin-tolerance in polyphagous grasshoppers would be associated with a lack of oxidation of tannic acid in their guts. However, conditions in the guts of both species favor oxidation. Oxidizing redox conditions have also been found in the guts of *Locusta migratoria* (Bignell, 1984) and *Ageneotettix deorum* (Barbehenn, unpublished data), suggesting that oxidizing conditions are a common feature of the grasshopper gut. Further evidence that tannins are oxidized in the guts of *M. sanguinipes* is provided by the observation of elevated levels of brown pigments in the frass of insects feeding on tannin-containing diets. These measurements cannot be used to calculate the actual amount of polyphenol oxidized, since the relationship between the amount of absorption at 420 nm and the amount of polyphenol oxidized has not been determined. However, if oxidation accounts for the difference between the amount of tannin ingested and the amount that can be recovered from the frass plus the amount determined to have been hydrolyzed, then the percent oxidized was substantial in *M. sanguinipes*: 28% in artificial diet and 83% on foliage. Thus, the ability of some polyphagous grasshoppers to tolerate ingested tannins results from their protection from the consequences of polyphenol oxidation, not from the absence of oxidation.

Antioxidant systems are well known in insects (Ahmad, 1995; Felton, 1995; Felton and Summers, 1995). Gut tissues may be protected from reactive oxygen species by enzymes, such as catalase and glutathione transferase peroxidase, and antioxidants, such as ascorbic acid. The polysaccharides and proteoglycans of the peritrophic envelope may also trap oxygen radicals generated in the midgut lumen (Felton and Summers, 1995). Felton and Summers (1995) showed that oxidative damage to midgut tissues was reduced *in vitro* in proportion to the mass of peritrophic envelope present. Thus, the hypothesis that the peritrophic envelope plays a critical role in protecting polyphagous grasshoppers from tannins remains a viable one, although the role may be to scavenge oxygen radicals, rather than to contain tannins within the endoperitrophic space. This possibility seems especially plausible considering the finding of Bernays and Simpson (1989) that the peritrophic envelopes of tannin-tolerant grasshoppers are thicker, and potentially capable of scavenging more oxygen radicals, than those of tannin-sensitive species.

Finally, we propose that the structural organization of digestion in grasshoppers may contribute to the ability of some species to tolerate ingested tannins. The foregut is not only a region in which a substantial amount of digestion occurs (Robinson, 1964; Price and Robinson, 1966; Evans and Payne, 1964; Droste and Zebe, 1974), but it is also the most active region for the hydrolysis, and probably the oxidation, of ingested tannic acid in *M. sanguinipes*. Grasshopper foreguts contain copious quantities of dark gut fluid ("tobacco juice"). The dark color of this fluid is consistent with the occurrence of polyphenol

oxidation in the foregut. In *M. sanguinipes*, most of the oxidation of tannic acid appears to take place in the foregut, since hydrolysis can account for only 45% of the tannic acid lost before ingested food reaches the anterior midgut. Because the foregut is lined with impermeable cuticle (Maddrell and Gardiner, 1980; Chapman, 1982), polyphenol oxidation in this region would not pose a threat to sensitive gut tissues, as it would in the midgut. Thus, the grasshopper foregut may function as an oxidation-resistant reaction vessel, in which the oxidation of ingested tannins occurs prior to their passage into the more sensitive midgut.

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REFERENCES

AHMAD, S. 1995. Antioxidant mechanisms of enzymes and proteins. pp. 238-272, *in* S. Ahmad (ed.). Oxidative Stress and Antioxidant Defenses in Biology. Chapman and Hall, New York.

APPEL, H. M. 1993. Phenolics in ecological interactions: the importance of oxidation. *J. Chem. Ecol.* 19:1521-1552.

APPEL, H. M., and MARTIN, M. M. 1990. Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.* 16:3277-3290.

BARBEHENN, R. V., and MARTIN, M. M. 1992. The protective role of the peritrophic membrane in the tannin-tolerant larvae of *Orgyia leucostigma* (Lepidoptera). *J. Insect Physiol.* 38:973-980.

BARBEHENN, R. V., and MARTIN, M. M. 1994. Tannin sensitivity in larvae of *Malacosoma disstria* (Lepidoptera): roles of the peritrophic envelope and midgut oxidation. *J. Chem. Ecol.* 20:1985-2001.

BERNAYS, E. A. 1978. Tannins: an alternative viewpoint. *Entomol. Exp. Appl.* 24:244-253.

BERNAYS, E. A., and CHAMBERLAIN, D. J. 1980. A study of tolerance of ingested tannin in *Schistocerca gregaria*. *J. Insect Physiol.* 26:415-420.

BERNAYS, E. A., CHAMBERLAIN, D., and MCCARTHY, P. 1980. The differential effects of ingested tannic acid on different species of Acridoidea. *Entomol. Exp. Appl.* 28:158-166.

BERNAYS, E. A., and SIMPSON, S. J. 1989. Nutrition. pp. 105-128, *in* R. F. Chapman and A. Joern (eds.). Biology of Grasshoppers. John Wiley and Sons, New York.

BIGNELL, D. E. 1984. Direct potentiometric determination of redox potentials of the gut contents in the termites *Zootermopsis nevadensis* and *Cubitermes severus* and in three other arthropods. *J. Insect Physiol.* 30:169-174.

CADENAS, E. 1995. Mechanisms of oxygen activation and reactive oxygen species detoxification. pp. 1-61, *in* S. Ahmad (ed.). Oxidative Stress and Antioxidant Defenses in Biology. Chapman and Hall, New York.

CHAPMAN, R. F. 1982. The Insects: Structure and Function. Harvard University Press, Cambridge, MA.

CILLIERS, J. J. L., and SINGLETON, V. L. 1989. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *J. Agric. Food Chem.* 37:390-396.

DADD, R. H. 1970. Digestion in insects. pp. 117-145, *in* M. Florkin and B. T. Scheer (eds.). Chemical Zoology. Academic Press, New York.

DROSTE, H. J., and ZEBE, E. 1974. Carbohydrasen und Kohlenhydratverdauung im Darmtrakt von *Locusta migratoria*. *J. Insect Physiol.* 20:1639-1657.

EVANS, W. A. L., and PAYNE, D. W. 1964. Carbohydrases of the alimentary tract of the desert locust, *Schistocerca gregaria Forsk. J. Insect Physiol.* 10:657–674.

FELTON, G. W. 1995. Oxidative stress of vertebrates and invertebrates. pp. 356–434, *in* S. Ahmad (ed.). Oxidative Stress and Antioxidant Defenses in Biology. Chapman and Hall, New York.

FELTON, G. W., and SUMMERS, C. 1995. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol.* 29:187–197.

GANGWERE, S. K., EVANS, F. C., and NELSON, M. L. 1976. The food-habits and biology of Acrididae in an old-field community of southeastern Michigan. *Great Lakes Entomol.* 9:83–123.

HAGERMAN, A. E., ROBBINS, C. T., WEERASURIYA, Y., WILSON, T. C., and MCARTHUR, C. 1992. Tannin chemistry in relation to digestion. *J. Range Manage.* 45:57–62.

HATHWAY, D. E., and SEAKINS, J. W. T. 1957. Autoxidation of polyphenols. Part 3. Autoxidation in neutral aqueous solution of flavans related to catechin. *J. Chem. Soc.* 2:1562–1566.

HOUSE, H. L. 1974. Digestion. pp. 63–117, *in* M. Rockstein (ed.). The Physiology of Insecta. Academic Press, New York.

IGARASHI, K., and YASUI, T. 1985. Oxidation of free methionine and methionine residues in protein involved in the browning reaction of phenolic compounds. *Agric. Biol. Chem.* 49:2309–2315.

ISELY, F. B. 1944. Correlation between mandibular morphology and food specificity in grasshoppers. *Ann. Entomol. Soc. Am.* 37:47–67.

JOERN, A. 1983. Host plant utilization by grasshoppers (Orthoptera: Acrididae) from a sandhills prairie. *J. Range Manage.* 36:793–797.

LEATHAM, G. F., KING, V., and STAHMANN, M. A. 1980. In vitro protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. *Phytopathology* 70:1134–1140.

MADDRELL, S. H. P., and GARDINER, B. O. C. 1980. The permeability of the cuticular lining of the insect alimentary canal. *J. Exp. Biol.* 85:227–237.

MOLE, S., and JOERN, A. 1994. Feeding behavior of graminivorous grasshoppers in response to host-plant extracts, alkaloids, and tannins. *J. Chem. Ecol.* 20:3097–3109.

OKUDA, T., YOSHIDA, T., and HATANO, T. 1993. Classification of oligomeric hydrolysable tannins and specificity of their occurrence in plants. *Phytochemistry* 32:507–521.

PRICE, R. G., and ROBINSON, D. 1966. A comparison of the β-D-glucosidase and β-D-galactosidase activities from eleven enzyme sources. *Comp. Biochem. Physiol.* 17:129–138.

RAUBENHEIMER, D. 1992. Tannic acid, protein, and digestible carbohydrate: dietary imbalance and nutritional compensation in locusts. *Ecology* 73:1012–1027.

ROBINSON, D. 1964. Fluorimetric determination of glycosidases in the locust (*Locusta migratoria*) and other insects. *Comp. Biochem. Physiol.* 12:95–105.

STEINLY, B. A., and BERENBAUM, M. 1985. Histopathological effects of tannins on the midgut epithelium of *Papilio polyxenes* and *Papilio glaucus*. *Entomol. Exp. Appl.* 39:3–9.

SUMMERS, C. B., and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Molec. Biol.* 24:943–953.

THOMSON, R. H. 1962. Some naturally occurring black pigments. pp. 99–113, *in* T. S. Gore, B. S. Joshi, S. V. Sunthankar, and B. D. Tilak (eds.). Recent Progress in the Chemistry of Natural and Synthetic Colouring Matters and Related Fields. Academic Press, New York.

WILKINSON, L. 1990. SYSTAT: the System for Statistics. SYSTAT Inc., Evanston, IL.

ZHAO, Y. 1995. Simultaneous determination of hydrolyzable tannin and condensed tannin. M.S. Thesis, Miami Univ., Oxford, Ohio.