

# Comparison of hepatic in vitro metabolism of the pyrrolizidine alkaloid senecionine in sheep and cattle

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**Objective**—To compare hepatic metabolism of pyrrolizidine alkaloids (PAs) between sheep and cattle and elucidate the protective mechanism of sheep.

**Sample Population**—Liver microsomes and cytosol from 8 sheep and 8 cattle.

**Procedure**—The PA senecionine, senecionine *N*-oxide (nontoxic metabolite) and 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP; toxic metabolite) were measured in microsomal incubations. The  $k_{cat}$  (turnover number) was determined for DHP and *N*-oxide formation. Chemical and immunochemical inhibitors were used to assess the role of cytochrome P450s, flavin-containing monooxygenases (FMOs), and carboxylesterases in senecionine metabolism. The CYP3A, CYP2B, and FMO concentrations and activities were determined, in addition to the role of glutathione (GSH) in senecionine metabolism.

**Results**—DHP concentration did not differ between species. Sheep formed more *N*-oxide, had higher *N*-oxide  $k_{cat}$ , and metabolized senecionine faster than cattle. The P450 concentrations and isoforms had a large influence on DHP formation, whereas FMOs had a large influence on *N*-oxide formation. In cattle, CYP3A played a larger role in DHP formation than in sheep. FMO activity was greater in sheep than in cattle. Addition of GSH to in vitro microsomal incubations decreased DHP formation; addition of cytosol decreased *N*-oxide formation.

**Conclusions and Clinical Relevance**—Hepatic metabolism differences alone do not account for the variation in susceptibility seen between these species. Rather, increased ruminal metabolism in sheep appears to be an important protective mechanism, with hepatic enzymes providing a secondary means to degrade any PAs that are absorbed from the rumen. (*Am J Vet Res* 2004;65:1563–1572)

**P**yrrolizidine alkaloids (PAs) are among the most common groups of plant toxins that affect livestock, wildlife, and human health throughout the world.<sup>1,2</sup> More than 350 PAs have been isolated from over 6,000 plant species.<sup>3</sup> It is estimated that 3% of the world's flowering plants contain toxic PAs.<sup>4,5</sup> Pyrrolizidine alkaloids are largely hepatotoxins and are

capable of causing acute and chronic toxicoses.<sup>6</sup> Acute toxicosis rarely occurs under natural conditions. It usually requires large doses of PAs, such as when PAs are rapidly absorbed because of IP or IV injection, and results in severe hemorrhagic necrosis of the liver and rapid death. Chronic toxicosis that follows oral ingestion of PAs usually results in the development of veno-occlusive disease of the liver, which occludes the small branches of hepatic veins. Veno-occlusive disease causes ascites, edema, reduced urinary output, and can eventually lead to cirrhosis and death. Pyrrolizidine alkaloids can also affect extrahepatic organs such as the lungs and heart, in addition to initiating cancer.<sup>6,7</sup>

Susceptibility to PA toxicity is influenced by many factors including species, age, and sex, as well as other temporary factors such as the mode of PA exposure and the biochemical, physiologic, and nutritional status of the individual.<sup>3</sup> Animal species resistant to PA toxicity include sheep, guinea pigs, rabbits, hamsters, gerbils, and Japanese quail, whereas rats, cattle, horses, and chickens are susceptible to PA toxicosis.<sup>8</sup> Cattle and horses develop chronic terminal hepatic disease after consuming as little as 3% to 10% of their weight in PA-containing plant material, whereas sheep are able to ingest up to 200% of their weight without adverse effects.<sup>9–11</sup> Thus, sheep appear to be at least 20 times more resistant than cattle to the toxic effects of PAs. The physiologic location of this protective mechanism in sheep (ie, ruminal versus hepatic metabolism of the PA toxins) has been a question of interest to researchers for more than 25 years.<sup>11–16</sup>

Substantial evidence supports ruminal microbe metabolism of PA toxins as a major mechanism accounting for the sheep's resistance to PA-induced toxicosis.<sup>13,14,17,18</sup> Bioactivation and detoxification processes in the liver, including the rate of formation of toxic and nontoxic metabolites, are also important in determining the ultimate response in the animal.<sup>19</sup> Metabolism of PAs occurs by 3 main pathways in the liver.<sup>6</sup> First, to induce toxicosis, PAs can be oxidized by cytochrome P450s (P450s) to reactive carbonium ion intermediates, which are unstable and either undergo rapid hydrolysis to 6,7-dihydro-7-hydroxy-1-hydrox-

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γ-methyl-5H-pyrrolizine (DHP), covalently bind to DNA and other cellular nucleophiles (Figure 1), or both. Thus, DHP is described as the primary toxic metabolite in PA toxicosis. Second, PAs can be oxidized to *N*-oxides by P450 and **flavin-containing monooxygenase (FMO)** enzymes. Pyrrolizidine alkaloid *N*-oxides are relatively unreactive and are believed to be excreted unchanged in the urine because of their high water solubility. Third, PAs can be hydrolyzed to nontoxic necine base and necic acid moieties via carboxylesterases. Thus, *N*-oxide formation and hydrolysis are identified as detoxification pathways. In addition, the toxic DHP metabolite can react with **glutathione (GSH)** to form a DHP-GSH conjugate,<sup>20</sup> which increases the water solubility of DHP. The conjugate is subsequently excreted into the bile or sinusoidal blood and ultimately into the urine,<sup>21</sup> thereby decreasing its potential for causing toxicosis.<sup>8</sup> Alternatively, the soluble DHP-GSH conjugate may serve as a transport vehicle to other organs such as the lung, in which toxicosis can be elicited if DHP is released from GSH.<sup>22</sup>

The purpose of the study reported here was to characterize the specific mechanisms of hepatic mixed-function oxidase bioactivation and detoxification of PAs in sheep and cattle. Our hypothesis was that the differences in susceptibility to PA-induced toxicosis of these 2 species are attributable in part to differences in the metabolic capabilities of the liver. In addition, males and females of both species were studied to

determine whether a sex-related difference in the metabolism of PAs exists. Side-by-side *in vitro* studies with sheep and cattle liver microsomes were carried out to determine the metabolic fate of PAs in each species. The addition of GSH to determine its effects on metabolite formation was studied as well.

## Materials and Methods

**Sheep and cattle**—Twenty grams of tissue from multiple locations in the liver was collected immediately after slaughter from 4 adult castrated male sheep, 4 adult female sheep, 4 adult castrated male cattle, and 4 adult female cattle from a commercial slaughterhouse at Oregon State University. Animals were raised on the farm of the Department of Animal Science at Oregon State University. Livers were determined to be healthy upon inspection for abscesses and flukes by a USDA inspector. Carcasses were then processed for human consumption.

**Preparation of samples**—The tissues were immediately rinsed in ice-cold 1.15% KCl buffer, placed in liquid nitrogen, and pulverized in a blender.<sup>9</sup> Ten grams of pulverized liver tissue was used to isolate microsomes and cytosol.<sup>23</sup> Aliquots of the microsomal mixture were stored at -80°C until used. Microsomal and cytosolic protein content were determined with Coomassie reagent<sup>b</sup> with bovine serum albumin as the standard.<sup>24</sup> Total P450 concentration was estimated spectrophotometrically.<sup>25</sup>

***In vitro* metabolism of senecionine**—All incubation experiments were carried out in triplicate. Liver microsomes (0.5 mg) from sheep and cattle were incubated with 0.5mM

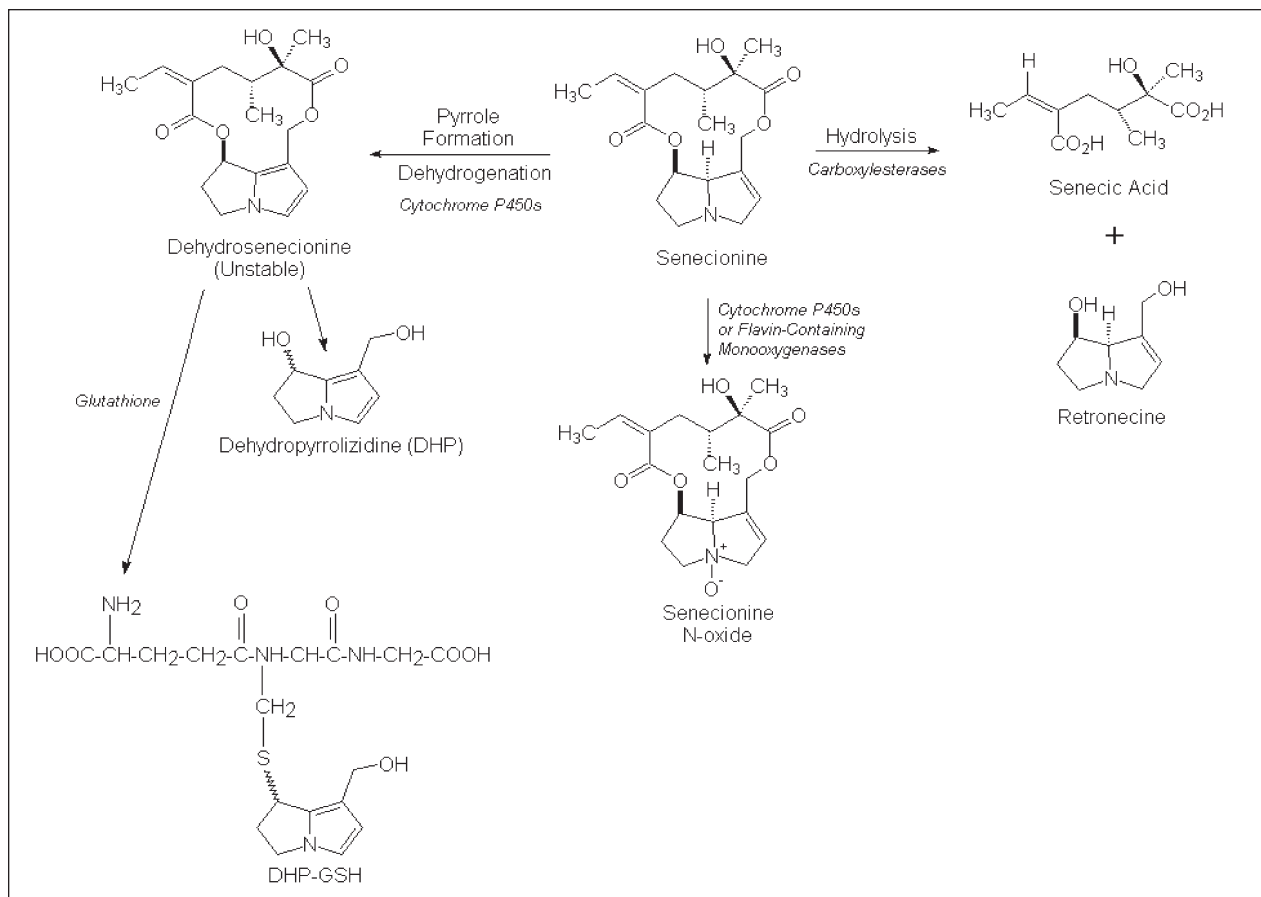


Figure 1—Schematic diagram of major pathways of hepatic metabolism of pyrrolizidine alkaloids. GSH = Glutathione.

senecionine<sup>cd</sup> (or 0.05 to 1mM for kinetic experiments) for 1 hour.<sup>8</sup> The incubation mixtures were analyzed by use of high performance liquid chromatography (HPLC)<sup>e</sup> for DHP, *N*-oxide, and senecionine with a polymeric reversed phase-1 column (150 × 4.1-mm, 10 μm pore size)<sup>f</sup> at λ = 220 nm.<sup>26</sup> *N*-oxide, DHP, and senecionine were identified and quantified by comparison with co-eluting standards. Incubation mixtures containing no nicotinamide adenine dinucleotide diphosphate (NADPH) were run as negative controls.

When chemical inhibitors (0.5mM β-diethylaminoethyl-2,2-diphenylpentanoate [SKF-525A], 0.25mM methimazole, 1.0mM phenylmethyl sulfonyl fluoride [PMSF], 0.1mM tri-*o*-cresyl-phosphate [TOCP] in dimethylsulfoxide [DMSO], 10μM triacetyloleandomycin [TAO] in acetone, and 0.25mM thiourea in H<sub>2</sub>O) were used, the microsomal mixture was incubated for 20 minutes at 37°C with inhibitor before addition of senecionine. Control incubation mixtures contained no NADPH, or an equal volume of DMSO, or acetone. The samples were analyzed for DHP and *N*-oxide and compared with control mixtures containing no chemical inhibitor.

Concentration of immunochemical inhibitors was 2.5, 5, and 10 mg of IgG/nmol of P450 for the mouse monoclonal antibodies raised against human CYP3A4/5/7<sup>8</sup> and the rabbit polyclonal antibodies raised against rat CYP2B1/2<sup>9</sup>; and 10, 20, and 30 mg of IgG/nmol of P450 for the goat polyclonal antibodies raised against rat NADPH cytochrome P450 reductase.<sup>8</sup> Each sample contained 0.1 nmol of P450. Microsomes were incubated with antibodies for 20 minutes at room temperature (22°C) before addition of incubation components and senecionine. Control incubation mixtures had an equivalent amount of preimmune rabbit IgG or no NADPH.<sup>b</sup> The samples were analyzed for DHP and *N*-oxide and compared with control mixtures containing no immunochemical inhibitor.

To determine the effect of GSH on metabolite formation, 2.0mM GSH, 100 μl cytosol, or 2.0mM GSH and 100 μl cytosol were added to the microsomal incubation mixtures. The samples were analyzed for DHP and *N*-oxide and compared with control mixtures containing no GSH or cytosol.

**Measurement of GSH and glutathione-S-transferase—**The GSH concentration of liver microsomes and cytosol samples was measured on a fluorometric plate reader<sup>i</sup> at λ<sub>em</sub> = 420 nm and λ<sub>ex</sub> = 350 nm against a GSH standard curve with *o*-phthalaldehyde as the substrate.<sup>27</sup> Glutathione-S-transferase (GST) was measured spectrophotometrically with 1-chloro-2,4-dinitrobenzene as the substrate at λ = 340 nm, with an extinction coefficient of 9.6mM<sup>-1</sup>·cm<sup>-1</sup>.<sup>28</sup>

**Measurement of FMO activity—**Buffer (100mM tricine and 1.0mM EDTA, pH 8.4) that had been aerated for 30 minutes was added to 2 cuvettes in addition to 0.5 mg of microsomal protein, H<sub>2</sub>O, and 0.1mM NADPH to a final volume of 0.5 mL and incubated at room temperature for 40 seconds.<sup>29</sup> *N*-Ethylmaleimide (25μM) in ultrapure formamide was added to inhibit non-FMO activity and incubated for 40 seconds at room temperature. Dithiothreitol (25μM) and 60μM 5,5'-dithio-(2-nitrobenzoic acid [TNB]) were added and incubated for 40 seconds at room temperature. Twenty microliters of H<sub>2</sub>O was added to the reference cuvette, and both cuvettes were incubated for 3 minutes at 37°C in a shaking metabolic incubator. Methimazole (1mM) was added to the sample cuvette, mixed, and read at λ = 412 nm for 4 minutes. The change in absorbance was used with an extinction coefficient of 28.2mM<sup>-1</sup>·cm<sup>-1</sup> (pH 8.4) to calculate nmol methimazole-enhanced TNB-oxidation/min/mg of protein.

**Measurement of CYP3A and CYP2B activity—**Total P450 (0.05 nmol), 0.1M sodium phosphate buffer (pH 7.4),

0.5mM EDTA, and H<sub>2</sub>O were added to a reaction vessel in duplicate for a final volume of 0.5 mL.<sup>30</sup> Carbon 14-testosterone (10 μCi/μmol)<sup>j</sup> was added to the reaction vessel for a final concentration of 100μM testosterone and was allowed to preincubate for 3 minutes at 30°C on a shaking metabolic incubator; NADPH (0.5mM) was added, and the mixture was incubated for 1 hour at 30°C. The reaction was stopped with 3 mL of methylene chloride and centrifuged at 2,000 × g for 10 minutes. A 2.4-ml aliquot of the organic layer was transferred to a new test tube and evaporated under argon gas. The residue was reconstituted in 100 μL of methanol. An aliquot was analyzed by use of a photodiode array detector (λ = 240 nm) attached to a flow scintillation counter<sup>k</sup> with a carbon 18 column (3.9 × 150-mm, 4 μm particle size)<sup>l</sup> and carbon 18 guard column.<sup>1</sup> Incubation mixtures containing no NADPH were run as negative controls. Testosterone, 6β- and 16β-hydroxytestosterone were identified by comparison with co-eluting standards and were quantified via radioactivity of the peaks.

**Western blot analysis—**Microsomal samples (20 μg/lane) and molecular weight standards<sup>m</sup> were separated by polyacrylamide gel electrophoresis with an 8% separating gel solution and a 3% stacking gel solution.<sup>31</sup> Proteins were electrophoretically transferred to a nitrocellulose membrane at 4°C overnight at 30V.<sup>32</sup> Nonspecific sites were blocked by incubating the membrane for 1 hour at room temperature in 2% bovine serum albumin in PBSS (160mM dibasic potassium phosphate, 40mM monobasic potassium phosphate, 8% NaCl, at pH 7.4). Anti-CYP3A4/5/7 (5 μg/mL),<sup>8</sup> 2.5 μg of anti-CYP2B<sup>9</sup>/mL (as described), rabbit whole serum antibody raised against human FMO1<sup>1</sup> (50 μg/mL), rabbit whole serum antibody raised against human FMO3<sup>8</sup> (24.8 μg/mL), or rabbit whole serum antibody raised against human FMO5<sup>8</sup> (25 μg/mL) made up in 2% bovine serum albumin in PBSS were incubated with the membrane for 1 hour at room temperature. The membrane was washed 4 times for 5 minutes with PBSS plus 0.05% Tween-20 (PBST). The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody<sup>n</sup> diluted 1:10,000 in secondary antibody buffer (0.01M Tris, 0.8% NaCl, 0.5% milk powder,<sup>o</sup> 0.05% Tween-20) for 1 hour at room temperature. The membrane was washed 3 times for 5 minutes and once for 25 minutes in PBST. Binding of the antibodies was detected with a commercially available electrochemiluminescence detection kit.<sup>m</sup> Bands were analyzed densitometrically with available software.<sup>p</sup>

**Statistical analyses—**Data were analyzed via linear regression and 2-tailed *t* test comparisons.<sup>q,t</sup> Values of *P* ≤ 0.05 were considered significant.

## Results

**In vitro metabolism of senecionine—**A reverse-phase HPLC profile of senecionine metabolized by sheep and cattle liver microsomes was obtained (Figure 2). The in vitro metabolism of senecionine and the formation of its metabolites in sheep and cattle liver microsomes were tabulated (Table 1).

Mean amounts of DHP produced did not differ between species. The amount of parent compound that remained after incubation, mean concentration of *N*-oxide formed, and DHP:*N*-oxide ratio differed between species. Sheep liver microsomes metabolized a greater proportion of the available senecionine than cattle liver microsomes did. Sheep liver microsomes formed more than 4 times the amount of *N*-oxide during incubation than equivalent amounts of cattle liver microsomes.

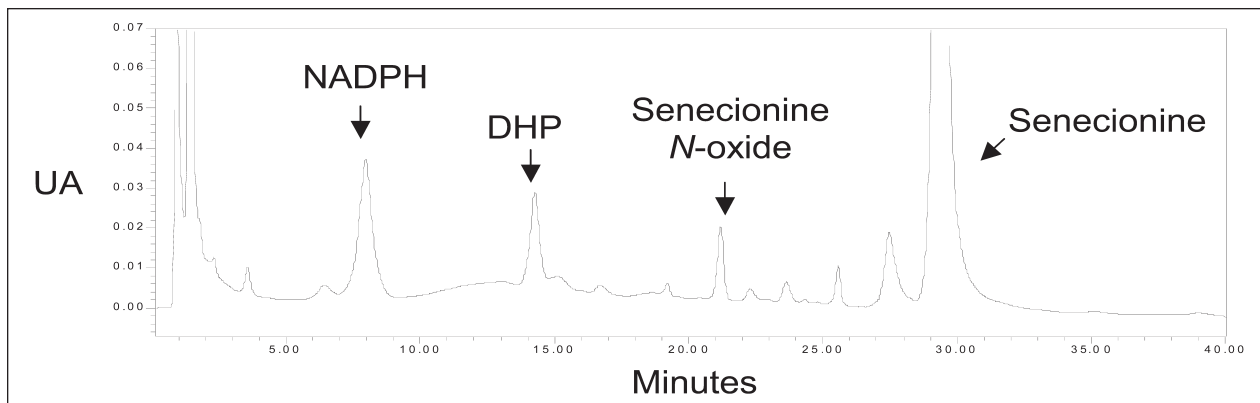


Figure 2—Representative chromatogram of reverse-phase high-performance liquid chromatography analysis of senecionine and its metabolites from sheep and cattle liver microsomes incubations. Ultraviolet absorbance (UA) was recorded at 220 nm. Nicotinamide adenine dinucleotide diphosphate (NADPH) = 8.0 minutes, 6,7-dihydro-7-hydroxymethyl-5H-pyrrolizine (DHP) = 14.5 minutes, senecionine *N*-oxide = 21.0 minutes and senecionine = 29.0 minutes.

This inversely affected the DHP:*N*-oxide ratio such that the ratio was approximately 4 times greater in cattle than in sheep. Thus, cattle appeared to form more DHP relative to *N*-oxide than sheep.

When animals were compared between sexes within a species, cattle liver microsomes had a difference in the ratio of DHP:*N*-oxide, in that castrated males formed more DHP relative to *N*-oxide than females. Sheep liver microsomes differed between sexes in the mean amount of DHP formed such that castrated male sheep formed more DHP than did female sheep. This affected the DHP:*N*-oxide ratio in that castrated male sheep formed more DHP relative to *N*-oxide than did female sheep. Thus, in general females appeared to form more *N*-oxide relative to DHP than males did in both species.

**Kinetics of senecionine metabolism in liver microsomes**—One representative microsomal preparation from each of the 4 groups (closest to the mean formation of DHP and *N*-oxide values for each group) was run in triplicate in incubation experiments with senecionine concentration ranging from 0.05 to 1.0mM. The kinetic parameters  $K_m$  and  $V_{max}$  were then determined for DHP and senecionine *N*-oxide formation from double reciprocal Lineweaver-Burke plots.

Catalytic efficiency of an enzyme is expressed through determination of its  $k_{cat}$ , which is a direct measure of the production of product under saturated conditions, and can be expressed as  $V_{max}/K_m$ . When animals were grouped by species, cattle had a  $k_{cat}$  of 5.75/s for DHP formation whereas sheep had a value of 3.36/s, a 1.7-times difference between the species. Measurement of *N*-oxide formation in both species resulted in  $k_{cat}$  values of 1.19/s for cattle and 3.69/s for sheep, a 3.1-times difference between the species. When animals were grouped by sex, the  $k_{cat}$  for DHP formation was determined to be 6.96/s for female cattle, 5.19/s for castrated male cattle, 3.93/s for female sheep, and 4.02/s for castrated male sheep. The  $k_{cat}$  for *N*-oxide formation was 1.40/s for female cattle, 0.89/s for castrated male cattle, 4.98/s for female sheep, and 2.92/s for castrated male sheep. Sex differences were most noticeable for *N*-oxide formation ( $k_{cat}$  was 1.6 times higher in female than in castrated male cattle and 1.7 times higher in female than in castrated male sheep).

#### Effect of chemical inhibitors on DHP and *N*-oxide formation

To determine the principal enzymes responsible for catalyzing the metabolism of senecionine to DHP and *N*-oxide, microsomal incubations were conducted in the presence of various chemical inhibitors (Table 2). The general cytochrome P450 inhibitor SKF-525A inhibited DHP formation in sheep and cattle. Triacetyloleandomycin, which specifically inhibits CYP3A,<sup>23,33</sup> had a greater effect on DHP formation in cattle than in sheep. The 2 FMO inhibitors, methimazole and thiourea, reduced *N*-oxide formation in both species. The carboxylesterase inhibitor PMSF had little change in metabolite formation, compared with controls. The carboxylesterase inhibitor TOCP inhibited DHP formation in all 4 groups.

#### Effect of immunochemical inhibitors on DHP and *N*-oxide formation

Antibodies produced against NADPH cytochrome P450 reductase inhibit all P450-catalyzed reactions, because NADPH cytochrome P450 reductase is a coenzyme required for the oxidation of substrates by P450s. Antibodies raised against NADPH cytochrome P450 reductase, CYP2B, or CYP3A were incubated with liver microsomes from the 4 representative metabolizers (as identified in the kinetic studies) to further characterize the contributions of general (NADPH cytochrome P450 reductase) and specific (CYP2B, CYP3A) enzymes in the metabolism of senecionine to DHP and *N*-oxide. Results (Figure 3) indicated that DHP formation was inhibited in all 4 groups when 30 mg of IgG/nmol of P450 anti-NADPH cytochrome P450 reductase was used. *N*-oxide formation was increased in female cattle, female sheep, and castrated male sheep liver microsomes, whereas castrated male cattle liver microsomes showed a decrease in *N*-oxide concentration. Incubations performed with anti-CYP2B and anti-CYP3A antibodies revealed no significant inhibition in DHP or *N*-oxide formation, possibly because of the low concentration of antibody used (maximum concentration was 10 mg of IgG/nmol of P450) or because of differences in interspecies specificity to the antibodies.

**FMO activity**—Sheep and cattle differed in FMO activity. Mean values were 3.1 nmol of methimazole-enhanced TNB-oxidation/min/mg of protein for

Table 1—In vitro metabolism of senecionine by sheep and cattle liver microsomes to 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) and senecionine *N*-oxide (mean  $\pm$  SEM).  $SN_B$ ,  $SN_A$  = Amount of senecionine before (B) and after (A) incubation.

Species	Sex	DHP (nmol/min/mg protein)	<i>N</i> -oxide (nmol/min/mg protein)	Ratio of DHP/ <i>N</i> -oxide	$SN_A/SN_B$ (%)
Cattle	Female	0.42 $\pm$ 0.13	0.33 $\pm$ 0.08	1.24 $\pm$ 0.19	88.6 $\pm$ 2.1
	Male	0.39 $\pm$ 0.08	0.19 $\pm$ 0.04	2.11 $\pm$ 0.18	87.0 $\pm$ 2.0
	Both	0.41 $\pm$ 0.01	0.26 $\pm$ 0.07	1.68 $\pm$ 0.29	87.8 $\pm$ 1.9
Sheep	Female	0.28 $\pm$ 0.09	1.11 $\pm$ 0.38	0.29 $\pm$ 0.05	76.4 $\pm$ 2.1
	Male	0.50 $\pm$ 0.10	1.08 $\pm$ 0.06	0.46 $\pm$ 0.09	73.5 $\pm$ 4.6
	Both	0.39 $\pm$ 0.11	1.10 $\pm$ 0.26	0.37 $\pm$ 0.08	74.9 $\pm$ 3.4

Table 2—Chemical inhibition of DHP and senecionine *N*-oxide (NO) formation (expressed as percentage of control values [mean  $\pm$  SEM]) from incubation with 0.5mM senecionine in sheep and cattle liver microsomes. \*Significantly ( $P \leq 0.05$ ) different from control value.

Species	Chemical inhibitor					
	SKF-525A	Triacetyl-oleandomycin	Methimazole	Thiourea	Phenylmethylsulfonyl fluoride	Tri- <i>o</i> -cresyl phosphate
	DHP (NO)	DHP (NO)	DHP (NO)	DHP (NO)	DHP (NO)	DHP (NO)
Female sheep	15.3 $\pm$ 3.1* (49.2 $\pm$ 10.6*)	61.4 $\pm$ 2.9* (67.4 $\pm$ 14.3*)	35.3 $\pm$ 11.7* (16.4 $\pm$ 2.7*)	35.4 $\pm$ 10.2* (26.0 $\pm$ 6.6*)	87.3 $\pm$ 4.1 (54.2 $\pm$ 13.7*)	23.8 $\pm$ 2.9* (46.5 $\pm$ 9.8*)
Male sheep	12.7 $\pm$ 4.1* (56.3 $\pm$ 3.3*)	50.9 $\pm$ 2.2* (83.6 $\pm$ 2.2*)	25.3 $\pm$ 6.4* (4.8 $\pm$ 0.3*)	25.4 $\pm$ 3.7* (19.6 $\pm$ 3.4*)	90.2 $\pm$ 4.2 (51.9 $\pm$ 7.3*)	19.2 $\pm$ 2.9* (48.8 $\pm$ 3.6*)
Female cattle	22.1 $\pm$ 10.6* (48.7 $\pm$ 7.9*)	17.6 $\pm$ 0.7* (55.9 $\pm$ 11.3*)	23.9 $\pm$ 8.3* (14.4 $\pm$ 4.4*)	59.1 $\pm$ 15.8* (24.2 $\pm$ 5.3*)	116.3 $\pm$ 9.1 (84.1 $\pm$ 19.1)	25.1 $\pm$ 2.2* (47.6 $\pm$ 7.8*)
Male cattle	12.6 $\pm$ 2.9* (35.9 $\pm$ 5.2*)	18.0 $\pm$ 2.7* (52.0 $\pm$ 9.7*)	39.1 $\pm$ 8.8* (22.8 $\pm$ 4.3*)	71.6 $\pm$ 8.9* (34.4 $\pm$ 4.3*)	113.7 $\pm$ 8.5 (84.7 $\pm$ 10.9)	21.4 $\pm$ 2.14* (51.3 $\pm$ 3.1*)

cattle and 7.3 nmol of methimazole-enhanced TNB-oxidation/min/mg of protein for sheep. Sex comparisons within a species revealed no differences. Mean values were 3.5 for female cattle, 3.0 for castrated male cattle, 7.2 for female sheep, and 7.2 nmol of methimazole-enhanced TNB-oxidation/min/mg of protein for castrated male sheep.

**CYP3A and CYP2B activity**—The 6 $\beta$ - and 16 $\beta$ -hydroxylation of testosterone is reflective of the activity of CYP3A and CYP2B enzymes, respectively. When both products were measured in sheep and cattle liver microsome incubations with testosterone, no difference was found either between species or between sexes. Cattle formed 0.16 nmol of 6 $\beta$ -hydroxytestosterone/min/nmol of P450 whereas sheep produced 0.21 nmol of 6 $\beta$ -hydroxytestosterone/min/nmol of P450. Cattle yielded 0.12 nmol of 16 $\beta$ -hydroxytestosterone/min/nmol of P450 and sheep formed 0.07 nmol of 16 $\beta$ -hydroxytestosterone/min/nmol of P450. When separated into sexes, female cattle produced 0.15 nmol/min/nmol of P450 of 6 $\beta$ -hydroxytestosterone whereas castrated male cattle, female sheep and castrated male sheep formed 0.17, 0.20 and 0.23 nmol/min/nmol of P450, respectively. 16 $\beta$ -Hydroxytestosterone was formed at a rate of 0.09, 0.11, 0.08, and 0.06 nmol/min/nmol of P450 for female cattle, castrated male cattle, female sheep, and castrated male sheep, respectively.

**Role of GSH in metabolism of senecionine**—The addition of GSH, cytosol that contains GSH in high concentrations, or both, to microsomal incubations

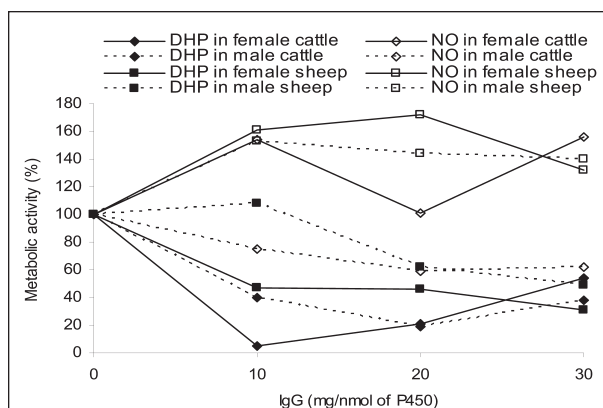


Figure 3—Immunoinhibition of senecionine metabolism to DHP and *N*-oxide (NO) by anti-rat NADPH cytochrome P450 reductase IgG in microsomes from male and female cattle and sheep. Mean values in each group were compared with control group mean values and expressed as a percentage.

measures the effect of GSH conjugation on senecionine metabolism. Addition of GSH alone resulted in an 80% reduction of DHP formation in female cattle, 73% in castrated male cattle, 74% in female sheep, and 79% in castrated male sheep liver microsomes and had no significant effect on *N*-oxide formation. Introduction of cytosol alone caused a 69% reduction in *N*-oxide formation in female cattle, 49% in castrated male cattle, 63% in female sheep, and 47% in castrated male cattle liver microsomes and had less of an effect on DHP formation. Inclusion of both GSH and cytosol caused a reduction in both products, similar to the reduction

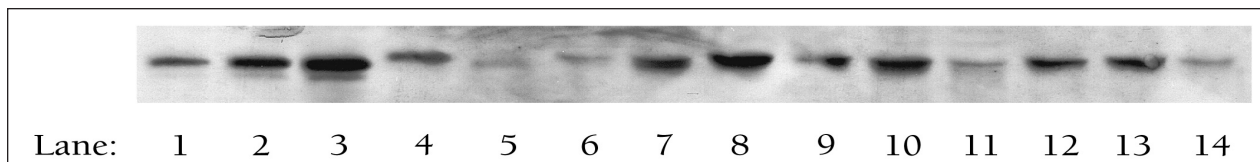


Figure 4—Western blot analysis of the cytochrome P450 CYP3A in sheep and cattle liver microsomes. Lanes 1 to 3 = 0.5, 1, and 2 pmol of CYP3A4 standard, respectively. Lane 4 = 1 pmol of CYP3A5 standard. Lane 5 = 1 pmol of CYP3A7 standard. Lanes 6, 10, 11, and 14 = male sheep. Lane 7 = female sheep. Lanes 8 and 13 = male cattle. Lanes 9 and 12 = female cattle.

that resulted from addition of each single component. Formation of DHP was reduced by 84% in female cattle, 77% in castrated male cattle, 78% in female sheep, and 82% in castrated male sheep liver microsomes. *N*-oxide formation was reduced by 63% in female cattle, 73% in castrated male cattle, 74% in female sheep, and 58% in castrated male sheep liver microsomes.

Mean GSH concentrations in 100  $\mu$ l of cytosol were 0.02mM for all 4 groups. When GST activity was measured, 39 nmol of GST/min/mg of protein was observed in female cattle liver microsomes, 46 nmol of GST/min/mg of protein for castrated male cattle, 83 nmol of GST/min/mg of protein for female sheep and 111 nmol of GST/min/mg of protein for castrated male sheep. Values in liver cytosol were as follows: 286 nmol of GST/min/mg of protein for female cattle, 351 nmol of GST/min/mg of protein for castrated male cattle, 1,053 nmol of GST/min/mg of protein for female sheep and 1,429 nmol of GST/min/mg of protein for castrated male sheep. Thus, cattle liver cytosol GST concentration was approximately 7.5 times higher than cattle liver microsome GST concentration. Sheep liver cytosol GST concentration was approximately 12.7 times higher than sheep liver microsome GST concentration.

**Western blot analysis**—Western blots were performed on all 16 microsomal samples with each of 5 antibodies (raised against CYP3A4/5/7, CYP2B1/2, FMO1, FMO3, or FMO5). A molecular weight ladder (14.4 to 97.0 kd) was run along with standards for the CYP3A4/5/7, CYP2B1/2, FMO1, FMO3, and FMO5 proteins. The standards each revealed a signal in the molecular weight range expected for P450 and FMO proteins (57 to 60 kDa). Measurement of CYP3A4/5/7 protein gave a mean of 0.04 nmol/mg of protein for cattle and 0.02 nmol/mg of protein for sheep (Figure 4). When sexes were compared within species, no differences were detected.

No signal was detected for the CYP2B1/2, FMO1, FMO3, or FMO5 proteins for any of the 16 sheep and cattle samples. Standards run for each isozyme were visible, so it was determined that the antibodies were responsive to the protein of interest.

## Discussion

The resistance of sheep and the susceptibility of cattle to PA toxicosis have been attributed to either differential transformation of PAs by ruminal microbes or to differences in the metabolism of PAs by liver mixed-function oxidase enzymes. The purpose of this study was to characterize the hepatic bioactivation and detoxification of PAs in sheep and cattle liver microsomes to determine if differences in liver metabolism

play a role in determining a species' susceptibility to toxicosis. Tansy ragwort (*Senecio jacobaea*) is widely distributed in the rangelands of the Pacific Northwest and has been described as the most significant toxic plant contributing to livestock loss there.<sup>9</sup> Because senecionine is found in high abundance in tansy ragwort, it was chosen as the model alkaloid for this study.

The mechanism of toxicosis is delineated as follows: *Senecio* plants containing approximately 0.2% PAs<sup>34</sup> are consumed by ruminants. The PAs diffuse out of the plant and come into contact with the rumen environment. If  $> 10^7$  PA-degrading bacteria are present, major amounts of the PA will be degraded.<sup>14,17,35</sup> If sufficient quantities of PA-degrading bacteria are not present, the PAs are transported to the liver where they are collected by the mesentery veins and pass through the portal triad to the central vein of the hepatocytes. In the hepatocytes, PAs encounter mixed-function oxidase enzymes, including P450s and FMOs. Here, PAs can follow various mechanisms of disposal and their eventual toxicologic effect depends on the balance of these pathways (Figure 1).<sup>6</sup>

Evidence that hepatic metabolism differences lead to varying susceptibilities in PA toxicosis amongst species has historically centered on the relative amount of DHP produced during metabolism of PAs.<sup>36</sup> In general, resistant species such as sheep are said to have lower rates of DHP formation than susceptible species such as cattle, suggesting that PAs are bioactivated at a slower rate in resistant species. Our study revealed that the mean amount of DHP formed and the catalytic efficiency of the enzymes responsible for DHP formation ( $k_{cat}$ ) did not differ between sheep and cattle, providing evidence that there is not a strong correlation between in vitro DHP formation from liver microsomes and a species' susceptibility to PA toxicosis.<sup>8</sup>

The ratio of DHP to *N*-oxide allows examination of the relative formation of DHP to *N*-oxide. This is important when comparing metabolite formation between groups because the relative activities of the bioactivation (DHP) and detoxification (*N*-oxide) pathways are important in determining a species' susceptibility to PAs. Sheep liver microsomes formed more *N*-oxide relative to DHP than cattle liver microsomes, as evidenced from the 4.5-times difference in the amount of *N*-oxide formed, and the DHP:*N*-oxide ratio detected between the species. When the  $k_{cat}$  for *N*-oxide formation was determined for both species, it appeared that the enzymes responsible for the *N*-oxidation of senecionine had a greater catalytic efficiency and thus used more of the parent compound for metabolism to *N*-oxide in sheep than in cattle. Coupled with a higher rate of senecionine metabolism, the ability to

form *N*-oxide more readily could aid sheep in eliminating a potentially toxic compound through a relatively nontoxic pathway, thereby increasing the sheep's resistance to PA toxicosis.<sup>8</sup>

Sex differences were also examined in the metabolism of senecionine in these species. In cattle and sheep with PA toxicosis, no clinical or histologic differences attributable to sex have been reported. However, the DHP:*N*-oxide ratio differed in both species, yielding an approximately 2 times higher value in castrated males than in females. Thus, castrated males form more DHP relative to *N*-oxide than females in these 2 species. In addition, the  $k_{cat}$  values for *N*-oxide were approximately 2 times greater in females than in castrated males, suggesting that the enzymes responsible for *N*-oxide formation have greater catalytic efficiency in females than in castrated males.

A previous study in rat liver microsomes detected a marked sex difference in the *N*-oxidation of senecionine, attributable to the male-specific enzyme CYP2C11.<sup>19</sup> The enzyme CYP2C11 is more efficient at senecionine *N*-oxidation than CYP3A1, which is present in both sexes in rats and carries out formation of both *N*-oxide and DHP. This suggests that female rats are more sensitive to PA toxicosis than male rats, because of their low rates of formation of *N*-oxide relative to DHP, which was confirmed in a later study.<sup>6</sup> The expression of CYP2C11 is dependent on testosterone, yet the mechanisms underlying *in vivo* regulation are not well understood.<sup>37</sup> Thus, metabolism of PAs by sexually intact and castrated males may differ if sex-specific P450 enzymes are responsible for the sex differences in our study. Given the low sample size in our study and the likelihood that sex differences in PA metabolism are regulated by a complex set of interrelated factors, further investigation of sex differences in cattle and sheep should be conducted to determine whether the apparent differences in *N*-oxide formation and kinetic measurements are consistent in these species.

Several studies have evaluated individual species to investigate which metabolic pathways confer resistance or susceptibility to PA toxicosis.<sup>6,38</sup> Huan et al<sup>8</sup> found that DHP formation was catalyzed mainly by P450s, whereas senecionine *N*-oxide was formed mainly by FMOs in sheep and hamsters (both resistant species). Sheep also used carboxylesterases more than hamsters, but both species had a high rate of DHP-GSH conjugation. Huan et al<sup>23</sup> concluded that species differences appeared to be dependent on the presence of species specific mixed-function oxidases and their catalytic capability. Therefore, identification of the specific enzymes that are involved in biotransformation of PAs may be an important step in understanding the potential hazards of PAs to animals and humans.<sup>23</sup>

Chemical and antibody inhibitors can be used to identify the relative metabolic contribution of the enzymes they inhibit and have been used in PA metabolism studies with various species.<sup>8,19,23,39-43</sup> A general cytochrome P450 inhibitor, SKF-525A, inhibited DHP formation so that only 13% to 22% of the DHP formed by control incubations was seen in sheep and cattle. Incubation with anti-NADPH cytochrome P450 reduc-

tase IgG, which inhibits all P450-catalyzed reactions, inhibited DHP formation in all 4 groups. These results suggest that P450s play a large role in the metabolism of senecionine to DHP in both species. To confirm the role of the CYP3A family in DHP formation, TAO was used as a more specific P450 inhibitor and had a large effect on cattle of both sexes, forming only 18% of the DHP seen in controls. It had less of an effect on sheep and was the only inhibitor that resulted in a marked difference between species. Thus, CYP3A appears to play a larger role in DHP formation in cattle than in sheep; CYP2B also plays an important role in the bioactivation of senecionine in some species.<sup>38,43,44</sup> To date, however, there are no confirmed specific chemical inhibitors of CYP2B in sheep and cattle.<sup>23</sup> Therefore, the role of CYP2B enzymes in senecionine metabolism could not be fully evaluated through the use of selective chemical inhibitors in our study.

The stereospecific sites of testosterone oxidation have been attributed to specific P450 isozymes. In our study, 6 $\beta$ - and 16 $\beta$ -hydroxylation of testosterone was used to measure the relative activities of CYP3A and CYP2B, respectively. No significant differences were found between species or sexes, suggesting that the activities of CYP3A and CYP2B were similar. Measurement of catalytic activities may miss enzyme-mediated activity if the animal has been previously exposed to dietary components that bind to the enzyme and inhibit its enzymatic capacity. Catalytic activity may also be too low and below the sensitivity of a given assay. Thus, methods such as western blotting are used to detect the nature and amount of enzyme protein. Because CYP3A is believed to be the main cytochrome P450 family responsible for metabolism of PAs to DHP, and in some instances to *N*-oxide,<sup>8,19,23,38,40,44</sup> its presence and activity were further studied by western blotting and catalytic activity assays. Because CYP2B may also play an important role in the bioactivation of senecionine,<sup>38,43,44</sup> the presence and activity of this isozyme were investigated as well.

Western blot analysis of CYP3A revealed a difference in enzyme concentration between species, with cattle liver microsomes containing more than twice the amount of CYP3A protein as sheep liver microsomes. This discrepancy between enzyme concentration and activity is an example of the effects other compounds can have on enzymatic activity. It is possible that other endogenous or exogenous components bound and partially inhibited CYP3A activity in cattle before they were euthanized. In effect, a proportion of the CYP3A enzyme in the microsomal preparation could have been bound by other components, which resulted in decreased activity in the testosterone hydroxylation assay relative to the amount of enzyme available for metabolism. Alternatively, the differences between species could be a reflection of the intrinsic catalytic characteristics of the 2 CYP3A forms.

Methimazole and thiourea were used to chemically inhibit FMOs and greatly reduced *N*-oxide formation in both species and sexes. This suggests that FMOs play an important role in the formation of *N*-oxide and therefore contribute to the detoxification of PAs in both species.<sup>8,39,41</sup> Activity of FMOs differed sig-

nificantly between sheep and cattle. Sheep had more than twice as much FMO activity as cattle. Coupled with the observations from the *in vitro* metabolism and kinetic experiments for *N*-oxide above, this could explain the sheep's ability to form *N*-oxide at a higher rate than cattle because *N*-oxide formation is thought to be catalyzed mainly by FMO enzymes.

No substantial detection of the anti-rabbit antibodies against FMO1, FMO3, FMO5, or the anti-rat CYP2B was found for the sheep and cattle liver samples assayed by western blots in this study. However, on the basis of information from previous studies of FMO<sup>8,39,41</sup> and CYP2B<sup>38,43,44</sup> enzymes in other species, and the FMO and 16 $\beta$ -testosterone hydroxylation activity in our study, it is possible that these proteins were present but were not being bound by the antibodies we used. The homology of the proteins between the species may not be conserved enough to enable specific binding of the non-ruminant antibodies (raised to rat and human antigens) to the separated sheep and cattle proteins. Because of the lack of availability of antibodies for P450 and FMO proteins in ruminant species at this time, detection of these proteins should be investigated at a later date when these antibodies can be purified.

Previous studies reveal that carboxylesterase hydrolysis may play an important role in PA metabolism.<sup>8,45,46</sup> An increase in DHP and *N*-oxide formation is to be expected if carboxylesterase activity is inhibited because more senecionine is available for metabolism to DHP or *N*-oxide.<sup>6</sup> In our study, however, the carboxylesterase inhibitor TOCP decreased DHP formation (20% of controls) in both species and sexes. The other carboxylesterase inhibitor used, PMSF, caused no significant change in DHP formation in either species. This suggests that neither TOCP nor PMSF are effective inhibitors of carboxylesterases in senecionine metabolism for these species. Therefore, alternative methods should be used to characterize the role of carboxylesterases in the metabolism of PAs in sheep and cattle.

Conjugation of GSH to electrophilic xenobiotics is an important detoxification route used by many organisms for a wide array of toxins. The cellular concentration of GSH is high (approx 10mM), making nonenzymatic conjugation of xenobiotics a relatively important pathway of elimination.<sup>47</sup> Results of our study support results of previous research, which found that GSH conjugation plays a role in the detoxification of PAs.<sup>8,20,21</sup> Addition of GSH to microsomal incubations resulted in a reduction in DHP formation. The decrease in DHP formation with the introduction of GSH supports similar results found by Huan et al.<sup>8</sup> This suggests that increasing GSH concentration *in vivo* may serve as a possible treatment or protective strategy against PA intoxication.

The decrease in *N*-oxide formation upon addition of cytosol was an interesting finding. Liver cytosol contains many metabolic components including GSH, GSTs, and carboxylesterases. It is possible that these components were present in the cytosol and were able to route senecionine through alternative metabolic pathways, which decreased the emphasis on the *N*-oxide detoxification pathway. In particular, GSTs catalyze the conjugation of some xenobiotics and can

comprise 10% of total cellular protein. The GST concentration differed substantially between liver microsomes and cytosol from cattle and sheep. Sheep cytosol contained 7.5 times more GST than sheep microsomes, whereas cattle cytosol had 12.7 times more GST than cattle microsomes. Alternatively, DHP formation did not markedly decrease with the addition of cytosol, so it is possible that a component in the cytosol inhibited FMOs or other enzymes from forming *N*-oxide.

Differences in PA concentrations reaching the liver and the detoxification of PAs in the digestive system by microbial flora<sup>13</sup> are alternative explanations for differences in toxicosis between species. This has been measured in 3 ways. In the first study,<sup>13</sup> similar concentrations of PA were infused directly into the hepatic portal vein of both sheep and cattle, bypassing the rumen and the detoxifying ability of ruminal microbes. The resultant liver cirrhosis in sheep suggests that the rumen plays a protective role in sheep's resistance to PA toxicosis. Further research was conducted, including isolation of PA-degrading microbes from the rumen contents of sheep and cattle.<sup>48,49</sup> Sheep ruminal fluid degraded inoculated PAs within 2 to 6 hours, whereas cattle ruminal fluid required 24 to 48 hours for degradation.<sup>49</sup> Thus, sheep ruminal fluid is able to degrade PAs approximately 10 times more efficiently than cattle ruminal fluid. The third measurement used most probable number analysis to count the number of PA-degrading bacteria in sheep and cattle ruminal fluid.<sup>49</sup> Sheep ruminal fluid contained  $3 \times 10^7$  PA-degrading bacteria/mL, whereas cattle ruminal fluid had  $1 \times 10^7$  PA-degrading bacteria/mL. Therefore, the slower rates of PA degradation and the lower concentration of PA-degrading microorganisms in cattle ruminal fluid may be partially responsible for the cattle's susceptibility to PA toxicosis, because there is no rapid detoxification prior to ruminal absorption of the toxin.

Research has indicated that there is a relationship between diet, bacterial status, and hepatic P450 content in rats.<sup>50</sup> Our study determined that the production of toxic and nontoxic metabolites differed by a factor of 4.5 between sheep and cattle. These differences alone are not enough to account for the variation in susceptibility to PAs between sheep and cattle, given the 20-times difference in the amount of PA-containing plant material required to cause chronic terminal hepatic disease in these species.<sup>9</sup> Rumen fluid degradation experiments reveal that sheep ruminal fluid can degrade PAs 10 times more efficiently than cattle ruminal fluid. Therefore, increased ruminal metabolism of PAs in sheep appears to be the primary protective mechanism, whereas detoxification of PAs by mixed-function oxidase enzymes in the liver provides a secondary means for elimination of PAs that are absorbed from the rumen.

<sup>a</sup>Robot Coupe Blixer RSI BX6, Ridgeland, Miss.

<sup>b</sup>Pierce Chemical Company, Rockford, Ill.

<sup>c</sup>Provided by Dr. J. N. Roitman, USDA-ARS, Western Regional Research Center, Albany, Calif.

<sup>d</sup>Indofine Chemical Company, Hillsborough, NJ.

<sup>e</sup>2690 Separations Module with a Photodiode Array Detector, Waters, Milford, Mass.

<sup>f</sup>Hamilton, Reno, Nev.

<sup>g</sup>BD Gentest, Bedford, Mass.



<sup>b</sup>Xenotech, Lenexa, Kan.

<sup>c</sup>Molecular Devices, Rockford, Ill.

<sup>d</sup>NEN, Boston, Mass.

<sup>e</sup>Packard, Meriden, Conn.

<sup>f</sup>NovaPak, Waters, Milford, Mass.

<sup>g</sup>Amersham, Piscataway, N.J.

<sup>h</sup>BioRad, Hercules, Calif.

<sup>i</sup>Carnation, Nestle, Glendale, Calif.

<sup>j</sup>ImageJ, National Institutes of Health, Bethesda, Md.

<sup>k</sup>Excel, Microsoft Corp, Redmond, Wash.

<sup>l</sup>SYSTAT, SPSS Inc, Chicago, Ill.

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### **Correction: Expression, bioactivity, and clinical assessment of recombinant feline erythropoietin**

In the article "Expression, bioactivity, and clinical assessment of recombinant feline erythropoietin," published October 2004 (2004;65:1355-1366), the following item was incorrect.

In the Results on page 1358, the second complete sentence in the first column incorrectly stated that the concentration was < 12 mg/mL. The correct concentration is < 12 µg/mL.