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COMPARISON OF DETOXIFICATION ENZYME mRNAS IN WOODRATS (*Neotoma lepida*) AND LABORATORY RATS

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Abstract—To understand how mammalian herbivores process plant secondary compounds, we examined differences in expression of biotransformation enzyme mRNAs among populations of wild woodrats (*Neotoma lepida*) and laboratory rats. We compared expression of mRNAs for 10 biotransforming enzymes in five families (CYP, mEH, QOR, GST, and UGT) by using Northern blot analysis. We found significant differences in eight of 10 mRNAs tested. We suggest that the differences in mRNA expression among populations of woodrats and laboratory rats may be due to differences in the secondary compound composition of their diets. Our results provide background for future studies of detoxification strategies in mammalian herbivores that combine pharmacological techniques with an ecological perspective.

Key Words—Detoxification, biotransforming enzymes, plant secondary compounds, *Neotoma*, woodrats, xenobiotics.

INTRODUCTION

Mammalian herbivores ingest and detoxify a wide variety of plant secondary compounds. Detoxification and elimination is carried out by a multitude of enzymes collectively referred to as "xenobiotic biotransforming enzymes" or less precisely, "drug-metabolizing enzymes." These enzymes transform hydrophobic molecules into hydrophilic compounds that can be excreted as metabolites in the urine or feces.

To understand how mammalian herbivores detoxify secondary compounds, ecologists have focused primarily on the end products of detoxification by examining metabolites in the urine. Most studies have focused on the excretion of a single compound, glucuronic acid, which is an endogenous compound that can be

conjugated to a diversity of secondary compounds (Dearing, 1997; Foley, 1992; Lindroth and Batzli, 1983). Focus on this pathway is due in part to the fact that relatively straightforward, colorimetric assays for glucuronic acid have been developed (Blumenkrantz and Asboe-Hansen, 1973). Quantification of metabolites from other enzymatic pathways often entail more involved chemical analyses, such as gas chromatography, high pressure liquid chromatography, or mass spectrometry (Mangione, 1999; McLean et al., 1993).

In contrast, contemporary pharmacological studies approach mechanisms of detoxification of foreign (xenobiotic) compounds differently. In addition to quantifying metabolites excreted in the urine, *in vitro* techniques are used to identify individual enzymes involved in drug metabolism (Batt et al., 1992; Kato, 1979). One such technique that uses Northern blot analysis (Sambrook et al., 1989) detects messenger RNAs (mRNA) that encode for numerous biotransformation enzymes. This analysis gives an indication of the level of expression of mRNA for a particular detoxification enzyme. Screens of mRNA for biotransformation enzymes typically are employed to determine the pathways of biotransformation that are induced when a known drug is administered.

We took a pharmacological approach to investigate the detoxification mechanisms of mammalian herbivores consuming their natural diet. As a first step, we used Northern blot analysis to quantify the expression of mRNAs for several biotransformation enzymes in the liver of desert woodrats, *Neotoma lepida*. This species occurs in a wide variety of habitats across the American Southwest. We selected two populations of *N. lepida* known to consume different diets. *N. lepida* from the Great Basin feeds primarily on Utah juniper (*Juniperus osteosperma*), whereas *N. lepida* from the Mojave Desert consumes desert almond (*Prunus fasciculata*) and creosote (*Larrea tridentata*) (Egoscue, 1957; Karasov, 1989; Mangione, 1999). These plant species contain high levels of various secondary compounds, particularly terpenes and phenolics (Dearing et al., 2000; Mangione, 1999; Mangione et al., 2000). We compared the results from *N. lepida* consuming natural diets to those from laboratory rats (*Rattus norvegicus*) consuming standard laboratory chow.

For each population, we examined levels of mRNA from five families of enzymes involved in xenobiotic biotransforming enzymes: cytochrome P450s (CYP), microsomal epoxide hydrolase (mEH), NAD(P)H quinone oxidoreductase (QOR), glutathione *S*-transferases (GST), and UDP-glucuronosyltransferases (UGT) (Figure 1). These five families encompass the majority of known detoxification enzymes and include both functionalization (phase I) and conjugation enzymes (phase II). In addition, we probed for the heavy subunit of γ-glutamylcysteine synthase (γ-GCS). CYP, mEH, and QOR encode for functionalization enzymes, whereas GST and UGT produce conjugation enzymes. For CYP and UGT families, we restricted our investigation to isoforms that are known to metabolize foreign compounds.

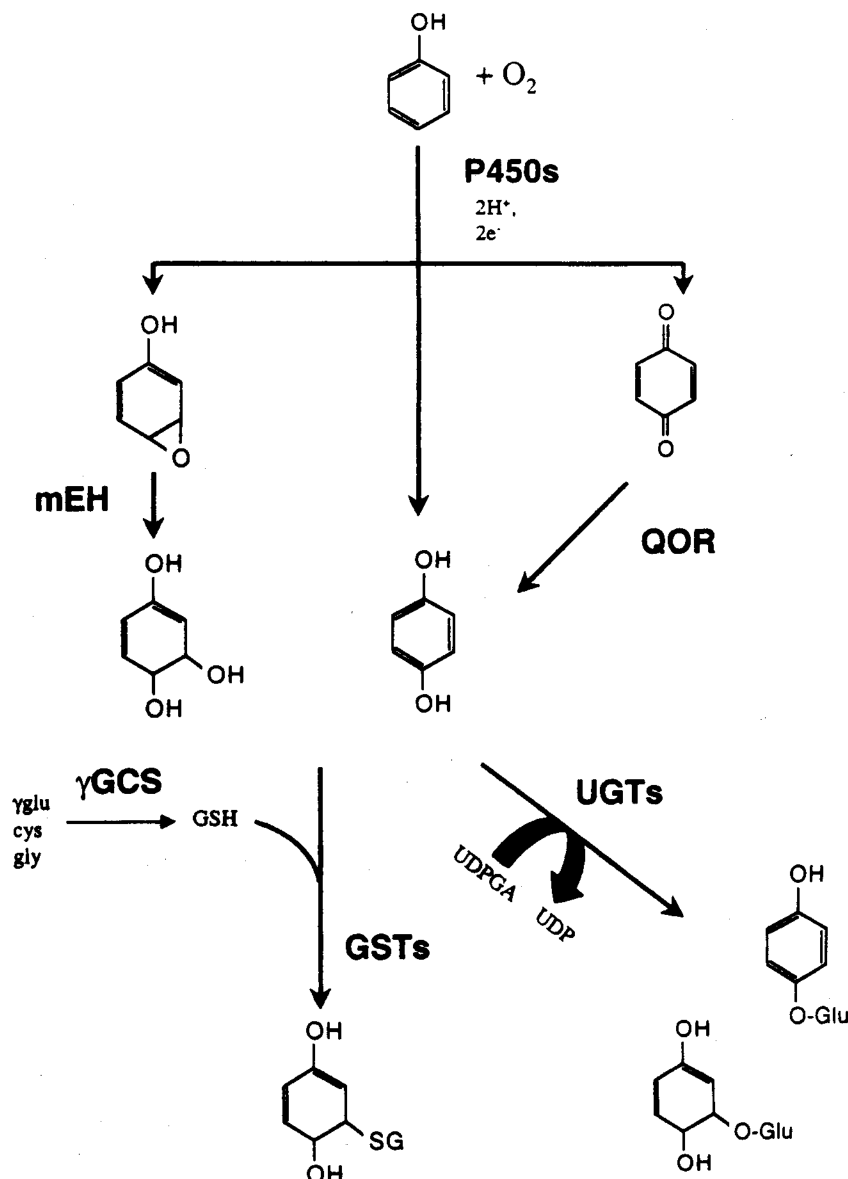

FIG. 1. General pathways of xenobiotic biotransformation. An example of a phenolic compound is shown. P450s: cytochrome P450 enzymes, mEH: microsomal epoxide hydrolase enzyme, QOR: NAD(P)H quinone oxidoreductase, UGTs: UDP-glucuronosyltransferase enzymes, γGCS: γ-glutamylcysteine synthase enzyme, GSH: glutathione, and GSTs: glutathione-S-transferase enzymes.

Enzymes from each of the five families modify xenobiotics in a specific way (Hardman and Limbird et al., 1996) (Figure 1). Cytochrome P450 enzymes catalyze a monooxygenation reaction by inserting one atom of molecular oxygen into the xenobiotic compound while the other oxygen atom is reduced to water. Microsomal epoxide hydrolase catalyzes the addition of water to alkane epoxides, which are commonly produced during cytochrome P450 reactions. QOR catalyzes the two-electron reduction of quinones by oxidation of NAD(P)H. GSTs catalyze conjugation of xenobiotic compounds with the tripeptide, glutathione. An

adequate supply of glutathione is critical for conjugation. The rate-limiting step in the production of glutathione is catalyzed by γ-GCS. UGTs conjugate xenobiotic compounds with a sugar acid moiety from the cosubstrate UDP-glucuronic acid (Dutton, 1980).

We had three objectives in this study: (1) to determine whether complementary DNA (cDNA) probes for biotransformation enzyme mRNAs in laboratory rats (*Rattus norvegicus*) were homologous to mRNAs in woodrats, (2) to determine detoxification pathways induced by woodrats consuming a natural diet, and (3) to compare levels of biotransformation enzyme mRNAs among populations of woodrats and laboratory rats. We hypothesized that *N. lepida* woodrats should express higher levels of mRNA for biotransformation enzymes compared to laboratory rats because woodrats consume plants with measurable quantities of secondary compounds, whereas laboratory rats consume formulated diets low in toxins.

METHODS AND MATERIALS

Laboratory Rats. Male laboratory rats (Muridae: Murinae: *Rattus norvegicus*: strain Sprague-Dawley; 160–180 g) were obtained from Simonson Laboratories and kept on a 12 hour light dark cycle and given free access to rat chow and water.

Collection of Neotoma. We collected *Neotoma lepida* (Muridae: Sigmodontinae) from two populations in Utah in October 1999. Four individuals were collected from a Mojave Desert habitat near Beaver Dam Wash, Utah (37°06'N, 113°58'W). The other four individuals were trapped in the Great Basin Desert near Jericho, Utah (39°57'N, 112°22'W). From here on, we refer to individuals trapped in the Mojave Desert as Mojave woodrats and those collected in the Great Basin Desert as Great Basin woodrats. For a description of each of these sites, see Dearing et al. (1998). Animals were live-trapped with Sherman traps and dispatched in the field with CO_2. Whole animal body mass was recorded. The liver was removed, weighed, and frozen quickly on dry ice. Samples of liver tissue were brought back to the laboratory for total RNA isolation.

Total RNA Isolation and Northern Blot Analysis. Total RNA was isolated from approximately 100 mg of frozen liver tissue by homogenization in 2 ml TRIzol (phenol–guanidine isothiocyante) reagent (Giboco/BRL) and isopropyl alcohol precipitation of the aqueous layer following chloroform treatment. Twenty micrograms of total RNA from each sample (concentration of total RNA was determined from 260 nm absorbance) was subjected to electrophoresis in 1% denaturing agarose gel containing formaldehyde. Sample integrity was determined by visualization of sharp 18S and 28S bands with ethidium bromide. Total RNA was transferred to a Nytran membrane (Schleicher and Schuell) by downward

TABLE 1. cDNAS USED FOR NORTHERN PROBES[a]

cDNA	Genbank	Region	Citation
CYP1A1	X00469	1511–2250	Yabusaki et al. (1984)
CYP2B	J00719	50–1567	Fujii-Kuriyama et al. (1981)
CYP3A	M10161	1079–2040	Gonzalez et al. (1985)
UGT1A1	U20551	2–870	Coffman et al. (1995)
UGT1A6	J02612	152–934	Iyanagi et al. (1986)
UGT2B1	M13506	8–710	Mackenzie (1986)
GSTYa	K00136	4–755	Pickett et al. (1984)
QOR	J02608	107–1495	Robertson et al. (1986)
mEH	M26125	107–1531	Porter et al. (1986)
γ-GCS	J05181	251–820	Yan and Meister (1990)

[a] The Genbank account numbers and approximate region of the cDNA are given.

alkaline transfer, cross-linked with UV light, and hybridized with cDNA probes labeled with [α-^{32}P]dCTP by random primed synthesis (Amersham Multiprime) according to the manufacturer's specifications. The rat cDNA probes used are given in Table 1.

Hybridized blots were washed twice for 30 min at 42°C in 2× SSC, 0.1% SDS, twice for 30 min at 42°C in 0.1× SSC, 0.1% SDS, and once for 45 min at 54°C in 0.1× SSC, 0.1% SDS. Autoradiographic film was exposed for 6–72 hr at −70°C with an intensifying screen, and the developed band intensity was determined by scanning densitometry with Molecular Analyst (Bio-Rad) software. To remove any effects of gel loading and transfer variations, all mRNA bands were normalized to a housekeeping gene, cyclophilin mRNA, in the same sample. Plasmids containing the cDNA probes were provided by Dr. J. Ritter, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.

Statistics. Statistical analyses were performed by using ANOVA, followed by Fisher's multiple range test.

RESULTS

cDNA Probes. In general, cDNA probes generated from mRNAs for rat biotransforming enzymes gave good results when used on woodrat livers (Figure 2). All probes gave bands that were similar in size to mRNA bands from laboratory rats, with the exception of UGT2B1, where a larger transcript was detected in woodrat samples (Figure 2).

mRNAs for Detoxification Enzymes. Levels of mRNAs varied between woodrats and lab rats and among woodrats. In eight mRNAs tested, there were significant differences between woodrats and laboratory rats and among woodrats, but no consistent pattern in levels of mRNAs emerged. We found no difference in

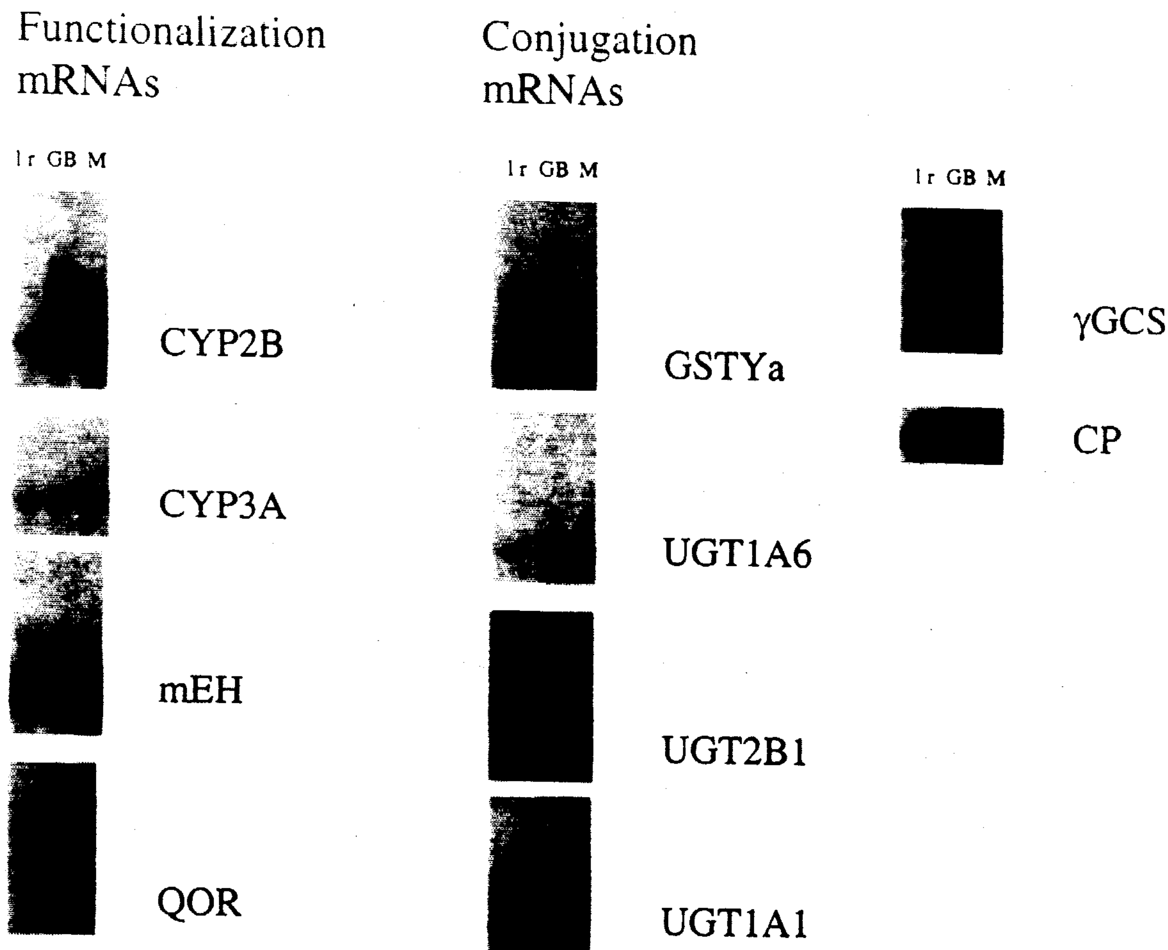

FIG. 2. Northern blots of liver mRNAs. Representative results are shown for each sample. Membranes were probed with the indicated ^{32}P-labeled cDNA probes (see Table 1) for each xenobiotic biotransformation enzyme. Membranes were also probed with ^{32}P-labeled cDNA for cyclophilin to compensate for gel loading and transfer variations. Band order is shown as laboratory rats (lr), Great Basin woodrats (GB), and Mojave woodrats (M).

mRNA levels for two of 10 mRNAs across the three animal groups tested. Details of the results for each mRNA are presented in the following sections.

mRNAs for Functionalization Enzymes. Among functionalization enzyme mRNAs, significant differences were present in mRNA levels for CYP2B and CYP3A. The level of mRNA for CYP2B was greater in Great Basin woodrats compared to Mojave woodrats and laboratory rats (Figure 3). Both populations of *N. lepida* had elevated levels of mRNA for CYP3A in comparison to laboratory rats. There was no detectable message for CYP1A1 in woodrat populations or laboratory rats (data not shown). The level of mEH mRNA was lower in Great Basin woodrats compared to laboratory rats and Mojave woodrats. The level of QOR mRNA was lower in Mojave woodrats than laboratory rats and Great Basin woodrats (Figure 3).

mRNA for γ-GCS and Conjugation Enzymes. The level of mRNA for γ-GCS differed among groups (Figure 4). Mojave *N. lepida* populations had higher

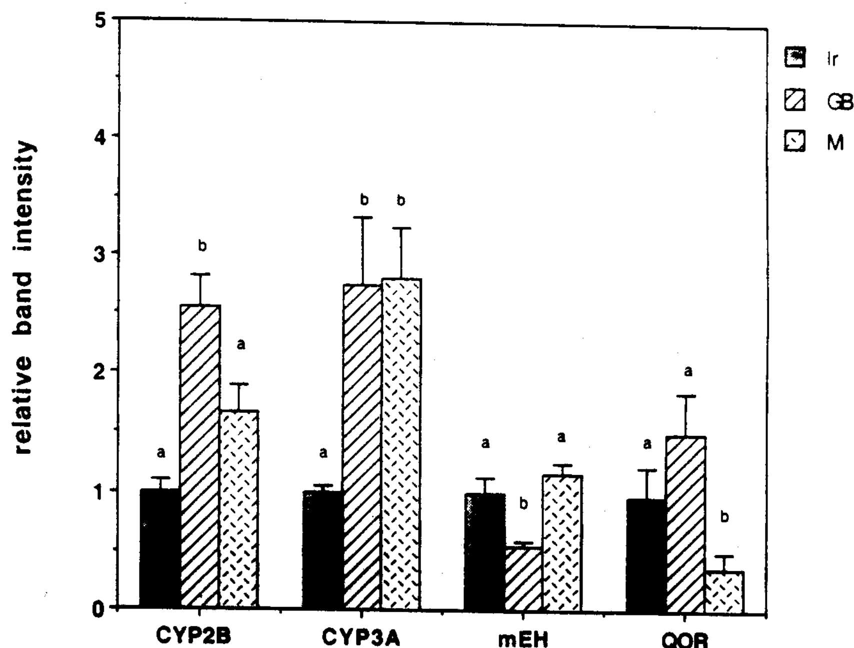

FIG. 3. Mean band intensities ± SE of northern blot analysis for functionalization enzyme mRNAs. Sample sizes were $N = 4$ for laboratory rats (lr), Great Basin woodrats (GB), and Mojave woodrats (M). Relative band intensities were normalized to laboratory rats. Different letters indicate significant differences among groups.

levels of γ-GCS compared to laboratory rats, but the difference between Great Basin woodrats and laboratory rats was not statistically significant. The Mojave population had γ-GCS levels five times that of laboratory rats and two times that of the Great Basin population. There was no difference in levels of mRNA for the GSTYa among woodrats and laboratory rats (Figure 4).

We found differences among animal groups with respect to mRNAs for glucuronic acid conjugation enzymes (Figure 4). Mojave woodrats had significantly higher levels of UGT1A6 mRNA compared to Great Basin woodrats and laboratory rats. Levels of mRNA for UGT1A6 were not significantly different between laboratory rats and Great Basin woodrats. Levels of mRNA for another glucuronidation enzyme, UGT2B1, showed the opposite pattern. Both populations of *N. lepida* exhibited significantly lower levels of mRNA for UGT2B1 compared to laboratory rats. Levels of UGT1A1 mRNA were also significantly lower in both *N. lepida* woodrat species compared to laboratory rats (Figure 4).

DISCUSSION

More than 25 years ago, Freeland and Janzen (1974) integrated the fields of pharmacology and ecology to generate numerous hypotheses on how herbivorous mammals cope with toxins in plants. Since then, diverse analytical techniques have been developed to understand the biotransformation pathways through which drugs

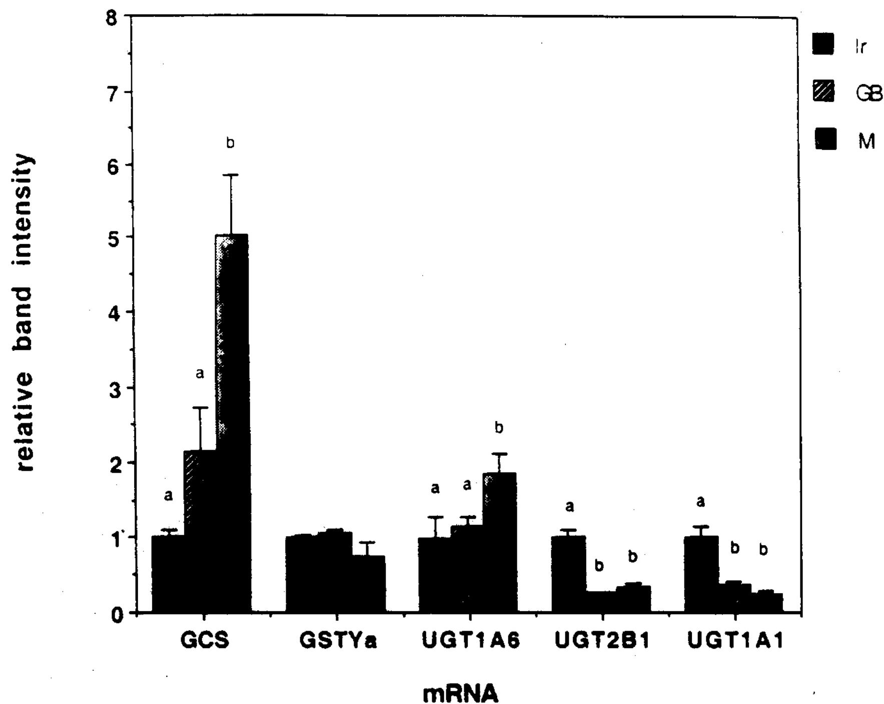

FIG. 4. Mean band intensities ± SE of northern blot analysis for γGCS and conjugation enzyme mRNAs. Sample sizes were $N = 4$ for laboratory rats (lr), Great Basin woodrats (GB), and Mojave woodrats (M). Relative band intensities were normalized to laboratory rats. Different letters indicate significant differences among groups.

and other xenobiotics are processed. Few of these techniques have been applied to studies of wild mammalian herbivores. A primary objective of our research was to join pharmacological knowledge and techniques to ecological questions of how wild herbivores detoxify secondary compounds in plants. Our results demonstrate that these approaches can be fruitful.

Use of cDNA Probes from Laboratory Rats in Woodrats. Overall, cDNA probes designed for detoxification mRNAs in laboratory rats gave good results in woodrats. Total RNA isolated from livers of woodrats and probed with cDNA for 10 biotransformation mRNAs of laboratory rats yielded bands of similar sizes to those found in laboratory rats (with the exception of UGT2B1). These results suggest that although laboratory rats and woodrats are different species, the sequences of their respective biotransforming enzyme mRNAs have been conserved. In the future, it would be valuable to have cDNA probes for the various biotransforming enzymes from *Neotoma* species. Knowledge of the exact DNA sequence of a specific biotransforming enzyme may yield valuable information on the evolution of detoxification systems (Lewis et al., 1998).

UGT2B1 did not yield results comparable to the other cDNA probes. The probe for UGT2B1 indicated a longer mRNA in woodrats than in laboratory rats. This result suggests that the mRNA for UGT2B1 in woodrats differs slightly from that of laboratory rats but is similar enough to hybridize to the probe.

Messenger RNA for CYP1A1 was not detected in either woodrat population. The CYP1A1 gene in woodrats may be significantly different from that of laboratory rats and, thus, was not detected by the probe for laboratory rats. This outcome, however, is unlikely given that species such as trout, which are more distantly related to laboratory rats than woodrats, exhibit a high degree of sequence similarity for CYP1A1 to laboratory rats (Heilman et al., 1988). It is more probable that levels of CYP1A1 mRNA in woodrats are undetectable, given that laboratory rats constitutively express CYP1A1 mRNA at low to undetectable levels.

mRNA for Functionalization Enzymes. The greater levels of mRNA for CYP2B in Great Basin woodrats compared to Mojave woodrats and laboratory rats may be attributable to high concentrations of α-pinene and/or other terpenes in the diet of Great Basin woodrats. α-Pinene is an abundant secondary compound in *Juniperus osteosperma*, the primary plant in the diet of Great Basin woodrats (Adams et al., 1981; Mangione, 1999; Mangione et al., 2000). This compound is not present in rat chow and occurs in low quantities in the diet of Mojave woodrats (Karasov, 1989; Mabry et al., 1977). In other studies, administration of α-pinene to laboratory rats increased quantities of the enzyme (P4502B) produced by CYP2B mRNA (Austin et al., 1988; Hiroi et al., 1995). The doses used in these experiments are comparable to those ingested by Great Basin woodrats in their natural diet (Dearing, personal observation). Thus, the elevated CYP2B mRNA in the Great Basin woodrats may be a response to α-pinene in its diet.

Both populations of woodrats had elevated levels of CYP3A mRNA compared to laboratory rats. In humans, the enzyme produced by CYP3A mRNA is responsible for the metabolism of over 60% of known pharmaceutical compounds (Gonzalez, 1989). Treatment of laboratory rats with essential wood oils, which are primarily terpenes, increased levels of the enzyme (P4503A) produced by CYP3A mRNA (Hiroi et al., 1995). It is plausible that the high levels of CYP3A mRNA in both populations of woodrats are induced by and required for detoxification of secondary compounds in their diets.

Neotoma lepida did not show higher levels of mEH and QOR mRNAs and in certain cases exhibited significantly lower levels than laboratory rats. The results imply that although both mEH and QOR mRNAs are subject to transcriptional induction by xenobiotic compounds (Hankinson, 1995; Daniel, 1993), the diets of *N. lepida* populations did not induce these two mRNAs.

Differences in Expression Levels of mRNA for Conjugation Enzymes. Conjugation with glutathione appears to be an important detoxification pathway for Mojave woodrats. Glutathione, a tripeptide thiol, is highly concentrated in liver cells (up to 10 mM) and is important in maintaining cellular redox status and metabolic and oxidative detoxification reactions. Synthesis of glutathione is rate-limited by the enzyme produced from γ-GCS mRNA (Packer, 1995). Mojave woodrats expressed significantly more γ-GCS mRNA than laboratory rats and Great Basin woodrats. However, there were no differences among woodrats and

laboratory rats in levels of mRNA GSTYa, which produces an enzyme that conjugates glutathione to xenobiotics. It is possible that the observed levels of GSTYa in woodrats produced sufficient quantities of enzyme for glutathione conjugation. In addition, there are several other forms of GST enzymes (GST μ and π) (Daniel, 1993) that we did not assay in this study. Thus, woodrats may be using other forms of GST enzymes for glutathione conjugation.

Conventional wisdom does not predict that mammalian herbivores would utilize glutathione (a tripeptide) as a primary detoxification pathway because nitrogen is typically a limiting resource to herbivores. Excretion of toxins conjugated to glutathione could result in marked losses of nitrogen. Utilization of the biochemically costly glutathione pathway by *N. lepida* suggests several intriguing possibilities. The amino acids in glutathione may not be limiting to *N. lepida*. If they are, the loss of amino acids can be minimized by recovering some of them in glutathione through transformation to mercapturic acids by kidney and intestinal γ-glutamyltransferase activity (Hardman and Limbird, 1996). Lastly, detoxification of the secondary compounds found in the diet of Mojave woodrats may be restricted to the glutathione pathway. Thus, elevated losses of glutathione may represent a biochemical cost of consuming plants containing toxins that can only be detoxified via the glutathione pathway. Further study of the use and costs of the glutathione pathway in Mojave woodrats is warranted.

It was interesting that both *N. lepida* populations expressed lower levels of mRNAs for two UGT mRNAs (UGT2B1 and UGT1A1) than laboratory rats. These two UGTs conjugate glucuronic acid to a number of plant secondary compounds common in the diets of *N. lepida*. UGT1A1 glucuronidates opiate alkaloids, while UGT2B1 conjugates "bulky" toxins, such as certain monoterpenes and phenolics (King et al., 1997). Several studies indicate that the glucuronic pathway is used extensively by woodrats (Mangione, 1999; Mangione et al., 2000). Mangione (1999) found that glucuronide conjugates comprised ~70% of the conjugates examined in the urine of *N. lepida*. The *N. lepida* used by Mangione (1999) were obtained from the same site in the Mojave as this study and were fed creosote resin, a mixture of secondary compounds present in their natural diet. Our results in conjunction with those from the literature (Mangione, 1999; Mangione et al., 2000) suggest that *N. lepida* utilize UGTs other than UGT2B1 and UGT1A1 for glucuronidation. There are ~19 different cDNA sequences for rat UGT enzymes (Mackenzie et al., 1997), and we have probed only for three.

Mojave woodrats did express slight but significantly higher levels of one UGT (UGT1A6) than either Great Basin woodrats or laboratory rats. The UGT1A6 enzyme metabolizes planar aromatic compounds (Jackson et al., 1988). The leaves of the creosote bush, a major component of the diet of Mojave woodrats, are coated with a resinous mixture of flavonoids and phenolics that can comprise up to 25% of the dry weight of the leaves (Mabry et al., 1977). It is possible that an increased

level of UGT1A6 expression is used to metabolize phenolic compounds in the diets of Mojave woodrats.

Detoxification in Woodrats and Laboratory Rats. The battery of detoxification enzymes used by woodrats differs from that in laboratory rats. Administration of phenobarbital to laboratory rats simultaneously induces CYP2B, mEH, and UGT2B1 mRNAs (Fujii-Kuriyama et al., 1981; Mackenzie, 1986; Porter et al., 1986). Great Basin woodrats exhibited high levels of CYP2B but had low expression of mEH and UGT2B1.

The pattern of mRNA expression in Mojave woodrats also differed from that of laboratory rats. In laboratory rats fed polycyclic aromatic hydrocarbons, transcription of UGT1A6 in the liver generally increases in concert with CYP1A1, GSTYa, and QOR (Hankinson, 1995). Mojave woodrats expressed slightly but significantly greater levels of UGT1A6 mRNA than Great Basin woodrats and laboratory rats. However, levels of GSTYa and QOR in Mojave woodrats were lower than or equal to those found in laboratory rats and Great Basin woodrats.

We propose two explanations for the differing patterns of functionalization and conjugation mRNAs in Great Basin and Mojave woodrats and laboratory rats. It is possible that the compounds woodrats consume in the wild are not processed through the same battery of enzymes as the drugs that have been given to laboratory rats. Alternatively, differences may be due to disparate constitutive expression of genes required to detoxify the various secondary compounds present in their typical diets. We plan to compare the detoxification series of laboratory rats and woodrats administered the same compound to distinguish between these two alternatives.

CONCLUSIONS

To our knowledge, this study is the first to examine biotransformation enzyme mRNAs of wild herbivores by using cDNA probes designed for laboratory rats. Overall, our results indicate that mRNA for biotransformation enzymes in woodrats and laboratory rats is similar enough that cDNA probes designed for laboratory rats work on woodrats. Our results also suggest that there are substantial differences in expression of biotransformation enzyme mRNAs between laboratory rats and woodrats and among populations of woodrats. Although more research is necessary to adequately address the ideas presented in this paper, the work provides a framework for future research.

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