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DETOXIFICATION IN RELATION TO TOXIN TOLERANCE IN DESERT WOODRATS EATING CREOSOTE BUSH

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Abstract—We studied the relationship between the use of three detoxification pathways and urine pH and the tolerance of desert woodrats from two populations to a mixture of naturally occurring plant secondary metabolites (mostly phenolics) in resin from creosote bush (*Larrea tridentata*). The two populations of desert woodrats came from the Mojave desert (Mojave woodrats), where woodrats consume creosote bush, and from the Great Basin desert (Great Basin woodrats), where the plant species is absent. We fed woodrats alfalfa pellets containing increasing levels of the phenolic resin and measured three detoxification pathways and urine pH that are related to detoxification of allelochemicals. We found that the excretion rate of two phase II detoxification conjugates, glucuronides and sulfides, increased with increasing resin intake, whereas excretion of hippuric acid was independent of resin intake, although it differed between populations. Urine pH declined with increasing resin ingestion. The molar proportion of glucuronides in urine was three times that of the other conjugates combined. Based on an evaluation of variation in the three detoxification pathways and urine pH in relation to resin intake, we rejected the hypotheses that woodrats' tolerance to resin intake is related to capacity for amination, sulfation, or pH regulation. However, Mojave woodrats had higher maximum glucuronide excretion rates, and we accepted the hypothesis that within and between populations woodrats tolerate more resin because they have a greater capacity for glucuronide excretion.

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INTRODUCTION

Herbivores that feed on plants containing plant secondary metabolites (PSMs) can manage owing to enzymes that metabolize the ingested toxins (Brattsten, 1992). Freeland and Jansen (1974) proposed that the amount of PSMs that an individual can ingest is related to its capacity to detoxify and eliminate PSMs. This capacity is ecologically important because it constitutes a constraint on how much of a food containing the PSM might be ingested, and so it can be used to predict diets (Belovsky and Schmitz, 1991).

The ability of herbivores to cope with PSMs can result from: (1) the capacity to habituate to aversive sensory properties of the PSM, (2) differential rates of PSM absorption, (3) variation in target site sensitivity, and (4) the capacity to detoxify PSMs, either by using different detoxification pathways or because of differences in the specific kinetics of detoxification (Lindroth, 1988). This study relates primarily to the fourth factor. Although there is a large body of literature on detoxification pathways used by mammals, and their relative affinities and capacities as enzymatic systems (Caldwell, 1986; Sipes and Gandolfi, 1991), there are three points that deserve more attention. First, almost all detoxification pathways have been studied with humans and laboratory and domestic mammals (Parke, 1968; Caldwell, 1986; Sipes and Gandolfi, 1991) and very little in wild mammal–allelochemical systems (McArthur and Sanson, 1993; Harju-Anu, 1996). Second, one of the major detoxification systems for the conjugation of phenolic compounds, glucuronidation, has been one of the more extensively studied in laboratory mammal–drug systems. A direct consequence of this is that glucuronidation usually is the only conjugation route studied in wild mammal–allelochemical systems (Lindroth and Batzli, 1983; MacCoubrey et al., 1997), although a few studies in birds measured other conjugation pathways along with glucuronidation (Jakubas et al., 1993; Guglielmo et al., 1996; Hewitt and Kirkpatrick, 1997). Third, researchers rarely determine the maximum capacities of conjugation pathways or which pathways are induced with toxin exposure and which are fixed at some constitutive level. Consequently, there are few tests of the idea that the amount of PSMs that an individual can ingest is related to its capacity to detoxify and eliminate them.

The general objective of this study was to establish the relationship between the use of three detoxification mechanisms and urine pH and the tolerance of desert woodrats (*Neotoma lepida*) from two populations to a mixture of naturally occurring PSMs (mostly phenolics) from creosote bush (*Larrea tridentata*). The detoxification mechanisms are phase II detoxification pathways that involve conjugation of endogenous substrates to toxin metabolites: glucuronidation, sulfation,

and amino acid (glycine) conjugation to form hippuric acid. These were chosen because: (1) they are the most common conjugation reactions in mammals; (2) all three can be detected in urine; and (3) their respective endogenous moieties (glucuronic acid, sulfate and glycine) can be limiting factors under nutritional stress and hence detoxification could be compromised, or, if the need for moieties exceeds their supply, the nutritional status of the animals may be compromised (Sipes and Gandolfi, 1991; Guglielmo et al., 1996).

Urine pH and the three detoxification pathways we studied relate to acid–base homoeostasis that may be affected by organic acids produced as a result of the biotransformation and metabolism of PSMs (Foley et al., 1995). These organic acids are ionized at physiological pH (Robinson et al., 1953). The resulting hydrogen ion can be excreted in the urine or buffered. However, if the animal's buffering capacity is saturated, the pH of the urine will decrease.

The two populations of desert woodrats subjected to study came from the Mojave desert (hereafter called Mojave woodrats) and from the Great Basin desert (hereafter called Great Basin woodrats). While the natural diet of Great Basin woodrats is mostly composed by Utah juniper (personal observation), the diet of Mojave woodrats is composed of at least 22% of creosote bush in some areas in the Mojave desert (Mangione et al., 2000). The leaves of creosote bush are covered with a resin (Mabry et al., 1977) that makes up 10–25% leaf dry mass. The resin is composed of 40% dry mass of nordihydroguaiaretic acid (NDGA) and the remainder of the resin is a complex mixture of partially *O*-methylated flavones and flavonols (Rhoades and Cates, 1976; Mabry et al., 1977). The resin is known to deter feeding by arthropods and to complex with protein in vitro. NDGA fed to laboratory rats at 0.5, 1, or 3% produced cysts in the kidney and vacuolation of kidney tubular epithelium (Grice et al., 1968; Goodman et al., 1970). In a previous study (Mangione et al., 2000), we found that there were differences in tolerance to creosote bush resin between these two populations; Mojave woodrats ingested more resin, and maintained body mass constant at higher concentrations of resin in the diet. Possibly, the difference in tolerance relates to differences in detoxification pathways used by woodrats from these two populations.

The specific goals of the study were: (1) to test whether desert woodrats rely more on glucuronidation compared to sulfation and glycine conjugation (hippuric acid), as occurs for other mammals; (2) to find the maximum capacities of each detoxification route and its relationship with the maximum tolerance of resin in the diet; (3) to establish whether the detoxification of the resin affects acid–base balance of woodrats; and (4) to test whether the differential use of detoxification routes explains the tolerance difference between the two populations of desert woodrats studied earlier (Mangione et al., 2000).

We investigated the overall hypothesis that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate the PSMs by testing a number of specific predictions, which are illustrated in Figure 1

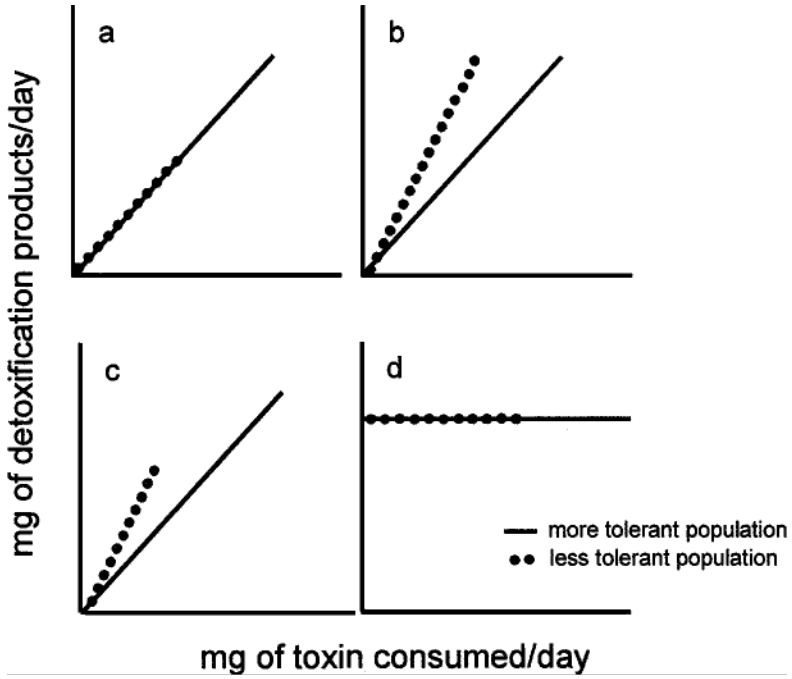

FIG. 1. Possible patterns between detoxification process(es) and toxin intake in two populations with different average maximum toxin tolerance. Plots a–c are based on the hypothesis that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate the toxin. Plot d is based on the assumption that the detoxification process plays no limiting role in determining toxin tolerance. (a) Animals from both populations absorb the same proportion of toxin in food (same regression line), but animals from the more tolerant population can ingest more resin and exhibit higher capacity for the detoxification process. (b) Animals from the more tolerant population absorb a smaller proportion of toxin in food (lower slope), excrete less detoxification products per unit resin ingested, and, therefore, the maximum excretion rate of detoxification products may not be markedly different even though tolerance to resin is different. (c) Animals from the more tolerant population absorb less toxin per unit resin ingested, but also excrete more detoxification products. (d) Detoxification process is unrelated to toxin ingestion in animals from both more and less tolerant populations because the process plays no limiting role in the maintenance of tolerance to the toxin.

for the case of glucuronidation. First, there should be a correlation between glucuronides excreted and resin ingested per day. If animals from both populations absorb the same proportion of toxin in food, then they will fall along the same regression line, animals from the more tolerant population will tolerate more resin (higher maximum tolerable resin intake) and exhibit higher maximum glucuronide

excretion rate (Figure 1a). Alternatively, tolerance to the resin in animals from the more tolerant population occurs because they absorb a smaller proportion of toxin in food, excrete less detoxification products per unit resin ingested, and therefore maximum glucuronide excretion rate may not be markedly different even though tolerance to resin is different (Figure 1b). If differences in both absorption and detoxification are involved, then animals from the more tolerant population not only absorb fewer toxins per unit ingested resin but also excrete more glucuronides (Figure 1c). In either case (Figure 1b and c), in addition to salivary or intestinal binding, a reduction in absorption rate of a toxin could be related to the presence of an intestinal multidrug transporter system that may retard the absorption of the toxin (Epel, 1998). If there were no correlation between resin ingestion rate and reliance on a particular elimination process (Figure 1d), then we would reject the hypothesis that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate toxin(s) by that process.

METHODS AND MATERIALS

Field Site and Sample Collection. Woodrats from the Mojave Desert were trapped at three different sites near Beaver Dam in Grand County, Utah (37±06′N, 113±58′W). Woodrats from the Great Basin were trapped in two locations 70 km apart at Jericho, Juab County, Utah (39±57′N 112±22′W) and Dugway, Tooele County, Utah (40±19′N 112±57′W) (see Mangione et al., 2000 for details on trapping methods). All woodrats were transported to the animal facility at the University of Utah and kept in quarantine for two to five months until they were tested for Sin Nombre hantavirus (Dearing et al., 1998), after which they were transported to the Department of Wildlife Ecology, University of Wisconsin, Madison, Wisconsin. Because detoxification enzymes require very short times for induction, hours to at most three to five days (Sipes and Gandolfi, 1991), and there was no induction of such enzymes, there was no concern for a reduction or alteration of the woodrats' capacity to detoxify creosote bush resin.

Animal Husbandry and Diet Preparation. Woodrats were housed in metal cages (47 £ 30 £ 21 cm) with screened bottoms. The animals were provided with cotton bedding and a ceramic bowl in which to nest. The room was kept at 21±C and 65% relative humidity. When not involved in experiments, woodrats were fed rabbit chow ad libitum [Harlan Teklad 8630; 17.5% crude protein, 22.0% acid detergent fiber (ADF), 2.5% fat] and 7.5% water. A slice of apple was given to the animals once a week.

Creosote bush collection and resin extraction were performed as indicated in Mangione et al. (2000) and resin was stored at 25±C for up to seven months. Resin-treated diets were prepared as follows: a known amount of resin (depending on the desired concentration of resin in the diet) was dissolved in a volume of

ethanol (95%) equal to 25% of the mass of the ground rabbit chow used in the treatment. Control diet was prepared in the same manner without addition of resin. The resin–ethanol solution and chow were thoroughly mixed, dried overnight at room temperature, and pelleted. To prevent alteration of the properties of the resin diet or palatability by the effects of heat and water (Price et al., 1980; Lindroth et al., 1984; Dietz et al., 1994), the pellet machine was refrigerated using a plastic bag filled with crushed ice and the amount of water added to make the pellets was reduced by using a 60% ethanol solution. The final pellets were dried at room temperature and stored in the freezer.

Feeding Trials. Two feeding trials were performed in this study. All experimental protocols were approved by the Research Animal Resources Center (RARC), University of Wisconsin-Madison.

Experiment 1. Nine woodrats from the Mojave Desert, six males and three females, and 8 woodrats from the Great Basin, three males and five females (five from Dugway, Tooele County, Utah and three from Jericho, Juab County, Utah), were exposed to increasing levels of resin following the procedure described by Mangione et al. (2000). At the beginning of the experiment, woodrats were offered rabbit chow with 0% resin and then switched sequentially to 1, 2, 3, 5, 7, and 9% resin. The animals were exposed to each concentration for six days. This sequence allowed the animals to habituate to the resin and permitted us to evaluate differences between populations at low and high concentrations of resin. Fresh food and water were offered daily. Body mass was measured daily, and animals were removed from the experiment if they lost 15% of their initial body mass or showed signs of sickness (e.g., swaying, not responding to sound or tactile stimuli, etc.). Water was supplied in bottles, and water intake was measured daily by weighing bottles and correcting for spillage and evaporation. Food intake was calculated daily by subtracting the dried orts (uneaten food) from the amount of food offered. Daily values of resin intake were calculated as the product of the amount of dry matter ingested per day multiplied by the proportion of resin in the diet at that particular level.

Urine was collected during days 2, 4, and six of each six-day treatment. On these days, woodrats were restricted to a portion of their cage (16 £ 19 £ 20 cm) with a funnel that allowed the collection of uncontaminated urine. Urine drained into an iced plastic vial to minimize bacterial growth and evaporation. The temperature of the vial remained between 0 and 5±C during a 24-hr period. Pilot experiments showed that there were no differences in food intake when woodrats were in either section of the cage (data not shown).

Experiment 2. Because some woodrats reduced their feeding rate and lost mass when fed diets with resin (Mangione et al., 2000), we performed a second, pair-fed control experiment to test whether variation in the excretion of glucuronides in urine of desert woodrats was due to variation in resin intake only and not the

reduction in food intake and mass loss. Four Mojave and six Great Basin woodrats were fed resin-free food daily (pair-fed control woodrats) at the same rate they were eating in experiment 1. Urine was collected for measurement of detoxification products.

Determination of Detoxification Products. Urine samples from the sixth day of each treatment (or from the last day a urine sample was taken if the animals had to be taken out of the experiment) were collected and used for analyses of conjugates. Urine samples were frozen at ¡ 25±C until they were used. Glucuronides were determined following the colorimetric assay described by Jakubas et al. (1993) and Blumenkrantz and Asboe-Hensen (1973). Briefly, urine samples were analyzed in duplicate. A urine sample (0.5 ml) was pipetted into a culture tube placed in an iced water bath along with 3 ml of sodium tetraborate–sulfuric acid solution (Blumenkrantz and Asboe-Hensen, 1973). The mix was shaken by Vortex and returned to the iced water bath. Tubes were then heated in a water bath at 100±C for 10 min. After cooling, 50 ¹ l of the reagent *m*-hydroxydiphenyl (Sigma, St. Louis, Missouri) was added to one set of samples (Blumenkrantz and Asboe-Hensen, 1973). A urine sample (blank) was prepared, but the reagent was replaced by 50 ¹ l of 0.5% NaOH. A standard curve was made with known concentrations of glucuronic acid (Sigma) between 25 and 250 ¹ mol. The absorbance was measured at 520 nm in a Beckman DU-64 spectrophotometer. All samples were diluted between 200- and 1000-fold, depending on the treatment.

Hippuric acid concentration was determined by high-pressure liquid chromatography (HPLC) (Chen et al., 1996) on a System Gold Nouveau (Beckman), equipped with a controller (Detector Module 168) and a pump (126 Solvent Module). The analytical column used was an Ultrasphere C_{18} (5 ¹ m) 250 £ 4.6 mm ID (Beckman). Two solvents—ethanol (15%) in 20 mmol acetic acid (A) and methanol (B)—were used (see Chen et al., 1996). The total run time was 29.5 min. The gradient used was (%B): 0% at 0 min, 50% at 7 min, and 100% at 14.5 min. Between 14.5 and 19.5 min, B was maintained at 100% and then changed back to 0% at 24.5 min. A 20-¹ l injection loop was filled with 50 ¹ l to ensure the delivery of a constant volume on every injection. Flow rate was 0.8 ml/min, and the separation was performed at room temperature. The HPLC settings, elution time, and solvent gradient were adapted from Chen et al. (1996) to ensure a good separation of hippuric acid in our samples. Urine samples were diluted with a 1 M sodium acetate buffer (pH 5.0 adjusted with glacial acetic acid) and filtered through 0.2-¹ m Acrodisc filters (HPLC certified; Fisher Scientific, Chicago, Illinois). The dilution factor varied with the level of resin in the diet. A standard curve was made with known concentrations of hippuric acid (Sigma) ranging from 25 to 250 mg/liter. Two standards were run with every batch of samples. To check for possible carryover during the runs, at least two blanks (mobile phase) were run every day, one

following three samples (six runs), and the other at the end of the day. Duplicate samples were monitored at 210 nm.

Sulfate esters were measured with a turbidimetric assay from Jakubas et al. (1993) and Lundquist et al. (1980). Briefly, 1.5 ml of diluted urine (dilution varied between 10 and 90 times the volume of urine sample available) was mixed with 300 ¹ l of an acidic barium chloride solution to precipitate inorganic sulfate. After 5 min, the solution was centrifuged at 3000 rpm for 10 min. The supernatant was decanted and 300 ¹ l of a 5% (mass per volume) solution of sodium carbonate was added to remove excess barium ions. The solution was centrifuged at 3000 rpm for 10 min, and 1.8 ml of the supernatant was pipetted into a culture tube with 675 ¹ l of 10% (v/v) hydrochloric acid. The tube was topped with nitrogen gas, sealed, and heated in an oil bath at 100±C for 30 min. The hydrolysate was cooled to room temperature, and three aliquots (750 ¹ l each) from each animal were pipetted into test tubes. Two of the aliquots were mixed with Ba-PEG-reagent (Lundquist et al., 1980), while the third was mixed with a PEG-8000 solution (150 g PEG 8000/liter of deionized water) and served as a sample blank. After mixing, samples were allowed to sit for 5 min before determining their absorbance at 600 nm. The 1%, 5%, and 7% treatments had to be discarded because of inconsistent sulfate values when different dilutions were made on the same sample. This could be an indicator that other factors may be influencing sulfate precipitation or hydrolysis. The molar proportion of total conjugates represented by glucuronides, sulfate, and hippuric acid was calculated for 0%, 2%, and 3% resin treatments, because we measured all three conjugates. Molecular weights used were 194.1 for glucuronides (glucuronic acid), 179.2 for hippuric acid, and 96 for sulfate conjugates.

An aliquot of the urine was used to measured pH (Acumet pH meter, model 15). All measurements were taken at 24±C with a pH pencil probe. Two Mojave and one Great Basin woodrat had to be excluded from pH analysis due to the low volume of urine collected.

Statistical Analysis. Relationships between detoxification product excretion rate and either resin intake rate (experiment 1, as in Figure 2) or feeding rate (experiment 2) were compared among the populations by analysis of covariance (ANCOVA, factor = population; covariate = resin or food intake). Residuals were normally distributed when values of excretion rate (milligrams per day) of glucuronides, hippuric acid and sulfates were natural log transformed; this transformation was not necessary in the case of urine pH. Interactions between covariate and factor (i.e., differences in slope) were not significant ($P > 0.05$) and are not reported.

Although regressions describe the relation between resin intake and conjugate excretion, each woodrat is considered three or more times [equal to the number of treatments (resin level)], and so the assumption of independency of data

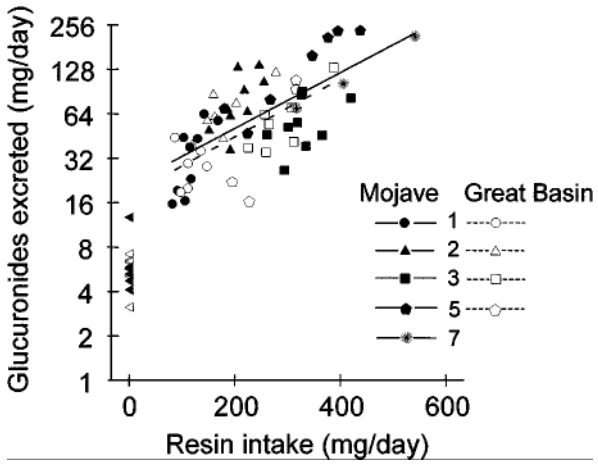

FIG. 2. Excretion of glucuronides as a function of resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed.

points is violated. Therefore, we followed each ANCOVA with a repeated measures one-factor analysis of variance (ANOVA; factor = population, repeated measure = treatment resin level). This test was more conservative because it avoided pseudoreplication and inflation of the degrees of freedom. Treatment resin levels were compared by post-hoc contrasts (Wilkinson, 1992).

Statistical operations were performed according to Wilkinson (1992). In all cases, values are expressed as mean § 1 standard error (SE) (N = number of woodrats). Comparisons of means between populations were made by using the t test. For all statistical comparisons, a value of $P < 0.05$ was considered significant, and $0.05 < P < 0.1$ was taken to indicate a trend.

RESULTS

Experiment 1: Effect of Resin Intake on Detoxification Pathways. Woodrats from both populations maintained a relatively constant food intake and body mass at a dietary level of 0% and 1% resin. Some Great Basin woodrats began losing body mass on 2% resin (see Mangione et al., 2000 for detailed mass records). Woodrats were removed from the experiment when eating diets with resin concentrations ranging from 3% to 9% (Table 1). Sixty percent of woodrats were removed from

TABLE 1. STATUS OF WOODRATS WHEN REMOVED FROM EXPERIMENT 1

Woodrat[a]	Treatment[b]	Body mass lost (% from initial)	Observations[c]
60 M	7	¡ 5.1	died[d]
53 M	3	¡ 5.5	non responsive[e]
59 M	9	¡ 5.7	non responsive
45 M	7	¡ 10.6	non responsive
48 M	7	¡ 12.1	swaying[f]
61 M	7	¡ 12.4	swaying
89 G	3	¡ 13.7	moved slowly[g]
118 G	3	¡ 15.0	non responsive
85 G	5	¡ 15.0	non responsive
87 G	3	¡ 15.6	moved slowly
34 G	5	¡ 15.8	moved slowly
88 G	5	¡ 16.8	moved slowly
49 M	7	¡ 17.1	died
51 M	3	¡ 17.4	moved slowly
33 G	5	¡ 17.5	swaying
42 M	5	¡ 20.1	moved slowly
94 G	5	¡ 20.7	moved slowly

[a] M, Mojave woodrat; G, Great Basin woodrats.
[b] Diet resin level (percent) at which woodrats were removed from the experiment.
[c] Status of each woodrat was found at the time it was removed from the experiment.
[d] Woodrat found dead in the cage.
[e] Woodrat did not respond to tactile or sound stimuli and did not move.
[f] Woodrat would sway when touched or when trying to move.
[g] Woodrat moved considerably slower than when it was in treatment 0% (control).

the experiment because they lost 15% or more of body mass, whereas 40% were removed because they exhibited signs of illness before they lost 15% of their body mass (Table 1). Some woodrats that lost only 5% of body mass were removed because they became nonresponsive to tactile or sound stimuli, a sign of possible postabsorptive toxicity.

The excretion of glucuronides was positively correlated with resin intake whether controls (i.e., zero resin intake) were excluded (ANCOVA $F_{1,59} = 50.22$, $P < 0.001$) or included ($F_{1,72} = 169$, $P < 0.001$) (Figure 2). Individual woodrats from both populations exhibited similar patterns of excretion in relation to intake because there was no significant difference by population in either elevation ($F_{1,59} = 0.98$, $P = 0.32$) or slope. The influence of resin ingestion on glucuronide excretion was also apparent in the repeated-measures ANOVA

($F_{3,36} = 43.7$, $P < 0.001$) where population as a factor had no effect ($F_{1,12,} = 1.4$, $P = 0.26$).

Excretion of hippuric acid was independent of resin intake whether controls were excluded (ANCOVA, $F_{1,59} = 0.44$, $P = 0.50$) or included (ANCOVA, $F_{1,72} = 0.6$, $P = 0.44$), but was nearly three times higher in Great Basin woodrats ($F_{1,59} = 18.05$, $P < 0.001$) (Figure 3).

Sulfate excretion rate was independent of resin intake over the range measured ($F_{1,20} = 2.75$, $P = 0.11$), and there was no difference between populations ($F_{1,20} = 0.48$, $P = 0.49$) (Figure 4). However, sulfate excretion increased with resin intake when controls (zero resin intake) were included (ANCOVA $F_{1,40} = 32.6$, $P < 0.001$) (Figure 4). The repeated measure analysis on the 0%, 2%, and 3% treatments suggested that this increment reflects a resin ingestion-dependent sulfate excretion up to a plateau value. The repeated measures analysis confirmed that there was a diet-dependent increase in sulfate excretion rate ($F_{2,20} = 18.2$, $P < 0.001$) with no difference by population ($F_{1,10} = 0.016$, $P = 0.9$). The significant diet effect was between controls and the 2% resin treatment ($F_{2,10} = 7.5$, $P = 0.01$), but not between the 2% and 3% resin treatments ($F_{2,10} = 0.75$, $P = 0.5$).

Urine pH declined with resin intake rate whether controls (zero resin intake) were excluded ($F_{1,63} = 15.9$, $P < 0.001$) or included ($F_{1,78} = 14.1$, $P < 0.001$)

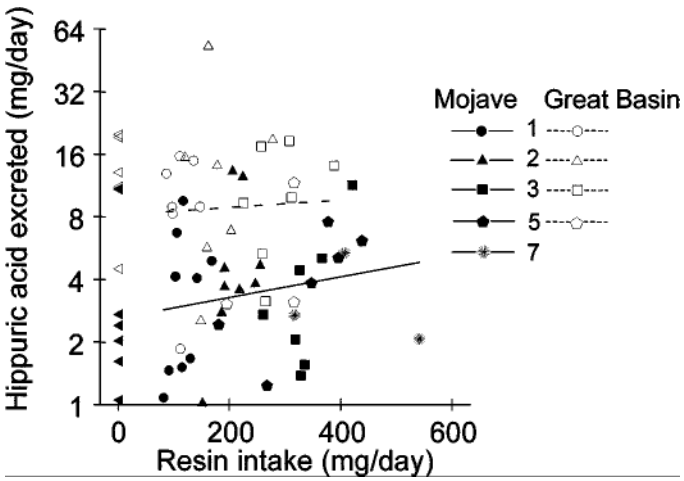

FIG. 3. Excretion of hippuric acid as a function of resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed.

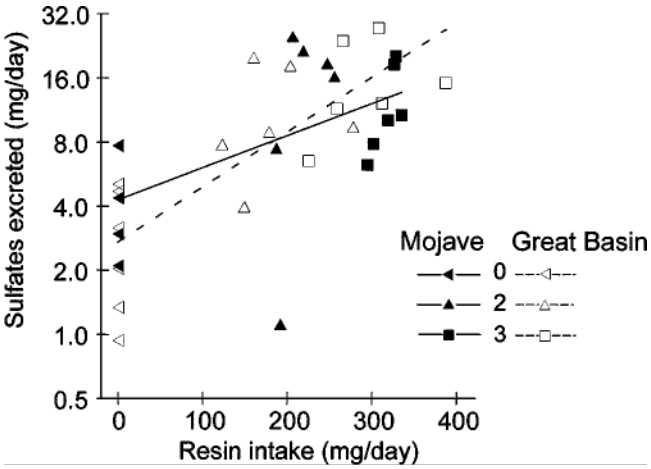

FIG. 4. Excretion of sulfates as a function of resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed. The very lowest value at around 200 mg of resin per day (filled triangle) was identified as an outlier, but the ANCOVA is not substantially different whether it is included or excluded. The datum was included in the final ANCOVA.

(Figure 5). Individual woodrats from both populations apparently exhibited the same urine pH pattern in relation to resin intake because there was no difference by population in either elevation ($F_{1,63} = 1.8$, $P = 0.19$) or slope. The influence of resin ingestion on urine pH was also apparent in the repeated-measures ANOVA ($F_{3,36} = 13.6$, $P < 0.001$), where population was not a significant factor ($F_{1,12} = 3.53$, $P = 0.085$).

Experiment 2: Effect of Food Intake on Detoxification Pathways. Woodrats fed diets with increasing resin concentration decreased their feeding rates by as much as 50% (Mangione et al., 2000). We tested whether the variation in detoxification pathways and urine pH with resin intake (e.g., Figures 2, 4, and 5) might be explained by decreases in feeding rate. Were this the case, glucuronide excretion would increase with decreasing feeding rate on resin-free diet, whereas the opposite pattern was found in woodrats from both populations (Figure 6, top), i.e., increasing glucuronide excretion with increasing feeding rate ($F_{1,34} = 16.5$, $P < 0.001$). Analogously, were this the case for urine pH, then it would decline with decreasing feeding on resin-free diet, whereas it did not change with intake rate in either population ($F_{1,34} = 0.05$, $P = 0.83$) (Figure 6, bottom). Overall, as feeding rate changed, the extent of variation in detoxification or urine pH was nil

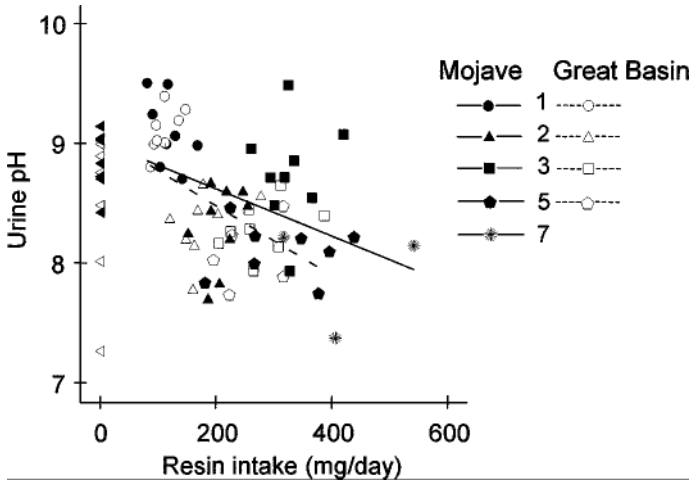

FIG. 5. Relationship between urine pH and resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed.

or in the wrong direction and was very small compared to the much larger changes in woodrats consuming increasing amounts of resin.

DISCUSSION

Detoxification Routes Used by Woodrats. Variation in detoxification can be due to qualitative and quantitative differences (Hansen and Shane, 1994). Qualitative differences correspond to the use of different metabolic routes and the activation or deactivation of metabolic pathways. Quantitative differences are due to variations in enzyme levels. Without simultaneous study of several possible detoxification pathways and the determination of their relative capacities, little progress can be made in understanding the role of detoxification in setting the limits of toxin ingestion (MacCoubrey et al., 1997).

In this study, we considerably advanced knowledge in these regards for one natural herbivore–PSM system: desert woodrats consuming resin from creosote bush. There were no major qualitative differences in detoxification pathways among individuals or populations; all three phase II detoxification routes (glucuronidation, sulfation, and amination) were used to some degree by both woodrat populations exposed to the resin. On resin-free diet, the molar excretion

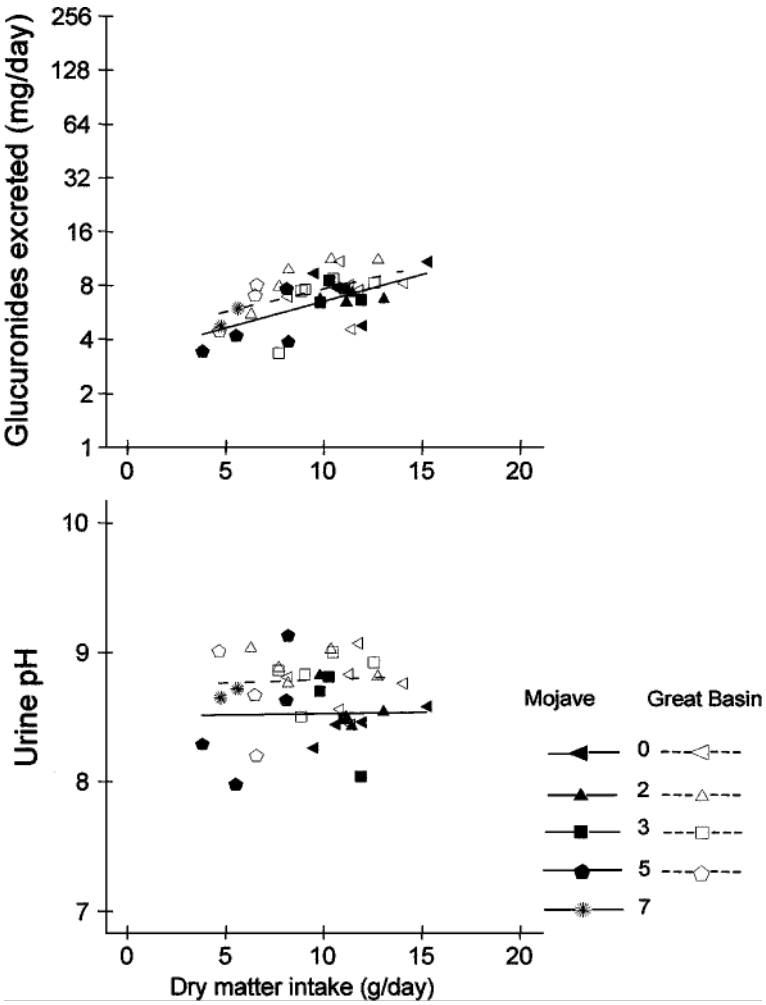

FIG. 6. Variation of glucuronides excreted (top) and urine pH (bottom) as a function of dry matter intake in pair-fed control woodrats (see Methods and Materials). The solid line represents the least-squares regression for Mojave woodrats, the dashed line is the least-squares regression for Great Basin woodrats. Each point represents one woodrat restricted to the same feeding rate it had during experiment 1 at a particular resin treatment. The numbers between the symbols in the legend represent the corresponding treatments (i.e., percentage of resin in the diet), although in this experiment there was no resin in the diets. The *y* axes of the figures match those in Figures 2 and 5 to permit comparison of these excretion rates in woodrats on resin-free diet with those of woodrats eating resin-containing diets.

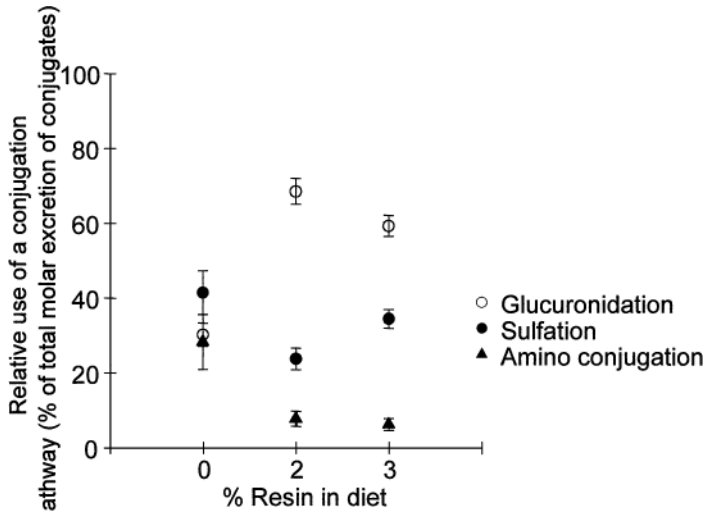

FIG. 7. Variation in the relative use of detoxification pathways versus percentage of resin in the diet. Percentages on the *y* axes were calculated as the ratio of the molar excretion rate of each conjugate to the sum of the molar excretion rates of the three conjugates times 100. Each value is the average of a pool of 12 individuals from both populations.

rates of the three conjugates (glucuronides, glycine, sulfate) were similar (Figure 7). Because glucuronides were the only conjugates that showed consistent increases in excretion with increasing resin intake (c.f. Figures 2–4), glucuronidation became proportionally more important than other conjugation pathways following resin ingestion, coming to represent about 75% of total moles of conjugates excreted (Figure 7). However, we do not know whether this is because of higher levels of specific constitutive enzymes or induction of the specific enzymes involved. Alternatively, reliance on glucuronidation may differ for woodrats reared on other diets or physiological conditions (Price and Jollow, 1988, 1989; Nanbo, 1993), or for woodrats possessing different gut microbial communities (Phipps et al., 1998).

Does Detoxification Capacity Set the Limit on Resin Intake Rate? We found no qualitative difference among woodrats to explain their differential tolerance to creosote bush resin. We also, can reject a number of hypothetical quantitative explanations.

We reject the hypothesis that the amount of resin that indiviuals can ingest is related to their capacity to detoxify and eliminate toxin(s), because hippuric acid excretion rates were independent of resin ingestion rates (Figure 3). Although the level of hippuric acid in urine of Great Basin woodrats was significantly higher than that in Mojave woodrats, this does not explain the latter's greater tolerance to resin.

Hippuric acid is a rather specific conjugate of benzoic acid. If benzoic acid is not a metabolic product of a toxic compound(s), then there would be little relationship between tolerance and hippuric acid excretion. It would be interesting to measure excretion of other benzoic acid metabolites, e.g., flavanoids with different patterns of hydroxylation such as *m*-hydroxybenzoic acid or *m*-hydroxphenyl propionic acid (Harborne, 1991). In addition, there might be other phase II conjugations that involve amino acids other than glycine, e.g., taurine (Caldwell, 1986). A good approach to test this might be to assay urine for glycine and taurine (Konishi et al., 1998; Phipps et al., 1998).

We reject the hypothesis that tolerance to resin is related to woodrat sulfation capacity because sulfate excretion increased with resin intake up to a plateau at 200 mg resin/day (Figure 4), while woodrats continued to consume resin at two to three times this level (Mangione et al., 2000) (Figure 2). The apparent saturation of sulfation in woodrats seems consistent with the observation in other mammals that conjugation with sulfate is a high affinity–medium capacity enzymatic system (Sipes and Galndolfi, 1991).

Two physiological features related to detoxification, glucuronide excretion and urine pH, were correlated with resin intake and consistent with our a priori predictions that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate the PSMs (Figure 1). These correlations were not artifactually related to variation in feeding rate (Figure 6). While Figures 2 and 5 illustrate how individual woodrats from the two populations respond to variation in resin intake with regard to glucuronide excretion and urine pH, they are not effective for testing whether a particular detoxification mechanism or buffer capacity might be limiting, and, thereby, explain observed differences in resin tolerance among individuals or between the two woodrat populations. To test this, we regressed each individual's maximum value for glucuronide elimination or minimum value for urine pH against its maximum tolerable resin intake rate—the latter values were taken from Mangione et al. (2000), although they correspond closely to maxima from Figures 2 and 5.

The maximum excretion of glucuronides was positively correlated with maximum resin intake (ANCOVA $F_{1,13} = 8.57$, $P < 0.012$) (Figure 8, top). Individual woodrats from both populations exhibited the same pattern of maximum glucuronide excretion in relation to maximum resin intake because there was no difference by population in either elevation ($F_{1,13} = 0.63$, $P = 0.44$) or slope. This corresponds with the prediction in Figure 1a. Furthermore, the mean maximum glucuronide excretion rate of Mojave desert woodrats, 143.8 § 23.3 mg/day ($N = 9$), exceeds that of the Great Basin desert woodrats, 67.0 § 10.8 mg/day ($N = 8$) ($t_{15} =$ ¡ 2.86, $P < 0.012$). Therefore, in accord with Figure 1a, we cannot reject the hypothesis that animals from both populations absorb the same proportion of toxin in food and that woodrats within and between populations that

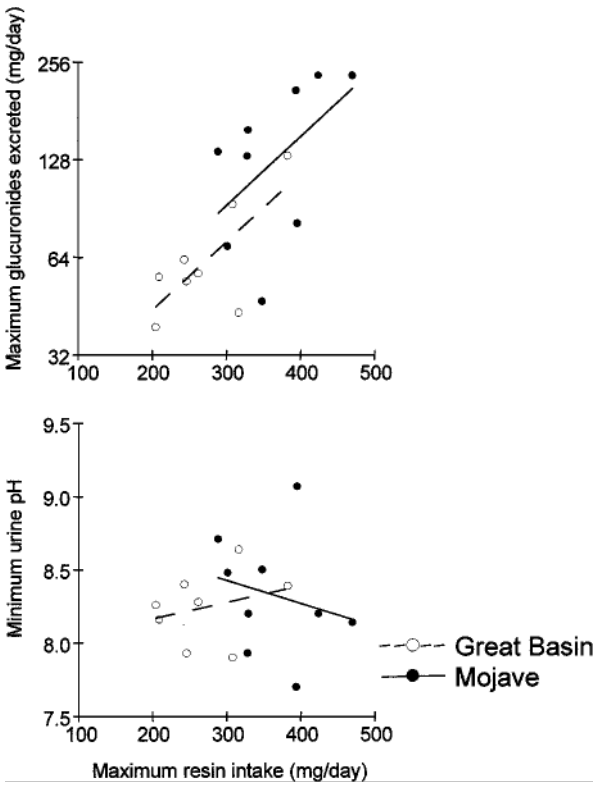

FIG. 8. Maximum glucuronides excreted (top) and minimum urine pH (bottom) as a function of resin intake. Each point represents an individual woodrat's maximum resin intake rate and corresponding maximum glucuronide excretion rate or minimum pH. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats.

tolerate more resin do so because they have a greater capacity for glucuronide excretion.

Instead urine pH can be considered an indicator of an animal's acid–base status and its capacity to detoxify and eliminate metabolites. These metabolites are strong acids that may challenge the animal's acid–base homeostasis (McLean et al., 1993; Foley et al., 1995). However, the tolerance for resin ingestion does not relate as well to urine pH as it does to glucuronide excretion. First, the lowest urine pH of each individual was not correlated with the individual's maximum tolerable resin intake rate (ANCOVA $F_{1,14} = 0.04$, $P = 0.84$) (Figure 8, bottom). Second, the reduction in urine pH to 8 observed in desert woodrats eating creosote resin

seems modest compared to reductions to pH 7.4 that have been observed in other woodrat species exposed to other PSMs (Dearing et al., 2000). Although urine pH was decreased as woodrats ingested greater amounts of resin (Figure 5), we reject the idea that they had to cease eating resin because they reached their capacity to buffer blood from organic acids or to excrete H^+ ions. Moreover, the small R^2 (0.22, Figure 5) suggests that some other factor might be involved in reducing of uirne pH, such as diuresis (García Matilla et al., 1999).

In conclusion, we studied the relationship between three detoxification mechanisms and urine pH and the tolerance of desert woodrats from two populations to a mixture of naturally occurring PSMs (mostly phenolics) in resin from creosote bush. The excretion rate of two phase II detoxification conjugates, glucuronides and sulfides, increased with increasing resin intake, while hippuric acid excretion was independent of resin intake. The molar proportion of glucuronides in urine was three times that of the other conjugates combined. Urine pH, a possible indicator of an animal's capacity to detoxify and eliminate metabolites, declined with increasing resin ingestion. Based on an evaluation of variation in the three detoxification pathways and urine pH in relation to resin intake, we rejected the hypotheses that tolerance to resin intake is related to woodrat capacity for amination, sulfation, or pH regulation. However, we could not reject the hypothesis that woodrats within and between populations that tolerate more resin do so because they have a greater capacity for glucuronide excretion. This hypothesis might be tested by feeding woodrats other compounds detoxified by this pathway, e.g., benzoic acid, and testing their ability to further increase glucuronide excretion rate. Another way to test whether glucuronidation can set the limits for tolerance to a PSM might be by using specific inhibitors of glucuronyl transferase.

Interactions between herbivores and the PSMs they ingest are complex. There may be other explanations for some of the patterns that we have observed. For example, we did not evaluate how differences in gut microbial community structure and function might influence detoxification, or whether different woodrats eating the same amount of resin excrete similar amounts of a conjugate (e.g., sulfates) even when they might actually differ in their metabolism of specific phenols in the resin. Other possible mechanisms that might explain differences in tolerance, such as differential absorption of PSMs, differences in other phase I and II detoxification pathways, differences in target site sensitivity, and differences in the capacity to habituate to aversive sensory properties of PSMs, remain to be studied.

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