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Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids

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Abstract

Species differences in pyrrolic metabolites and senecionine (SN) N-oxide formation among eight animal species (sheep, cattle, gerbils, rabbits, hamsters, Japanese quail, chickens, rats) varying in susceptibility to pyrrolizidine alkaloid (PA) intoxication were measured in vitro by hepatic microsomal incubations. The results suggested that there is not a strong correlation between the production of pyrrolic metabolites and susceptibility of animals to PA toxicity. The rate of PA activation in hamsters, a resistant species, measured by formation of (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) far exceeded the rate of SN N-oxide formation (detoxification) (DHP/N-oxide = 2.29). In contrast, SN N-oxide was the major metabolite in sheep, another resistant species, with much lower production of DHP (DHP/N-oxide = 0.26). The roles of cytochrome P450s and flavin-containing monooxygenases (FMO) in bioactivation and detoxification of pyrrolizidine alkaloids (PA) were studied in vitro using sheep and hamster hepatic microsomes. Chemical and immunochemical inhibition data suggested that the conversion of SN to DHP is catalyzed mainly by cytochrome P450s (68–82%), whereas the formation of SN N-oxide is carried out largely by FMO (55–71%). There also appeared to be a high rate of glutathione–DHP conjugation in hamster (63%) and sheep (79%) liver microsomal incubation mixtures. Therefore, low rates of pyrrole metabolite production coupled with glutathione conjugation in sheep may explain the resistance of sheep to SN, whereas the high rate of GSH-DHP conjugation may be one of the factors contributing to the resistance of hamsters to intoxication by this PA.

Keywords: Pyrrolizidine alkaloids; Cytochrome P450s; Flavin-containing monooxygenases; Glutathione conjugation; Species

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1. Introduction

The pyrrolizidine alkaloids (PAs) are a large and important family of natural toxicants produced by a variety of plant species. Most PA which produce toxic effects in livestock and humans are in the genera *Senecio*, *Crotalaria*, *Heliotropium* and *Echium* (Cheeke, 1998). The primary routes of PA metabolism have been established in laboratory animals: dehydrogenation to pyrrolic derivatives, conversion to N-oxides and hydrolysis (Mattocks, 1986). PAs such as senecionine require metabolic activation to produce toxicity. Dehydrogenation of PA to yield unstable dehydropyrrolizidine alkaloids (PA pyrroles) by hepatic monooxygenases located in the endoplasmic reticulum is the classic pathway of PA metabolism. The PA pyrroles either react with cellular macromolecules or are hydrolyzed to form a secondary pyrrole such as (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP). Hydrolysis of the ester groups by esterases such as carboxylesterase with excretion of the acid and amino alcohol products, and formation and excretion of highly water soluble N-oxides are detoxification mechanisms (Mattocks, 1986).

There is a marked variation in the susceptibility of animal species to the toxic effects of PA exposure. Sheep, guinea pigs, gerbils, rabbits, hamsters and Japanese quail are highly resistant to PA whereas rats, cattle, horses and chickens are highly susceptible (Cheeke, 1998). The species differences in resistance or susceptibility appear to be primarily a consequence of differences in hepatic PA metabolism rather than to microbial PA metabolism in the rumen or intestine (Cheeke, 1998). In general, those susceptible species such as rats, mice, cattle and horses have a high rate of pyrrole production, whereas PA-resistant animals such as sheep, guinea pigs and Japanese quail have low rates of pyrrole formation (White et al., 1973; Shull et al., 1976), as assessed by colorimetric analysis of pyrroles. In some cases, however, for example with the rabbit, the in vitro pyrrole production of a resistant species is high, whereas with chickens, a relatively susceptible species, the pyrrole formation is relatively low (Cheeke, 1998). However, data from other research (Winter et al., 1988) using gas chromatography/mass spectrometry (GS/MS) and MS/MS to identify the pyrrolic metabolite (DHP), PA N-oxide, and hydrolytic metabolite from hepatic microsomal incubation showed that there was a high rate of DHP production in guinea pigs, whereas the DHP production in rats was relatively low. They suggested that the resistance to certain PA such as SN in the guinea pig is due to resistance to pyrrole toxicity rather than low pyrrole formation. It seems that the relative levels of DHP or N-oxide produced from in vitro hepatic microsomal metabolism do not reflect the resistance or susceptibility among the animals. Furthermore, flavin-containing monooxygenase (FMO), another hepatic microsomal monooxygenase, accounted for no more than 20% of the PA N-oxidase activity in Sprague–Dawley rat liver microsomes (Williams et al., 1989b), but this enzyme played a major role in N-oxidation of SN in guinea pigs (Miranda et al., 1991).

A number of indirect methods have been utilized in studies aimed at determination of the relative role of cytochrome P450s and FMO in the metabolism of PA. These include the use of chemical inhibitors (such as SKF-525A, etc.); pH or thermal optima determination; and immunochemical inhibition. Evidence based on chemical and immunochemical inhibition data from guinea pigs (Miranda et al., 1991) suggested that SN N-oxide production was carried out largely by FMO in guinea pig liver, whereas DHP formation from SN was catalyzed mainly by liver cytochrome P450.

Hydrolysis of a toxic pyrrolizidine ester to the necine and necic acid moieties is another primary detoxification pathway (Mattocks, 1986). Evidence from chemical inhibition data (Dueker et al., 1992a,b) showed that esterase hydrolysis contributed 92% of moncrotaline metabolism in the guinea pig, but only minimal activity was seen for SN. Moreover, Chung and Buhler (1995) using purified guinea pig hepatic carboxylesterases showed that hepatic carboxylesterase GPH1 hydrolyzed [^{3}H]-labeled jacobine (JB) and SN at rates of 4.5 and 11.5 nmol/min per mg protein, respectively, while carboxylesterase GPL1 had no activity toward PAs.

There are conflicting reports on the correlation of pyrrolic metabolite formation and the susceptibility of animals to PA toxicity. In order to better understand the mechanisms of species differences in PA metabolism and the relative contribution of hepatic microsomal enzymes involved in PA metabolism as well as other possible biotransformation pathway, in vitro hepatic microsomal metabolism and HPLC analytic methods developed by Kedzierski and Buhler (1986) were used in this study to allow for the specific identification and quantitation of important hepatic microsomal metabolites of the PA senecionine. Hamsters and sheep hepatic tissues were used for further study because while both species are resistant to PA toxicity, the mechanisms of resistance appear to be different.

2. Materials and Methods

2.1. Chemicals and antibodies

Senecionine (purified from extracts of *Senecio triangularis*) was provided by Dr J.N. Roitman (USDA-ARS, Western Regional Research Laboratory, Albany, CA). Synthetic standards of DHP and SN N-oxide were prepared as described previously (Kedzierski and Buhler, 1986). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, glutathione (GSH), SKF-525A, methimazole, phenylmethylsulfonyl fluoride (PMSF) and thiourea were obtained from Sigma (St. Louis, MO). Tri-*o*-cresyl phosphate (TOCP) was from Eastman Kodak (Rochester, NY). The antibody for rat liver NADPH cytochrome P450 reductase was raised in rabbits and has been characterized previously (Williams et al., 1989a).

2.2. Animals

Golden Syrian hamsters (100–120 g), Sprague–Dawley rats (330 ~ 350 g), New Zealand white rabbits (2000 ~ 2300 g) and gerbils (22 ~ 25 g) were obtained from the Laboratory Animal Research Center of the Oregon State University. Broiler chickens (2400 ~ 3000 g) and Japanese quail (85 ~ 110 g) from the Poultry Facility of the Department of Animal Science in the Oregon State University were used. All animals were housed in 12 h cycles of light and dark. They were allowed food and tap water ad libitum. Sheep (75 ~ 90 kg) and cattle (500 ~ 600 kg) were raised on the farm of the Department of Animal Science at the Oregon State University.

2.3. Microsome preparation

Four adult male animals (except there were eight gerbils used, with livers from two animals combined for each sample because of small liver size) from each species were killed by cervical dislocation. The livers were immediately removed and homogenized in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 1.15% KCl, and 1.0 mM EDTA in a Potter-Elvehjem homogenizer. Liver tissues of four male sheep and cattle were collected immediately after animals were slaughtered at the Meat Science Laboratory of Oregon State University, and liver samples were homogenized immediately. The homogenate was centrifuged at $9000 \times g$ for 30 min at 4°C and the supernatant was then centrifuged at $105000 \times g$ at 4°C for 60 min. The centrifuged supernatant was saved as the cytosol fraction and the microsomal pellet was washed once by resuspension in the above homogenizing buffer and recentrifuged at $105000 \times g$ as before. The washed microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v) and 0.1 mM EDTA and stored at -80°C until used. All the experiments were conducted within an 8-month time period.

2.4. In vitro metabolism of SN by microsomes

Microsomal incubations were carried out in a mixture containing 0.5 mg microsomal protein with the final concentration of 0.1 M potassium phosphate buffer (pH 7.6), 1.0 mM EDTA, 0.5 mM SN and a NADPH-generating system (10.0 mM glucose-6-phosphate, 1.0 U/ml glucose-6-phosphate dehydrogenase and 1.0 mM $NADP^+$ in a total volume of 0.5 ml. The mixture was equilibrated to 37°C for 5 min and the reaction was initiated by the addition of SN (SN was

dissolved in 0.1 N HCl/NaOH mixture with adjusting pH between 6.5 ~ 6.8. It was sonicated to aid its solubility before use). After 1 h incubation at 37°C with shaking (100 cycle/min), the reaction was terminated by rapid cooling with ice water. The reaction mixture was then centrifuged at $46000 \times g$ for 45 min at 4°C and an aliquot of the supernatant was analyzed by HPLC with a PRP-1 column at $\lambda = 220$ nm wavelength as previously described (Kedzierski and Buhler, 1986). When chemical inhibitors (SKF-525A, methimazole, PMSF, TOCP, all dissolved in DMSO and thiourea in dH_2O) were applied to inhibit the reaction, the mixture was preincubated with inhibitor at 37°C for 20 min before the addition of SN. An equivalent volume of DMSO was used in the control reactions. This protocol was also slightly modified when rabbit anti-rat NADPH cytochrome P450 reductase IgG was used to inhibit the microsomal enzyme activity. The concentrations of antibodies used in hepatic microsome inhibition were 10, 20 and 30 mg/nmol total P450, respectively. The concentration of hepatic microsomes used in incubations were 0.25 and 0.35 nmol of total P450 from hamsters and sheep, respectively. The hepatic microsomes were preincubated with various concentrations of anti-rat NADPH cytochrome P450 reductase antibodies for 20 min at room temperature, after which the buffer system and SN were added as described above. Control incubations with an equivalent amount of preimmune rabbit IgG were also preincubated as described above.

2.5. Effect of GSH in the metabolism of SN

To assay the effect of GSH in the metabolism of SN, hepatic microsomal incubations with different concentration of GSH were carried out. Microsomes from sheep or hamsters were incubated in the presence of 2.0 mM GSH in final incubation mixture, 100 μl cytosol with average GSH concentrations of 4856.3 μg/ml for hamsters and 4731 μg/ml for sheep in the total of 500 μl incubation mixture or 2.0 mM GSH plus 100 μl cytosol in final incubation mixture, respectively. After incubation, the production of DHP and SN N-oxide was assayed by HPLC as described above. For the controls, the microsomal incubations were carried out in the absence of GSH or cytosol fraction.

2.6. Statistics

Statistical analyses were performed using the statistical software base SAS (SAS Institute). Data were assessed for homogeneity of variance using analysis of variance procedure with means compared by Student-Newman–Keuls (SNK) test at $P < 0.05$. Other data were assessed by using two-T-test from Statgragh (Statistical Graphics Corporation).

2.7. Other assays

Microsomal protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Total cytochrome P450 content was estimated using the method of Omura and Sato (1964). The concentration of reduced glutathione in liver cytosol was measured with the method of Hissin and Hilf (1976).

3. Results

3.1. Species differences in PA metabolism

There is no strong correlation between PA pyrrole formation and species susceptibility to PA intoxication among tested species ($P < 0.05$) (Table 1). In some animals such as the hamster, hepatic microsome DHP formation far exceeded the rate of SN N-oxide formation. In contrast, SN N-oxide was the major metabolite in other species such as sheep. There appeared to be a different rate in metabolizing the parent compound. The data from Table 1 show that the remaining parent SN expressed as SNA/SNB (SNA and SNB are expressed as amount of SN after and before microsomal metabolism) varies from 23.1 to 91.5% in hamster and Japanese quail, respectively. The variations among species in DHP and N-oxide formation are mainly dependent on the efficiency of catalytic capability of liver microsomes to particular PA compounds.

Table 1
In vitro metabolism of senecionine by liver microsomes to form DHP and N-oxide in different species

Species (four, male)	DHP (nmol/min per mg)	N-oxide (nmol/min per mg)	Ratio of DHP/N-oxide	SNA/SNB (%)
Susceptible:				
Cattle	$0.23 \pm 0.03^{a,b}$	$0.59 \pm 0.04^{b,c}$	$0.38 \pm 0.02^{a,b}$	61.1 ± 4.3^{c}
Chicken	$0.22 \pm 0.03^{a,b}$	$0.52 \pm 0.05^{a,b}$	0.44 ± 0.06^{b}	84.5 ± 1.2^{d}
Rat	0.85 ± 0.22^{c}	$0.70 \pm 0.20^{b,c}$	1.25 ± 0.08^{c}	69.7 ± 9.3^{c}
Resistant:				
Sheep	0.45 ± 0.07^{b}	1.76 ± 0.08^{e}	0.26 ± 0.05^{a}	27.5 ± 1.5^{a}
Hamster	3.55 ± 0.08^{f}	1.55 ± 0.01^{e}	2.29 ± 0.07^{d}	23.1 ± 1.2^{a}
Rabbit	1.71 ± 0.14^{e}	$0.81 \pm 0.05^{c,d}$	2.11 ± 0.07^{d}	30.0 ± 2.3^{a}
Japanese quail	0.08 ± 0.03^{a}	0.28 ± 0.05^{a}	$0.30 \pm 0.05^{a,b}$	91.5 ± 2.3^{d}
Gerbil	1.34 ± 0.06^{d}	0.97 ± 0.01^{d}	1.39 ± 0.08^{c}	42.2 ± 3.0^{b}
Guinea pig[a]	0.46	0.87	0.53	—

Mean $\pm$ S.E. in the same column followed by different superscripts are different ($P < 0.05$). Hepatic microsomal protein was 0.5 mg in the microsomal mixtures. SNA and SNB are expressed as amount of SN after and before microsomal metabolism.
[a] Data were adapted from Chung and Buhler (1995).

3.2. Effect of chemical inhibitors in DHP and N-oxide formation

In order to examine the relative contribution of cytochrome P450, FMO and esterase in the metabolism of SN by sheep and hamster hepatic microsomes, several chemicals have been used with in vitro microsomal incubation. Table 2 shows that there was an almost complete inhibition in DHP formation with SKF-525A ($P < 0.05$) in both species, whereas SN N-oxidation was only reduced by 7.6% in sheep and 34% in hamsters. With two FMO inhibitors (methimazole and thiourea), the SN N-oxide formation was reduced 80 and 71% in hamsters, respectively, whereas in sheep SN N-oxide production was reduced about 37.7 and 55.8%, respectively. The involvement of hepatic esterase hydrolysis in PA metabolism has also been examined. With TOCP, an esterase inhibitor, DHP formation was reduced dramatically in sheep (90.8%), but with only 23% reduction in hamsters. In contrast, with PMSF, another esterase inhibitor, the DHP production was decreased by 30.8 and 10.4% in sheep and hamsters, respectively. There were no marked decreases in SN N-oxide formation with the two inhibitors in either species (Table 2).

3.3. Effect of anti-NADPH cytochrome P450 IgG in DHP and N-oxide formation

In order to further determine the relative contribution of cytochrome P450 and FMO in the metabolism of PA, an immunoinhibition experiment with rabbit anti-rat NADPH cytochrome P450 reductase IgG was carried out. The results from this experiment show (Fig. 1) that there was a maximum inhibition in DHP production with 67.2 and 82.2% in sheep and hamsters ($P < 0.05$), respectively, whereas only 8.1 and 54.7% of SN N-oxide formation was eliminated in sheep and hamsters, respectively.

3.4. Effect of GSH conjugation in DHP and N-oxide formation

An indirect method for determining the effect of GSH conjugation on DHP and N-oxide formation was carried out by adding the reduced form of GSH to the incubation mixture and then comparing the DHP or N-oxide formation with controls (Figs. 2 and 3). With 2.0 mM GSH in the incubation mixture, the DHP production was reduced 63 and 79% in hamsters and sheep ($P < 0.05$), respectively. In contrast, there was little effect on SN N-oxide formation ($P > 0.05$).

Table 2
Chemical and antibody inhibition of DHP and N-oxide formation from senecionine by sheep and hamster liver microsomes

Inhibitors	DHP (as % of control)		N-oxide (as % of control)	
	Sheep	Hamster	Sheep	Hamster
SKF-525A (0.5 mM)	N.D.	1.8 ± 0.2*	92.4 ± 3.5	65.9 ± 3.0*
Methimazole (0.25 mM)	6.1 ± 0.7*	N.D.	62.3 ± 4.9*	20.1 ± 1.9*
Thiourea (0.25 mM)	30.8 ± 4.5*	14.8 ± 0.8*	44.2 ± 5.1*	29.0 ± 2.2*
PMSF (1.0 mM)	69.2 ± 6.4*	89.6 ± 1.3	74.0 ± 3.7*	105.7 ± 5.2
TOCP (0.1 mM)	9.2 ± 1.5*	76.9 ± 2.5*	72.9 ± 2.4*	84.4 ± 2.2
Anti-P450 reductase IgG[a]	32.7*	17.8*	91.9	45.3*

Results are given as the mean ± S.E. of four animal liver microsomal incubations containing 0.5 and 1.0 mg microsomal protein from hamster and sheep, respectively. The data are expressed as percentage of control. In control, the average production of DHP and SN N-oxide from four hamster samples was 3.95 and 1.54 nmol/min per mg, respectively, the average values of DHP and SN N-oxide from four sheep samples were 0.42 and 1.73 nmol/min per mg, respectively. The data with '*' are significantly different from the control ($P < 0.05$). N.D. means the values are non-detectable.

[a] Each incubation was done in duplicate at a concentration of 30 mg/nmol P450 with preimmune IgG used as control.

4. Discussion

Species differences in the metabolism of PAs and other xenobiotics are well known. Previous studies (Chesney and Allen, 1973; White et al., 1973; Shull et al., 1976; Cheeke, 1998) have indicated that there was a correlation between the pyrrolic metabolites from PA metabolism and susceptibility among various species. Our data (Table 1) in this study showed that some susceptible species such cattle and chickens have a low DHP rather than a high DHP production, while some resistant animals such as hamsters, rabbits and gerbils have a relatively high pyrrole formation instead of low DHP production. These results suggested that there is no strong correlation, at least in the in vitro hepatic microsomal metabolism of SN using an HPLC method (Kedzierski and Buhler, 1986), between the pyrrole or N-oxide production and the resistance or susceptibility among tested species ($P < 0.05$). It is interesting (Table 1) that resistant animals such as sheep, hamsters, and rabbits catalyzed the highest proportion of parent compound, whereas those susceptible species such as cattle, chickens and rats have a low capability toward metabolizing parent compounds. The dose of PA reaching the target site and the catalytic capability of individual species may play an important role in susceptibility to PA toxicosis. As a good example, the hamster is resistant to oral administration of PA (Shull et al., 1976), while this species is susceptible to i.p. injection of PA (White et al., 1973). With the data shown on Table 1, the hamster has the highest capability to catalyze the SN to yield the high rate of pyrrolic metabolites. The combination of these results could be explained such that with i.p injection of PA and high capability to metabolize the PA toward toxic metabolites, the animal may accumulate a large amount of toxic pyrrolic metabolites in a relatively short time period, overwhelming the animal's detoxification system such as phase II conjugation enzymes, resulting in hepatic cytotoxicity. With the oral administration of PA and high rate of pyrrolic metabolite formation, the animal may be capable of rapidly eliminating toxic metabolites over the more prolonged period of PA absorption without causing the cytotoxic effects. Another example of PA resistance is that of Japanese quail. In the case of this species, very little metabolism of the parent alkaloids occurs (Buckmaster et al., 1997), as shown also in the present experiment (Table 1); 91.5% of SN remained as parent compound with Japanese quail microsomes. Thus Japanese quail are resistant to PA because they form the pyrrolic metabolites at very slow rate.

The use of chemicals or antibodies as inhibitors to identify the relative contribution of cytochrome

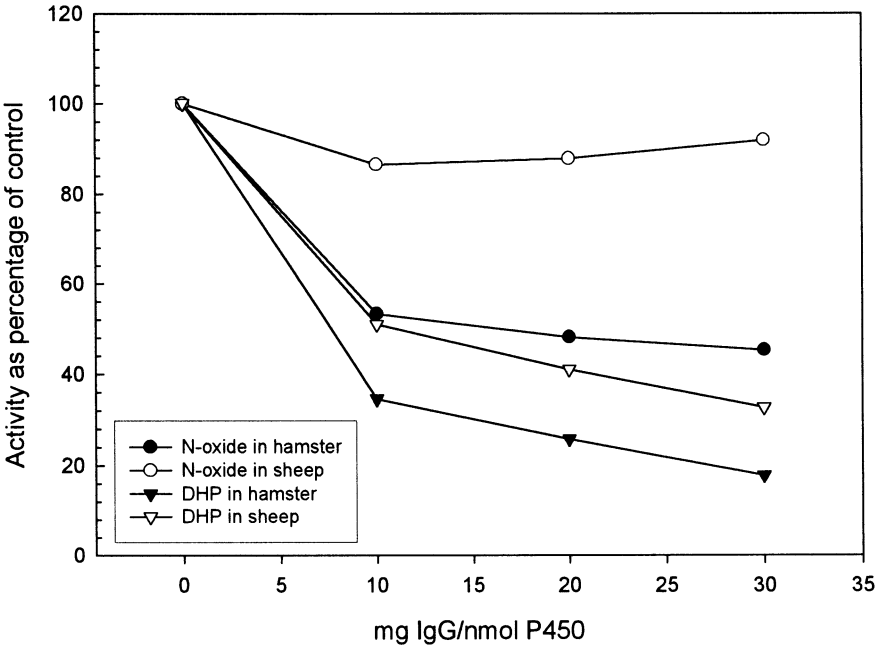

Fig. 1. Immunoinhibition of SN metabolism in sheep and hamster liver microsomes by rabbit anti-rat NADPH cytochrome P450 reductase IgG. Hepatic microsomes from male adult sheep (0.35 nmol of total P450) or hamster (0.25 nmol of total P450) were preincubated with rabbit anti-rat NADPH cytochrome P450 reductase IgG (10, 20 and 30 mg/nmol total P450, respectively), and DHP and SN N-oxide were measured as described in Section 2. The means of experimental data were compared to their respective control group mean. Results were expressed as a percentage of controls (preimmune rabbit IgG was used as control). In control, the average production of DHP and SN N-oxide from sheep samples was 0.78 and 2.57 nmol/min per mg, respectively. The average production of DHP and SN N-oxide from hamster samples was 2.06 and 0.89 nmol/min per mg, respectively.

P450 and FMO has been reported in several studies (Williams et al., 1989a,b; Miranda et al., 1991). We found that 0.5 mM SKF-525A, a traditional P450 inhibitor, almost completely blocked the DHP formation in both sheep and hamsters ($P < 0.05$), while SN N-oxide was only reduced by 7.6% in sheep and 34% in hamsters. This result may suggest that cytochrome P450s mainly contribute to the hepatic metabolism of SN to DHP. The result also is in agreement with the study of Miranda et al. (1991) in which they reported that DHP formation was inhibited completely by SKF-525A, but there was lack of inhibitory effect on SN N-oxide formation in guinea pigs. These results were further supported by immunoinhibition results from Table 2. In summary, we suggest that the conversion of the SN to DHP is catalyzed mainly by cytochrome P450s, and that these hepatic microsomal enzymes have much less contribution toward the N-oxide formation. In an earlier study, Miranda et al. (1991) reported that in the guinea pig DHP formation was largely due to cytochrome P450s. They also suggested that the NADPH-dependent microsomal N-oxidation of SN was carried out mainly by FMO in guinea pig liver, lung and kidney. However, in another study FMO accounted for no more than 20% of the PA N-oxidase activity in Sprague–Dawley rats (Williams et al., 1989b). In the present study two chemical FMO inhibitors were used to determine the involvement of FMO in SN metabolism. The data from Table 2 show that either methimazole or thiourea significantly reduced SN N-oxide production in sheep and hamsters ($P < 0.05$). These results demonstrate that FMO may play a major role in detoxification of PA in both species.

The hepatic esterase hydrolysis has been shown to play an important role in PA metabolism

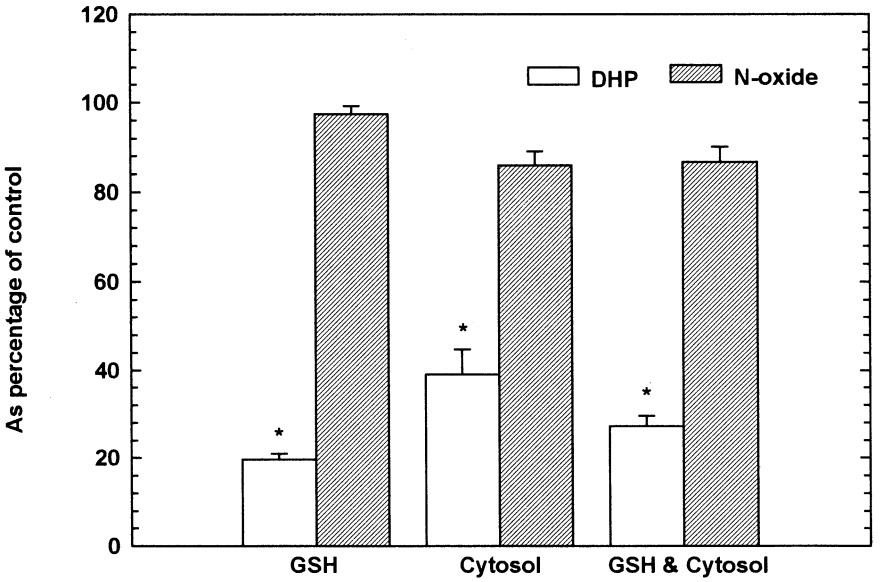

Fig. 2. The DHP and N-oxide formation from SN after incubation with sheep hepatic microsomes in presence of GSH, cytosol, or GSH plus cytosol. Hepatic microsomes (0.35 nmol of total P450) from four male adult sheep were incubated with 0.5 mM senecionine in presence of 2.0 mM GSH, 100 μl cytosol, or 2.0 mM GSH plus 100 μl cytosol, respectively, as described in Section 2. Results were given as the mean $\pm$ S.E. of four animals and expressed as a percentage of controls. In control, the average production of DHP and SN N-oxide from four sheep samples was 0.58 and 2.14 nmol/min per mg, respectively. The bars with '*' are significantly different from the control ($P < 0.05$).

(Dueker et al., 1992a). Previous research has shown that hepatic microsomes hydrolyze monocrotaline into retronecine and monocrotalic acid in guinea pigs (Dueker et al., 1992b), while the rat exhibits no such hydrolytic capabilities (Lame et al., 1991). Chung and Buhler (1995) in a recent study showed that hepatic carboxylesterase GPH1 hydrolyzed [^{3}H]-JB and [^{3}H]-SN at rates of 4.5 and 11.5 nmol/min per mg protein, respectively, while carboxylesterase GPL1 has no activity toward PA. In this study, two chemical esterase inhibitors were used to identify the involvement of hepatic esterases on PA metabolism in sheep and hamsters. The results from Table 2 show that DHP formation was reduced dramatically (90.2%) in sheep and only 23% in the hamsters with TOCP at a concentration of 0.1 mM. Only slight effects were seen in N-oxide formation from SN. This may suggest that sheep hepatic microsomes possess significant hydrolytic activity toward PA. However, when PMSF was used as an inhibitor at the concentration of 1.0 mM, the DHP production was only decreased 30.8 and 10.4% in sheep and hamsters, respectively. The SN N-oxide formation was inhibited about 26% by PMSF in sheep whereas the production of SN N-oxide was unaffected by this inhibitor. PMSF has been presented earlier to be a potent inhibitor for carboxylesterase (Dueker et al., 1992a). It was shown that with 0.1 mM PMSF, 99% of guinea pig hepatic carboxylesterase GPH1 activity was inhibited. The differences in inhibition of esterase hydrolysis to PA metabolism in two species may be due to different specificity of the inhibitors toward the different esterases in sheep and hamster hepatic microsomes. The presence of esterlytic cleavage in PA metabolism may partially explain the sheep's resistance to PA toxicosis. The high rate of esterase hydrolysis may decrease the proportion of PAs that are available for conversion to toxic metabolites, and subsequently, a lower toxicity is exhibited by the PAs. The absence of significant hydrolytic activity in hamsters may correlate to susceptibility in this animal when i.p.

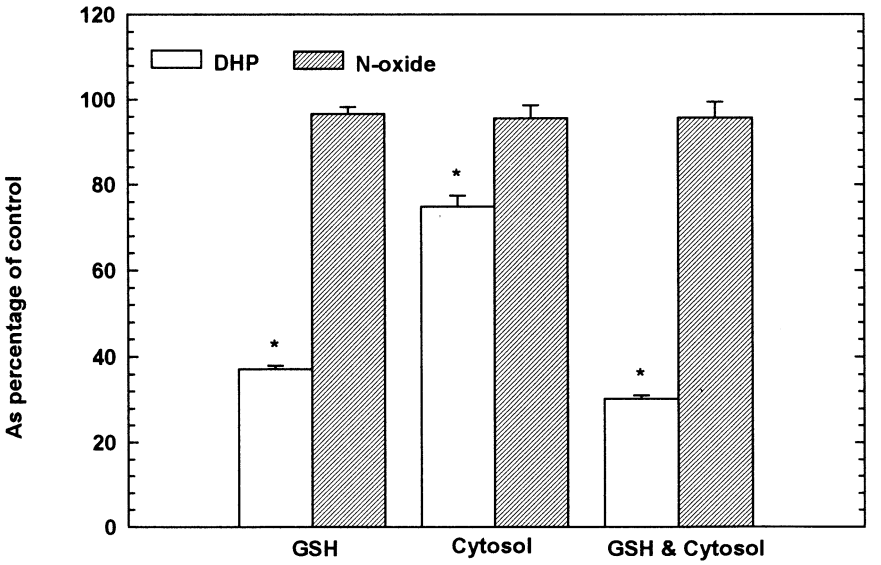

Fig. 3. The DHP and N-oxide formation from SN after incubation with hamster hepatic microsomes in presence of GSH, cytosol, or GSH plus cytosol. Hepatic microsomes (0.25 nmol of total P450) from four male adult hamsters were incubated with 0.5 mM senecionine in presence of 2.0 mM GSH, 100 μl cytosol or 2.0 mM GSH plus 100 μl cytosol, respectively, as described in Section 2. Results were given as the mean $\pm$ S.E. of four animals and expressed as a percentage of controls. In control, the average production of DHP and SN N-oxide from four hamster samples was 5.89 and 2.60 nmol/min per mg, respectively. The bars with '*' are significantly different from the control ($P < 0.05$).

administration of PAs was given (White et al., 1973).

Conjugation with cellular nucleophiles such as GSH has been shown to be an important detoxification process with respect to pyrrolic metabolites (Lame et al., 1990; Reed et al., 1992; Dueker et al., 1994; Yan and Huxtable, 1995). The results from this study (Figs. 2 and 3) show that when 2.0 mM GSH was added in hepatic microsomal incubation mixtures, the DHP formation was significantly reduced in sheep and hamster ($P < 0.05$), while SN N-oxide formation from SN was unaffected ($P > 0.05$). The presence of the cytosol fraction alone without additional GSH in the incubation mixtures also significantly decreased the production of pyrrolic metabolites in sheep and hamster ($P < 0.05$). However, the presence of cytosol fraction with additional GSH compared to the absence of the cytosol fraction with additional GSH did not alter the reduction of pyrrole formation in either species ($P > 0.05$). Similar results have been reported by Dueker et al. (1994). Using purified rat hepatic GST, they found that there was no significant difference between the in vitro rate of reaction of GSH conjugation with JB in the presence of rat GST and the non-enzymatic rate. In contrast, the guinea pig GST catalyzed the formation of JB-GSH at approximately twice the non-enzymatic rate. Furthermore, the levels of DHP and SN N-oxide in the different treatment groups were also compared (Figs. 2 and 3). It appears that the relative GSH concentration present in the incubation mixture could significantly affect the production of DHP ($P < 0.05$) but not SN N-oxide ($P > 0.05$) in both species. Moreover, the earlier study reported that GSH could react with DHP directly in the chemical synthesis of the conjugate (Robertson et al., 1977). This suggests that when an adequate concentration of GSH is present, GSH is capable of directly reacting with pyrrolic metabolites even if there is a low GST activity. Reed et al. (1992) reported that less than 20% of DHP was conjugated with GSH in vitro, whereas 95–100% of dehydrosenecionine was conjugated with GSH in a very short time period. From these results, they

suggested that SN could be catalyzed initially into dehydrosenecionine, which then is either hydrolyzed to DHP or reacts with GSH to form the DHP-GSH conjugate with cleavage of the senecic acid. Thus, our results shown in Figs. 2 and 3 suggest that GSH conjugation may play an important biological role in the detoxification of PA in sheep and hamsters.

In conclusion, our study demonstrates that there is no strong correlation between the production of pyrrolic metabolites and the susceptibility of animals to PA toxicity among the tested species. The species differences in susceptibility to PA toxicity appear to be dependent on the individual species hepatic microsomal enzymes catalytic capability. Chemical and antibody inhibition data suggest that the conversion of SN to DHP is catalyzed mainly by cytochrome P450s, whereas the formation of SN N-oxide is carried out largely by FMO in sheep and hamsters. The involvement of esterase hydrolysis may have a significant impact on DHP formation in sheep with much less effect in hamsters. The indirect evidence shows that there is a high rate of GSH-DHP conjugation in both species. It has been proposed (Wachenheim et al., 1992) that the resistance of sheep to PA toxicosis is due to microbial metabolism of PA in the rumen. In the present study, we worked with a variety of PA resistant species, most of which (hamsters, gerbils, Japanese quail, rabbits) are not ruminants. We believe that in these species, resistance to PA toxicosis is associated with hepatic PA metabolism. Cheeke (1994, 1998) discussed in detail his viewpoints on mechanisms of PA resistance as a reflection of evolutionary history and feeding strategies of herbivores.

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