Species differences in hepatic microsomal enzymes involved in metabolism of the pyrrolizidine alkaloids (PA), including cytochrome P450s, flavin-containing monooxygenases (FMO) and hepatic esterases, and the roles of CYP3A and CYP2B isozymes in bioactivation and detoxication of the PA senecionine (SN) have been investigated. There was no strong relationship between the pyrrolic metabolites and the susceptibility of animals to PA toxicity among tested species. In some resistant species such as hamsters, the rate of PA activation measured by (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) formation far exceeded the rate of senecionine N-oxide formation (detoxication), while other resistant animals such as sheep had a much lower DHP production with relatively high of SN N-oxide formation. Thus, species differences in susceptibility to PA toxicity may be mainly dependent on the species-specific patterns of hepatic microsomal enzymes that metabolize PA. Chemical and antibody inhibition data suggested that the conversion of senecionine to DHP is catalyzed mainly by cytochrome P450s (68-82%), whereas the formation of senecionine
N-oxide is carried out largely by FMO (55-71%). The involvement of hepatic esterase hydrolysis may have a significant impact on DHP formation in sheep but much less effect in hamsters. There was a high rate of glutathione-DHP conjugation in hamster (63%) and sheep (79%) liver microsomal incubation mixtures. Therefore, low rates of pyrrolic metabolite production coupled with glutathione (GSH) conjugation in sheep may explain the apparent resistance of sheep to senecionine, whereas the high rate of GSH-DHP conjugation may be one of the factors in resistance of hamsters to senecionine intoxication.

Immunoinhibition data show that CYP3A played the major role in the conversion of PA to pyrrolic metabolites in both species (over 90% in sheep; 68% in hamsters). This enzyme also contributed 38.8% and 41.3% of SN N-oxidation in sheep and hamster, respectively. In contrast, CYP2B had a limited capacity toward DHP formation in both species (47% in sheep; and 32% in hamster), while this enzyme catalyzed only 24.6% and 35.4% SN N-oxidation in sheep and hamsters, respectively. These results suggested that CYP3A has an important role in bioactivation and detoxication of PA in both sheep and hamsters, whereas CYP2B is less efficient in biotransformation of PA.
Species Difference in Bioactivation and Detoxification of Pyrrolizidine (Senecionine) Alkaloids

by

Jianya Huan

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Completed March 31, 1995
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CONTRIBUTION OF AUTHORS

Drs. Cheeke and Buhler were involved in the design, interpretation, and revision of each manuscript. Dr. Miranda assisted in design, analysis, and interpretation of this study. Dr. Galtier was involved in the interpretation of data and provided experimental materials.
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Species Differences in Bioactivation and Detoxification of Pyrrolizidine (Senecionine) Alkaloid

1. Introduction

1.1 Pyrrolizidine Alkaloids

The pyrrolizidine alkaloids (PAs) are a large class of naturally occurring toxicants that are produced by numerous plant species worldwide. As many as 6000 plant species in the world have been estimated to contain PA (Smith and Culvenor, 1981); these plants have been shown to produce toxic effects in grazing animals, rodents, poultry and humans (Cheeke and Shull, 1985). Most PA-containing plants that have been implicated in livestock poisoning are in genera Senecio, Crotalaria, Heliotropium and Echium.

Chemical structure and metabolism of PAs

The PA constitute a large group of alkaloids containing the pyrrolizidine nucleus. Most PA are esters of hydroxylated 1-methylpyrrolizidine. The amino alcohols are called necine bases, and the acid moieties necic acids (Mattocks, 1986). PA toxicity is influenced by chemical structure. Most cytotoxic PAs are esters of the necine base retronecine and heliotridine (Figure 1.1) which are diestereomers with opposite configuration at C7. For hepatotoxicity, there must be a 1,2-double bond in the pyrrolizidine ring and a branch in an esterified side chain. PA cyclic diesters are the most toxic, noncyclic diesters are of intermediate toxicity and monoesters are the least toxic (Mattocks, 1986). Rates of metabolism of different PAs by hepatic microsomes
in vitro vary widely, depending on the structure and physical properties of the alkaloid (Mattocks and Bird, 1983a). The lipophilic PAs are metabolized at the fastest rates. There are also considerable differences between the proportions of pyrroles and N-oxides formed from PAs with different ester groups, and these appear to be due to the different degrees of steric hindrance caused by the acid moieties at the sites of these metabolic reactions. PAs which are open diesters and able to give the greatest hindrance at the C-8 position yield the highest proportion of N-oxide compared with pyrrole, whereas monoesters and closed diesters, in which movement of the acid moiety is restricted, give relatively more pyrrole.

![Chemical Structures]

Figure 1.1 Typical PA necine bases

The tissue distribution of PAs in animals after the administration of radiolabeled PAs has been studied. Eastman et al. (1982) investigated the distribution of [14C]-labeled senecionine and seneciphylline in lactating mice. At 16 hr. after an i.p. injection of senecionine (82 mg/kg), about 96% of the radioactivity had been recovered in the urine (75%), feces (14%), expired CO₂ (0.21%) and the milk (0.04%). A relatively high level in liver (1.92%) reflects the potential for hepatotoxicity by this
PA, whereas smaller amounts occurred in the blood (0.32%), kidney (0.05%) and lung (0.03%). Some of the radioactivity in liver from senecionine or seneciphylline is bound to proteins, RNA and DNA. The main metabolic routes (Figure 1.2) for PA that have been established in laboratory animals are dehydrogenation to pyrrolic derivatives, conversion to N-oxides and hydrolysis (Mattocks, 1986).

![Chemical structures](image)

**Figure 1.2 Major pathways for the metabolism of the PA senecionine**
(adapted from Winter and Segall, 1989)
Dehydrogenation of PA to yield pyrrolic derivatives is one of the major pathways of PA metabolism. The parent PA are nontoxic, but cytochrome P450-containing monooxygenases in the liver dehydrogenate the 1,2-dehydropyrrolizidine ring, forming highly reactive pyrrolic metabolites that are unstable and either undergo rapid hydrolysis to yield the most stable necine pyrrole [(±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP)] or covalently bind to DNA or other tissue nucleophiles (Mattocks and White, 1971). DHP is also a strong electrophile capable of alkylating tissue constituents. The cytochrome P450 system (localized in the endoplasmic reticulum) functions to oxidize the alkaloids to the more water soluble derivatives which can be more readily excreted, but the chemical instability of these intermediates leads to the formation of a more lipophilic and highly toxic metabolite. The formation of N-oxide from PA metabolism is catalyzed both by cytochrome P450s and flavin-containing monooxygenase (FMO) that are also localized in the endoplasmic reticulum of primarily the liver. The N-oxides once formed are more hydrophilic than their parent alkaloids and readily excreted. In addition, the N-oxides of PAs are not converted to pyrrolic metabolites by microsomal enzymes (Mattocks, 1971).

**Toxicology of pyrrolizidine alkaloids**

The PA are metabolized to a proximal toxin pyrrole in hepatic parenchymal cells and this toxin causes necrosis in those cells. Some pyrroles escape to damage the endothelium of the hepatic vein leading to cell proliferation and veno-occlusion. Some of the reactive metabolite may proceed further by way of the bloodstream to damage other organs such as lung (Estep et al., 1991). Most of the other tissue lesions and
signs of toxicity, including mortality, can be related to impaired liver function. General characteristic signs of PA toxicosis include swelling of hepatocytes, centrilobular necrosis, megalocytosis of the parenchymal cells, karyomegaly, progressive fibrosis, bile duct proliferation, veno-occlusion and loss of hepatic metabolic functions. Other common clinical signs of PA toxicity are rough unkempt appearance, diarrhea, prolapsed rectum, ascites, edema of tissues of the digestive tract, lassitude and dullness, photosensitization reactions and abnormal behavior (Cheeke and Shull, 1985). A number of PAs such as fulvine, heliotrine, jacobine, monocrotaline, retrorsine and senecionine have been shown to be mutagens in bacterial test systems (Mattocks, 1986). The N-oxides of heliotrine, lasiocarpine and monocrotaline are also mutagens (Clark, 1960). Heliotrine-induced chromosome damage has been observed in human lymphocytes (Kraus et al., 1985). Lasiocarpine and its pyrrolic metabolites can exert antimitotic activity which results in megalocytosis (Samuel and Jago, 1975).

The toxicity of PAs in animals can be influenced by nutritional status and various chemical treatments. Rats fed a low protein diet are more susceptible to the toxicity of *Senecio jacobaea* (SJ) PA whereas high protein diets afford some protection (Cheeke and Garman, 1974). The acute hepatotoxicity of retrorsine was decreased more than three-fold in rats fed only sucrose for 4 days prior to injection of the PA and then returned to a normal diet (Mattocks, 1972). Incorporation of high levels of copper in the diet of rats and chicken increased the hepatotoxicity of SJ (Miranda et al., 1981; Huan et al., 1992). Cysteine pretreatment doubles the liver glutathione level and halves the toxicity of retrorsine whereas 2-chloroethanol reduces the liver GSH concentrations to one-fourth of control values and doubles the PA toxicity (White, 1976). Elevated
zinc levels in the diet also lowers the ability of rat liver microsomes to convert PAs to pyrrolic metabolites and gives some protection against hepatotoxicity (Miranda et al., 1982). Pretreatment of rats with phenobarbital caused an increase in susceptibility to PAs under conditions where the rate of metabolism in rats was normally low, but phenobarbital was protective where metabolism was normally fast, even though pyrrole production was increased (Mattocks, 1972). Male rats pretreated with SKF-525A had reduced susceptibility to retrorsine toxicity, whereas the animals pretreated with tri-orthocresyl phosphate, an inhibitor of esterase, had a large proportion of such alkaloids being converted to pyrrolic metabolites in vivo (Mattocks, 1981).

Species differences in PA toxicity

Hepatotoxic effects of PA have been demonstrated in several animal species but major differences exist in species susceptibility to PA poisoning. Among animal species, sheep, goats, guinea pigs, rabbits and hamsters are highly resistant to PA whereas rats, mice, cattle and horses are highly susceptible (Table 1.1) (Cheeke and Shull, 1985). Chickens and turkeys are susceptible to PA toxicosis (Cheeke and Pierson-Goeger, 1983) but Japanese quail are resistant to a tansy ragwort cumulative intake of several thousand percent of body weight (Buckmaster et al., 1977). Although numerous studies have shown species differences in susceptibility to PA toxicity, the major determinants of susceptibility or resistance to PA toxicity are still not well understood. Several factors that have been suggested to account for the species differences to PA toxicity include: 1) poor absorption of PAs from digestion system; 2)
rumenal biodegradation of PAs; 3) differences in rate of hepatic formation of pyrroles; 4) conjugation and excretion of PAs metabolites.

Table 1.1 Characterization of animal species by susceptibility to pyrrolizidine alkaloid toxicity in vitro hepatic pyrrole production rate

<table>
<thead>
<tr>
<th>Species</th>
<th>Susceptibility to PA toxicosis</th>
<th>In vitro pyrrole production rate</th>
<th>Lethal dose of SJ* (as % of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>high</td>
<td>high</td>
<td>3.6</td>
</tr>
<tr>
<td>Horse</td>
<td>high</td>
<td>high</td>
<td>7.3</td>
</tr>
<tr>
<td>Sheep</td>
<td>low</td>
<td>low</td>
<td>302</td>
</tr>
<tr>
<td>Goat</td>
<td>low</td>
<td>?</td>
<td>205</td>
</tr>
<tr>
<td>Hamster</td>
<td>low</td>
<td>high</td>
<td>338</td>
</tr>
<tr>
<td>Rat</td>
<td>high</td>
<td>high</td>
<td>21</td>
</tr>
<tr>
<td>Mouse</td>
<td>intermediate</td>
<td>high</td>
<td>?</td>
</tr>
<tr>
<td>Rabbit</td>
<td>low</td>
<td>high</td>
<td>113</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>low</td>
<td>low</td>
<td>119</td>
</tr>
<tr>
<td>Gerbil</td>
<td>low</td>
<td>?</td>
<td>3640</td>
</tr>
<tr>
<td>Chicken</td>
<td>high</td>
<td>low</td>
<td>39</td>
</tr>
<tr>
<td>Japanese quail</td>
<td>low</td>
<td>low</td>
<td>2450</td>
</tr>
</tbody>
</table>

Adapted from Cheeke and Shull, 1985. SJ: *Senecio jacobaea*

Although hepatic metabolism probably explains the majority of species differences in the resistance to PA, ruminal metabolism of PA may be relevant. A number of experiments supported the evidence of rumen detoxification of PA. In those studies, the alkaloids were primarily monoester or open di-ester PA from *Heliotropium*
*europaeum* and *Echium plantagineum*. The early studies of Lanigan (1972; 1976) showed that feeding methanogens, which increase rumen hydrogen, increased the detoxification of heliotrope PA by reduction to 1-methyl derivatives. Moreover, he also isolated a rumen microbe, *Peptococcus heliotrinreductans*, which metabolized heliotrope and echium PA to non-toxic 1-methyl derivatives. A recent *in vitro* study (Wachenheim et al., 1992) has shown that the rates of *Senecio jacobaea* (SJ) biotransformation were more rapid in sheep and goat rumen contents compared to cattle rumen contents, and it was concluded that rumen bacteria in animals with larger numbers of PA-biotransforming bacteria are responsible for the increase rates of PA-biotransformation and protection from PA toxicosis. In contrast, Swick et al. (1983) incubated SJ in sheep rumen fluid from both *Senecio*- and non-*Senecio* -fed sheep and did not find a change in the PA spectrum before and after incubation, as determined by HPLC and mass spectrometry analysis. Incubation of SJ with sheep rumen fluid did not alter its toxicity to rats in the studies of Swick et al. (1983) and Shull et al. (1976). Therefore, a complete understanding of the mechanism of resistance remains to be elucidated.

Several research results showed that susceptible species such as rats, cattle and horses had a high rate of pyrrole production whereas PA-resistant animals such as sheep and Japanese quail had low pyrrole production (White et al., 1973; Shull et al., 1976; Winter et al., 1988). In some cases, however, as for example with rabbits, the *in vitro* pyrrole production rate is high (Shull et al., 1976; Pierson et al., 1977) but the animals are very resistant to PA toxicity. Their resistance might be due to an ability to rapidly detoxify pyrroles, a suggestion supported by a high rate of urinary excretion of
PA metabolites in the rabbit (Swick et al., 1982b). It appears that the major determinant of susceptibility to PA toxicity may be the extent to which the different species convert PAs to pyrrolic metabolites through the action of hepatic microsomal enzymes. Winter et al. (1988), using a GC/MS analytic method, reported that the guinea pig, a resistant animal to PA toxicity, has a high rate of DHP production from senecionine in vitro metabolism whereas rats, a susceptible species, have relatively low rates of DHP production. They suggested that the resistance of the guinea pig to PA poisoning may be due to resistance of the animal to the toxic effects of pyrroles rather than from low metabolic pyrrole formation. In other studies, it has been shown that in Sprague-Dawley rats and guinea pigs, the P450 isozymes and FMO enzymes play different roles in the bioactivation and detoxication of PAs. In Sprague-Dawley rats, the liver microsomal conversion of senecionine to DHP is catalyzed primarily by the male-dominant CYP3A subfamily (Williams et al., 1989a), while the N-oxidation of senecionine is metabolized mainly by CYP2C11. In guinea pigs, however, the orthologues of CYP2B1 and CYP3A participate in DHP formation by hepatic microsomes (Miranda et al., 1992). These results have been confirmed in the study of Chung and Buhler (1994) in which CYP3A played an important role in senecionine bioactivation in rats but not in guinea pigs. Furthermore, FMO, another hepatic microsomal enzyme system, has been demonstrated to be a major catalyst for the NADPH-dependent microsomal N-oxidation of senecionine in guinea pigs (Miranda et al., 1991), but it accounted for no more than 20% of the PA N-oxidase activity of Sprague-Dawley rat liver microsomes (Williams et al., 1989b). There are marked
species differences in esterase hydrolysis, another principal pathway of hepatic PA metabolism. Dueker et al. (1992b) reported that guinea pig hepatic microsomes were capable of hydrolyzing monocrotaline into retronecine and monocrotalic acid, while the rat exhibited no such hydrolytic capabilities (Lame et al., 1991). Purified carboxylesterase (GPH1) from guinea pig hydrolyzed \([^{3}\text{H}]\)-JB and \([^{3}\text{H}]\)-SN at rates of 4.5 and 11.5 nmol/min/mg protein, respectively, while carboxylesterase GPL1 had no activity toward PA (Chung and Buhler, 1995). Thus, the eventual biological effects of the PAs in a given animal species may depend upon a balance of these various hepatic metabolic pathways.

Conjugation of pyrrolic metabolites is of interest because this could be a major pathway for detoxication in liver and other tissues after PA are bioactivated. (R)-DHP has been found to react chemically \textit{in vitro} under acidic conditions with cysteine and GSH to form DHP-cysteine and DHP-GSH adducts, probably by reaction at C-7 hydroxyl (Robertson et al., 1977). DHP-GSH conjugates have also been identified in the bile of monocrotaline-treated-rats (Lame et al., 1990; Mattocks et al., 1991). Reed et al. (1992) reported that there was formation of DHP-GSH conjugate following incubation of senecionine with rat liver microsomes \textit{in vitro} in the presence of GSH. Other conjugates such as N-acetylcysteine-conjugated pyrrole have also been identified in rat urine following administration of monocrotaline and senecionine (Estep et al., 1990). However, the DHP-GSH conjugate is pneumotoxic (Huxtable et al., 1990). In addition to its direct toxicity, the GSH-pyrrole conjugate could also serve as a transport
vehicle to other organs where toxicity is elicited after cysteine-conjugate β-lyase-dependent bioactivation or release of the GSH (Vamvakas and Anders, 1991)

1.2 Cytochrome P450 in Xenobiotic Metabolism

Cytochrome P450, the carbon monoxide-binding pigment of microsomes, is a superfamily of hemoproteins that are the terminal oxidases of the monooxygenase system. It is named from the wavelength of the carbon monoxide derivative of the reduced cytochrome which has an absorption maximum at 450 nm (Sato and Omura, 1961). Now, the Nomenclature Committee of the International Union of Biochemistry prefers the term “heme-thiolate protein” instead of “cytochrome” for P450 (Palmer and Reedijk, 1991). At the last official count (Nelson et al., 1993), the P450 superfamily was comprised of 221 genes found in 31 eukaryotes (including 11 mammalian and 3 plant species) and 11 prokaryotes. The mammalian families are divided further into subfamilies, which are comprised of those forms that are at least 55% related by their deduced amino acid sequences. Only 3 P450 gene families (i.e., CYP1, CYP2, and CYP3) of the 36 families identified are currently thought to be responsible for the majority of hepatic xenobiotic metabolism (Wrighton and Stevens, 1992). The molecular weights of all cytochrome P450 enzymes characterized fall in range of 45-60 kd (Guengerich, 1990).

Cytochrome P450 enzymes are important in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biogenic amines (Omura et al., 1993). Many of these enzymes also metabolize a wide range of foreign chemicals including
drugs, environmental pollutants, natural plant products, and alcohols. The metabolism of foreign chemicals can frequently produce toxic metabolites, some of which have been implicated as agents that may be responsible for tumor initiation, promotion, and tumor progression (Guengerich, 1992). The mechanism of P450 function has not been established unequivocally; in general, the initial step consists of the binding of substrate to oxidized P450 followed by a one-electron reduction catalyzed by NADPH cytochrome P450 reductase to form a reduced cytochrome-substrate complex. This complex can interact with carbon monoxide to form the CO-complex, which gives rise to the well-known difference spectrum with a peak at 450 nm. Then, the cytochrome-substrate complex interacts with molecular oxygen to form a ternary oxygenated complex. This ternary complex accepts a second electron from either NADH through cytochrome b5 reductase or NADPH through cytochrome P450 reductase, resulting in the further formation of one or more poorly understood complexes. One of these, however, is probably the equivalent of the peroxide anion derivative of the substrate-bound hemoprotein. Under some circumstances this complex may break down to yield hydrogen peroxide and the substrate-oxidized cytochrome complex. After the transfer of one atom of oxygen to the substrate and the other to form water, dismutation reactions occur which lead to the formation of the hydroxylated product, water and the oxidized cytochrome (Figure 1.3) (Hodgson and Levi, 1994).
Figure 1.3 Generalized scheme showing sequence of events for cytochrome P450 monooxygenations (adapted from Hodgson and Levi, 1994)
The hepatic microsomal P450s catalyze the metabolism of an amazingly large number of lipophilic endogenous and exogenous compounds. The P450s actually catalyze only a limited number of reactions including carbon hydroxylation, heteroatom oxygenation, dealkylation, and epoxidation, as reviewed by Guengerich (1990). In addition, the P450s can catalyze reductive reactions, and with certain compounds, metabolites can be formed that inactivate the P450s (Guengerich, 1990). The metabolites formed from the majority of substrates by the P450s are more hydrophilic than the parent compound and are thus more readily excreted from the body. In some cases, however, electrophilic metabolites are formed, which can react with cellular nucleophiles resulting in toxic or carcinogenic insult (Nebert et al, 1987; Guengerich, 1988).

The studies with the human hepatic P450s have reinforced the notion that drug metabolism and the regulation of the expression of the drug-metabolizing enzymes are often quite different in humans compared to that found in experimental animals. That is, it has become apparent that significant differences exist between humans and experimental species with respect to the catalytic activities and regulation of the expression of the hepatic drug-metabolizing P450s (Wrighton and Stevens, 1992). In general terms, three basic differences were identified. First, different P450s in the various species may perform, with high specificity, the same metabolic function. Second, the regulation of expression of related forms of P450 can vary among the various species, including humans. The classic case of this is the sexual bimorphism observed for the metabolism of many compounds by rats that is not observed with the other species, including humans (Ryan and Levin, 1990). In addition, strain differences
were observed in rats and mice with respect to their hepatic drug-metabolizing capabilities (Wrighton and Stevens, 1992). The third general difference is that, through gene duplication, species-specific P450s have evolved (Gonzalez, 1988; Nebert, 1987). Therefore, only through a complete understanding of these differences can the best extrapolation to man be performed.

To elucidate the catalytic functions and regulation of expression of the hepatic P450s, particularly in CYP1, CYP2 and CYP3, involved in xenobiotic metabolism will help us to understand polymorphism in drug disposition and risk assessment in human.

The CYP1 family

The CYP1 family is described as the most straightforward P450 gene family to be studied thus far in that only two genes (CYP1A1 and CYP1A2) to date have been identified among species (Nelson et al., 1993). They were previously recognized as P448 since both CYP1A1 and 1A2 show peaks at 448nm in carbon monoxide difference spectra. These cytochromal proteins share high similarity in their primary structures as well as chemical and physical properties, and are induced by treatment of animals with 3-methylcholanthrene (3-MC), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other polycyclic aromatic hydrocarbons (PAH). They also show clear differences in their catalytic properties.

CYP1A1 accounts for approximately 90% of the aryl hydrocarbon hydroxylase (AHH) activity in both humans and rodents previously exposed to PAH (Song et al., 1985), an activity resulting in the transformation of these compounds to their ultimate pathological forms. As such, it was hypothesized that a higher levels of inducible AHH
activity, or perhaps a more active enzyme, would result in an increased risk of PAH-induced cancer. CYP1A1 is constitutively expressed at extremely low levels (Fujino et al., 1982) in the liver and in extrahepatic tissues such as lung, and known to be strongly induced by cigarette smoking in extrahepatic tissues such as placenta, lung, and peripheral blood cells and in cultured cells (Fujino et al., 1982; McLemore et al., 1989). The induction is mediated by a receptor protein, termed the aryl hydrocarbon receptor (AhR) (Poland et al., 1974). CYP1A1 catalyzes several oxidations of polycyclic hydrocarbons, forming both epoxides and phenols, and the N-oxygenation of some arylamines (McManus, 1990).

CYP1A2, originally purified by Disterlath et al. (1985) as the phenacetin O-deethylase, was found to be constitutively expressed in human liver (Wrighton et al., 1986). It has a primary sequence 80% identical with that of CYP1A1 (Quattrochi, 1985). This enzyme is the major one involved in the oxidation of a variety of arylamines and arylacetamides to their respective hydroxylamines and hydroxamic acids (Guengerich and Shimada, 1991). Other substrates for human CYP1A2 include phenacetin (O-deethylation), 7-ethoxyresorufin (O-deethylation), caffeine (3-demethylation) and aflatoxin B₁ (9a-hydroxylation). It also appears to be involved in the oxidation of acetaminophen to an iminoquinone (Guengerich and Shimada, 1991). Higher 1A2 levels and associated enzyme activities have been positively correlated with increased cigarette smoking (Kale and Tang, 1991). In vivo studies using caffeine metabolism as a marker for 1A2 found that other factors influencing 1A2 levels include enzyme induction by physical exercise and the ingestion of cruciferous vegetables and charbroiled meats (Vistisen et al., 1991). It has been hypothesized that those
individuals with higher levels of CYP1A2 are at increased risk for several cancers because of the increased capacity for bioactivation of carcinogens (Kadlubar et al., 1990). However, there are some evidences linking CYP1A2 induction by dietary factors to a lower incidence of breast cancer (Michnovicz and Braldow, 1990, 1991). The proposed mechanism for the decrease in breast cancer incidence is that increased 1A2-catalyzed estrogen 2-hydroxylation decreases the levels of endogenous estrogens, which have been associated with metastatic breast cancer. Unlike CYP1A1, CYP1A2 does not appear to be expressed in extrahepatic tissues, so that larger, more comprehensive studies are needed in the area of chemoprevention by the alteration of endogenous metabolic pathways by dietary changes (Wrighton and Stevens, 1992).

The CYP2 family

This family is known to be the largest and most diverse of the P450 families, consisting of ten subfamilies (2A-2K) (Nelson et al., 1993). Some of the P450 subfamilies (2B1, 2B2, 2C1, 2C2, 2C4, 2C6, 2C16, 2H1 and 2H2) are induced by phenobarbital (PB) and other “foreign” compounds (Hansen et al., 1989; Omura et al., 1993). The sex specific expression seen in this family has been considered to be apparently caused by sex hormones (Kamataki et al., 1983) and more directly by the secretion pattern of growth hormone (Morgan et al., 1985). Because of broad aspects and diversity of this superfamily, in this brief review it is really not possible to deal with each subfamily and all isozymes in this family. Here only a few of them will be reviewed.
The CYP2B subfamily

The major forms of P450 induced by phenobarbital comprise the CYP2B gene subfamily. In rat CYP2B1 and CYP2B2 genes are coordinately regulated by PB and the mRNAs exhibit 97% nucleotide similarity (Giachelli et al., 1989). Low levels of CYP2B2 but not CYP2B1 are constitutively expressed in the rat liver (Omiecinski, 1985). The human gene referred to as 2B6, which codes for a P450 76% similar to rat 2B1, was determined by their deduced amino acid sequences (Yamano et al., 1989). The regulation of this subfamily has not been clear yet. The studies showed that heme, the prosthetic group of P450, may function as a positive regulatory factor of CYP2B1/B2 transcription (Ravishankar and Padmanaban, 1985). The induction of CYP2B1/B2 mRNAs by PB in rat liver is significantly inhibited by the simultaneous administration of inhibitors of heme biosynthesis such as CoCl₂. Furthermore, dexamethasone at pharmacological doses has been reported to increase CYP2B1/B2 mRNA without changing transcription rates (Simmons et al., 1987). These enzymes show a loose substrate specificity compared with all other cytochrome P450 forms and can catalyze the oxidation of a wide variety of foreign substrates (Omura, 1993).

Benzphetamine is N-demethylated by the isozyme from rats (Kamataki et al., 1976) and rabbits (Haugen et al., 1975) at very efficient rates. 7-ethoxycoumarin has been reported to be specifically metabolized by the CYP2B isozyme (Lubet et al., 1985). Biphenyl is hydroxylated at the 4-position by this enzyme (Johnson et al., 1979). It has been suggested that CYP2B may play an important role in the bioactivation and detoxification of pyrrolizidine alkaloids (Miranda et al., 1992; Chung and Buhler, 1994). The data showed that PB treatment increased DHP formation from
senecionine by 274% in male guinea pig (Miranda et al., 1992). This result has been further confirmed by Chung and Buhler (1995) that CYP2B played the most important role (over 70%) in bioactivation of senecionine in both untreated or PB-treated guinea pig. CYP2B also exhibited its ability to contribute along with FMO to detoxify senecionine in both untreated (34%) and PB-treated (40%) guinea pigs.

The CYP2C subfamily

This subfamily shares a considerable amount of the total P450 in normal liver of experimental animals and humans. CYP2Cs are constitutively expressed in liver and are not known to be affected by inducers (Gonzalez, 1992). However, they exhibit a high degree of interindividual variability in expression in human liver specimens (Furuya et al., 1991). Expression of this subfamily has not been detected in extrahepatic tissues. Several rat CYP2C isozymes, but not human CYP2C isozymes, are expressed in a sex-specific manner (Gonzalez, 1992). The members of this subfamily metabolize several clinically important drugs including S-warfarin, tolbutamide and S-mephenytoin. CYP2C11, which is expressed sex-specifically in the liver of male rats, showed diverse catalytic activity for different types of carcinogens. The 2-aminoimidazoles derived from food pyrolysates (Yamazoe et al., 1988), aflatoxin B1 (Ishii et al., 1986) and acetaminophen (Harvison et al., 1988) have been shown to be activated by CYP2C11 using the purified preparation. Further, Williams et al. (1989a) reported that CYP2C11 is also involved in metabolism of PA. Their studies showed that the N-oxidation of senecionine in male Sprague-Dawley rats is catalyzed primarily by CYP2C11 and CYP3A2. The data from Chung and Buhler (1994) showed that spironolactone (SPL), a
known inducer of CYP3A, was subjected to increased DHP formation from senecionine and decreased the senecionine N-oxidation by 49% in male Sprague-Dawley rats. They suggested that the reduction of N-oxidation was due to the loss (69%) of CYP2C11 activity by SPL treatment in male rats.

The CYP2E subfamily

The CYP2E subfamily is toxicologically a very important metabolic system. CYP2Es are among the best conserved P450 forms in the CYP2 family. A single CYP2E gene exists in human, rats and two very similar genes in rabbits (Nelson et al., 1993). The catalytic activities of this enzyme across species are quite similar, and it may be expected that substrates found for the animal enzymes will also be substrates for the human enzymes (Guengerich and Shimada, 1991). CYP2E1 has been shown to play a role in metabolism of ketone bodies in the propandiol pathway of gluconeogenesis (Koop and Casazza, 1985). CYP2E1 is also a major xenobiotic metabolizing enzyme capable of metabolically activating numerous low-molecular-weight toxins and carcinogens including ethanol, diethyl ether, pyridine, halothane, benzene carbon tetrachloride, ethyl carbamate and N,N-dimethylnitrosamine (Guengerich and Shimada, 1991). Normally, the hepatic CYP2E1 is constitutively expressed in humans and can be induced by the presence of inducers, many of which are CYP2E1 substrates such as ethanol (Koop and Tierney, 1990). P450 2E protein is also known to be elevated by physiologic states that result in the accumulation of acetone or ketones such as fasting, diabetes, and obesity as well as after ingestion of the anti-tuberculosis drug, isoniazid (Wrighton and Stevens, 1992).
The CYP3 family

The glucocorticoid-inducible CYP3A subfamily, the only member of the CYP3 family, is the most abundantly expressed in human liver (Guengerich and Shimada, 1991). Multiple forms of CYP3A proteins are detected in liver of rats (Nagata et al., 1990) and humans (Beaune et al., 1986). All forms share high immunochemical similarity and catalyze testosterone 6β-hydroxylation (Nagata et al., 1990). CYP3As were shown to be responsible for the metabolism of a wide array of clinically and toxicologically important agents, and to be inducible by steroids, macrolide antibiotics, imidazole antifungals, and PB (Gonzalez, 1988). CYP3A1/A2 have been identified in the rat (Gonzalez et al., 1986). It was found that rat CYP3A1 is induced by treatment with glucocorticoids and is not expressed in untreated adult animals. The studies also showed that CYP3A2 is not induced by glucocorticoids and is expressed in only adult male rats, but both CYP3A1 and CYP3A2 are induced by PB (Gonzalez et al., 1986). The most abundant form in human liver is CYP3A4. CYP3A3 is very similar to CYP3A4, having only eleven amino acid substitutions and no known differences in catalytic activity (Gonzalez, 1992). Form CYP3A5, which shows similar substrate specificity to CYP3A4 is expressed only in a minority of individuals (Cholerton et al., 1992). Human CYP3A type of P450s are responsible for the metabolism of a number of clinically important drugs including nifedipine and other dihydropyridines, ciclosporin, erythromycin, the benzodiazepines midazolam and triazolam, and quinidine (Cholerton et al., 1992). This is also applicable to carcinogen metabolism. In humans, this subfamily is reported to catalyze various types of bulky carcinogens such
as 1-nitropyrene and 6-aminochrysens (Omura et al., 1993), and also is highly capable of activating mycotoxins including aflatoxin B₁ (Shimada et al., 1989). The endogenous compounds metabolized by the human 3A subfamily are various steroids including the 6β-hydroxylation of testosterone, progesterone, cortisol, androstenedione and the 2- and 4- hydroxylation of estradiol (Wrighton and Stevens, 1992). CYP3A7 is the major form of P450 expressed in human fetal liver and this single P450 comprises 30 to 50% of the total fetal P450 (Wrighton and VandenBranden, 1989). In contrast, rodents do not express fetal liver CYP3A P450s (Gonzalez, 1992). The role of CYP3A7 in the human fetus is not entirely clear but it may be involved in physiologically significant steroid metabolism (Wrighton and VandenBranden, 1989). The CYP3As have been suggested to play a very important role in PA metabolism in experimental animals. The PA metabolism by P450 enzymes vary among animal species. The studies in guinea pigs showed that CYP2B1 is more important than CYP3A in the bioactivation of senecionine (Miranda et al., 1992), whereas the data from the Sprague-Dawley rats study indicated that the CYP3A2 play a important role in bioactivation of senecionine (Williams et al., 1989a). In vitro studies using human CYP3A4 showed that this enzyme is the major enzyme catalyzing both of the bioactivation and detoxication of senecionine (Miranda et al., 1991a). Further, the studies in both rats and guinea pigs showed that the CYP3A play an important role in senecionine bioactivation in rats, but not in guinea pigs (Chung and Buhler, 1994).
Species differences

As the cytochrome P450s exhibit different substrate specificities, and stereo- and regio-selectivities (Guengerich, 1990; Omura et al., 1993; Oguri and Yamada, 1994), the biological fate of a chemical depends not only on the total levels of the cytochrome P450s, but also on the relative abundance of the various isozymes. The nature of the cytochrome P450 population will therefore determine if a chemical will be detoxicated and excreted, or will be activated to interact covalently with cellular components, thus giving rise to toxicity, mutations, and malignancy (Ioannides and Parke, 1990). The well-known differences in the susceptibility of different animal species to chemical and natural plant toxicity may simply represent the presence of different cytochrome P450 isozymes. An appreciation of the substrate specificities of the component cytochrome P450s, and their relative abundance in laboratory animals and man, will no doubt make extrapolation of animal toxicity data to humans a more precise exercise.

Structural Aspects

The mouse, rat and rabbit are the three most extensively studied animal species with respect to the mixed-function oxidase system. Very similar forms of the cytochrome P450s are induced in different species by the various inducing agents. A comparison of the partial cDNA sequence of rat CYP1A1 and CYP1A2 with MC inducible mouse CYP1A1 and CYP1A2 revealed high homology between rat CYP1A1 and mouse CYP1A1 DNA, and between rat CYP1A2 and mouse CYP1A2 (Kimura et al., 1984). Furthermore, rabbit CYP1A1 mRNA is orthologous to rat CYP1A2 and
mouse CYP1A2 (Kimura et al., 1984). Rat CYP2B1 and rabbit CYP2B4 have 80% similarity in their primary structures (Black et al., 1982), and amino acid and DNA sequence analysis revealed homology between the rat CYP2B1, CYP2B2, and rabbit CYP2B4 (Ryan et al., 1986).

Immunological Aspects

The advent of monoclonal antibodies has allowed the rapid identification and quantification of antigenically similar cytochrome P450 proteins, such as cytochromes belonging to the same gene family. Thomas et al. (1984), using monospecific polyclonal antibodies, detected CYP1A1 at low levels in the liver of rats and at relatively higher levels in rabbit and guinea pig livers, whereas no protein was detectable in the mouse and hamster. One protein related to CYP1A2 was present in all animals (Thomas et al., 1984). 7-ethoxyresorufin O-deethylase (EROD) activity was significantly higher in hamster than in rat (Hyde et al., 1987), but as EROD activity measures mostly CYP1A1, and to a much lesser extent CYP1A2, it can not be decided which CYP1A1 isozyme is responsible for the high activity. The possibility that EROD activity and the monoclonal antibodies determine different but immunologically related proteins cannot be excluded. Using the same monoclonal antibodies, both cytochrome CYP1A isozymes were induced by 3-methylcholanthrene in rat, hamster, guinea pig, rabbit, and “responsive” mice, but not in “nonresponsive” mice (Thomas et al., 1984). Similarly, isosafrole induced CYP1A2 in all animals, but CYP1A1 in rat, rabbit, and hamster only.
Extent of Induction

Species differences also occur in the extent of induction; 3-methylcholanthrene induced CYP1A1 markedly in rat and responsive mice, and to a lesser extent in hamster, rat, and guinea pig. Using two monoclonals against CYP1A1, similar findings have been reported by Cheng et. al. (1984). When EROD activity was determined, induction was much higher in rat than hamster (Astrom et al., 1986). CYP1A2 was most extensively induced in the responsive mouse, followed by rat, guinea pig, hamster, rabbit, and nonresponsive mouse (Thomas et al., 1984). Induction of CYP1A1 by isosafrole was highest in rat, followed by guinea pig.

A marked species difference is seen in the induction of CYP2E1 which in the rabbit, but not in the rat, is induced by imidazole (Koop et al., 1985).

Substrate Criteria

A similar comparison of hepatic CYP1A1 enzymes activity, using the EROD assay and undertaken with various inducing agents, including 3MC, 2-aminoanthracene, and Aroclor 1254, showed that hepatic CYP1A1 activity is poorly induced in hamster, but markedly induced in the rat. A single i.p. administration of Aroclor 1254 caused a 150-fold increase in EROD activity in rat liver but only a 3-fold increase in hamster (Iwasaki et al., 1986; Hyde et al., 1987). Similarly, liver AHH activity was not induced in the hamster by 3MC (Thorgeirsson et al., 1979). These findings indicate that the CYP1A1 isozymes induced in hamster share common epitopes with the rat proteins but have different affinities toward certain substrates, such as 7-ethoxyresorufin and benzo(a)pyrene (Ioannides and Parke, 1990). Furthermore, the
guinea pig, similar to hamster, is relatively refractory to AHH induction by 3MC (Abe and Watanabe, 1982); TCDD treatment produced a 500-fold increase in EROD activity in rat hepatocytes but only a 6-fold increase in the guinea pig (Wroblewski and Olson, 1985). Moreover, TCDD induced its own CYP1A1 mediated metabolism in rats but not in guinea pig. The poor response of the guinea pigs to CYP1A1 inducers may be at least partly responsible for its well-known resistance to 2-acetylaminofluorene (2-AAF) hepatocarcinogenesis, since the activating N-hydroxylation is catalyzed by CYP1A1 (Astrom and DePierre, 1985), which in the rat is self-induced by 2-AAF (Astrom et al., 1983).

Age

In animal species including man, it has been established, using a limited number of “standard” mixed-function oxidase substrates, that activities are very low at birth but increase rapidly to adult levels (Kato et al., 1964) and then subsequently decline in geriatric animals (Schmucker and Wang, 1981). Even in these initial studies it was observed that not all substrates followed the same developmental pattern, indicating that cytochrome P450 isozymes may not develop coordinately; for example, the CYP1A1 mediated 2-hydroxylation of biphenyl was present in neonatal rat and rabbit livers but was undetectable during adulthood (Basu et al., 1971; Atlas et al., 1977). These observations have been confirmed recently using the more specific EROD assay, showing that CYP1A1 activity is relatively high in the neonatal rat, reaching a maximum 2 weeks after birth, and then decreasing with age. In contrast, the PB-cytochrome P450-mediated N-demethylation of benzphetamine was low at birth and
increase with age (Lum et al., 1985). Similar to CYP1A1 isozyme, the alcohol-inducible cytochrome CYP2E1 appears to be higher at around 3 weeks after birth, constituting a quarter of the total cytochrome P450 content, and then declines rapidly (Thomas et al., 1987). In other studies, employing Western blotting analyses, this protein was undetectable in the newborn rat but increased rapidly within a week and remained at this level for at least 12 weeks. In contrast, the same workers have shown that CYP2B1 was detectable in the neonate and increased with age, reaching a maximum at 4 weeks of age.

Nutrition

Nutrition and diet may modulate the levels of cytochrome P-450 isozymes, resulting in changes in the metabolic fate of xenobiotics (Parke and Ioannides 1981). The diet-mediated effects may be due to chemicals inherent in the diet, to contaminants or food additives, or to compounds generated during the process of cooking. Some types of food have high contents of natural xenobiotics which are potent inducers of the mixed-function oxidase system, such as safrole, flavones, and indoles. Rats maintained on a diet containing cruciferous vegetables, such as sprouts and cabbage, exhibited both high levels of intestinal mixed-function oxidase activity (Pantuck et al., 1976) and an altered response to chemical carcinogens (Wattenberg 1975; Wattenberg et al., 1976). Similar effects were observed in humans maintained on such diets (Pantuck et al., 1979). These effects are probably due to the high content of indoles in crucifers. Indeed, indole-3-acetonitrile, indole-3-carbinol, and 3,3'-di-indolylmethanes, present in cruciferous vegetables, have been identified as naturally occurring inducers of hepatic
and intestinal AHH, and of other enzyme activities (Branfield and Bjeldanes 1984); these compounds selectively induced the cytochromes CYP1A1 (Shertzer 1982). Liver and intestinal EROD activities have been induced in rats by feeding cabbage-containing diets (McDanell et al., 1987); indole-3-carbinol was a potent inducer of liver activity and to a lesser extent of intestinal activity. Other indoles also induced liver activity, although weakly, while ascorbigen (a product of indole-3-carbinol and ascorbic acid) stimulated only intestinal activity.

Rats maintained on these indole-supplemented diets showed protection against PAH-induced carcinogenicity (Wattenberg et al., 1976), possibly because the indoles may act as competitive substrates preventing activation of the carcinogen, or they may be even more potent inducers of the phase II detoxication pathways. Pretreatment of animals with indole-3-carbinol decreased the covalent binding of benzo(a)pyrene metabolites to DNA and protein (Shertzer 1982), which accords with both hypotheses. The observed stimulation of hepatic glutathione-S-transferase activities by these brassica-supplemented diets (Aspry and Bjeldanes 1983) makes an increase in the detoxication pathways the more likely mechanism of the protective effect against chemical carcinogenesis.

1.3 Objectives

The objectives of this study were to examine the species differences in the hepatic microsomal enzymes including cytochrome P450s, FMO and hepatic esterases in metabolism of the PA as well as the role of cytochrome P450 isozymes in the bioactivation and detoxication of PA. Although the early studies in both in vivo and in

vitro PA metabolism (Cheeke and Shull, 1985; White et al., 1973; Chesney and Allen; 1973; Shull et al., 1976) have shown that the susceptible animals such as rat have a high rate of pyrrole formation, while the resistant animals such as guinea pig has a low rate of pyrrole production, the methodology that they used were nonspecific colorimetric assays which fail to identify the pyrrole and other PA metabolites specifically. In this study an HPLC method, a much more sensitive and specific method developed by Kedzierski and Buhler (1986a), was used to allow for the specific identification and quantitation of important metabolites of the PA senecionine from hepatic microsomal incubations. Sheep are very resistant to the PA toxicosis, while hamsters are resistant to orally dosed PA (Cheeke and Pierson-Goeger, 1983), but susceptible to i.p. injected PAs (White et al., 1973). Sheep have a low pyrrole production rate and relatively high N-oxide production (Shull et al., 1976; Winter et al., 1988), while hamsters have a relatively high pyrrole formation and low N-oxide production (White et al., 1973). We chose sheep and hamsters as animal models for resistant species which may have different pathways of PA metabolism.

The over-all goals of this research were to understand the roles that hepatic microsomal enzymes play in metabolism of PA among the different species. The specific goals of this research are:

1. To screen for species differences in the metabolism of the senecionine among cattle, sheep, rabbits, chickens, Japanese quail, hamsters and gerbils.

2. To identify the role of CYP3A and CYP2B involved in the bioactivation and detoxication of the senecionine in sheep and hamsters.
3. To determine the function of cytochrome P450s and FMO in the metabolism of PA in sheep and hamsters.

4. To investigate the involvement of sheep and hamsters hepatic esterase in PA metabolism.
2. Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids

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2.1 Abstract

Species differences in pyrrolic metabolites and senecionine (SN) N-oxide formation among eight animal species varying in susceptibility to PA intoxication were measured in vitro with liver microsomal incubation. The results suggested that there is not a strong relationship between the production of pyrrolic metabolites and susceptibility of animals to PA toxicity. The rate of PA activation measured by (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) formation far exceeded the rate of senecionine N-oxide formation (detoxification) (DHP/N-oxide=2.29) in hamster, a resistant species. In contrast, senecionine 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 antibody inhibition data suggested that the conversion of senecionine to DHP is catalyzed mainly by cytochrome P450s (68-82%), whereas the formation of senecionine N-oxide is carried out largely by FMO (55-71%). There 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 apparent resistance of sheep to senecionine, whereas the high rate of GSH-DHP conjugation may be one of the factors explaining the resistance of hamsters to senecionine intoxication.
2.2 Introduction

The pyrrolizidine alkaloids (PA) 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 and Shull, 1985). 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 SN require metabolic activation to produce toxicity. Dehydrogenation of PA to yield unstable dehydropyrrolizidine alkaloids (PA pyrroles) by hepatic monoxygenases 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 thought to represent detoxication mechanism (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 and Shull, 1985). The species differences in resistance or susceptibility appear to be primarily a consequence of differences in hepatic PA metabolism. In general, those susceptible species such as rat, mice, cattle and horse have a high rate of pyrrole production, whereas PA-resistant animals such as sheep,
guinea pig, 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 and Shull, 1985). 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), other 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 formation 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 detoxication pathway (Mattocks, 1986). Evidence from chemical inhibition data (Dueker et al., 1992a, b) showed that esterase hydrolysis contributed 92% of monocrotaline metabolism in the guinea pig, but only minimal activity was seen for SN. Moreover, a recent study (Chung and Buhler, 1994) with purified guinea pig hepatic carboxylesterases showed that hepatic carboxylesterase GPH1 hydrolyzed [3H]-JB and [3H]-SN at rates of 4.5 and 11.5 nmol/min/mg protein, respectively, while carboxylesterase GPL1 had no activity toward PAs.

There are some conflicting reports on the correlation of pyrrolic metabolite formation and the susceptibility of animals to PA toxicity. In order to better understand the mechanism 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 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.
2.3 Materials and Methods

Chemicals and antibodies

Senecionine (SN) (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 senecionine N-oxide were prepared as described previously (Kedzierski and Buhler, 1986). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP+, glutathione, SKF-525A, methimazole, phenylmethylsulfonyl fluoride (PMSF) and thiourea were obtained from Sigma Chemical Co. (St. Louis, MO). Tri-o-cresyl phosphate (TOCP) was from Eastman Kodak Co. (Rochester, NY). Antibody for rat liver NADPH cytochrome P450 reductase was purified from rabbits as described previously (Williams et al, 1989).

Microsome Preparation

Four adult male animals from each species including hamster, rat, guinea pig, gerbil, rabbit, chicken and Japanese quail were killed by cervical dislocation. Tissue was obtained from sheep and cattle slaughtered in a commercial meat processing facility. The livers were immediately removed and homogenized in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 1.15% KCl, 1.0 mM EDTA in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9,000g for 30 min at 4 °C, and the supernatant was then centrifuged at 105,000g 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 105,000g 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.

**In vitro metabolism of SN by microsomes**

Microsomal incubations were carried out in a mixture containing 0.25-0.70 mg microsome protein, 0.1 M potassium phosphate buffer (pH 7.6), 1.0 mM EDTA, 0.5 mM senecionine and a NADPH-generating system (10.0 mM glucose-6-phosphate, 1.0 unit/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. After one hour incubation at 37 °C with shaking (100 cycle/min), the reaction was terminated by rapid cooling on ice water. The reaction mixture was then centrifuged at 46,000g for 45 min at 4 °C, and an aliquot of the supernatant was analyzed by HPLC with PRP-1 column at λ=220 nm wavelength as previously described (Kedzierski and Buhler, 1986). When chemical inhibitors (SKF-525A, methimazole, thiourea, PMSF and TOCP) were applied to inhibit the reaction, the mixture was preincubated with inhibitor at 37 °C for 20 min before the addition of SN. This protocol was also slightly modified when anti-NADPH cytochrome P450 reductase IgG was used to inhibit the microsomal enzyme activity. The antibody (0-30 mg/nmol P450) and microsome (0.25-0.5 mg) were preincubated for 20 min at room temperature, after which buffer system and SN were added as described above.
Effect of GSH in the SN metabolism

The standard microsomal incubation system was carried out as described above with individual treatment: 2.0 mM GSH in final concentration; 100 μl cytosol with a average concentration at 20.2 mg protein /ml in total of 500 μl final incubation mixture; and 2.0 mM GSH plus 100 μl cytosol, respectively.

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).

2.4 Results

Species differences in PA metabolism

A screen of the HPLC method of analysis showed that there is no strong correlation between pyrrole formation and species susceptibility among tested species (Table 2.1). In some species such as hamster, the DHP formation far exceeded the rate of SN N-oxide formation in liver microsomes. 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 2.1. show that the remaining parent SN expressed as SNA/SNB (SNA and SNB are expressed as amount of SN after and before microsomeal meatbolism) varies from 23.1% to 91.5% in hamster and
Japanese quail, respectively. It seems that the variation among species on DHP and N-oxide formation are mainly dependent on the efficiency of catalytic capability of liver microsomes to particular PA compound.

Table 2.1  
**In vitro** metabolism of senecionine by liver microsome to form DHP and N-oxide in the different species

<table>
<thead>
<tr>
<th>Species</th>
<th>DHP nmol/min/mg</th>
<th>N-oxide nmol/min/mg</th>
<th>Ratio of DHP/N-oxide</th>
<th>SNA/SNB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Susceptible:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>0.23±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.59±0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.38±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>61.1±4.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.22±0.03&lt;sup&gt;a6&lt;/sup&gt;</td>
<td>0.52±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.44±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84.5±1.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat</td>
<td>0.85±0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.70±0.20&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.25±0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>69.7±9.3&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Resistant:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>0.45±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.76±0.08&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.26±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.5±1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hamster</td>
<td>3.55±0.08&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.55±0.01&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.29±0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23.1±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.71±0.14&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.81±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.11±0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30.0±2.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Japanese quail</td>
<td>0.08±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>91.5±2.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gerbil</td>
<td>1.34±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.97±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.39±0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42.2±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Guinea pig*</td>
<td>0.46</td>
<td>0.87</td>
<td>0.53</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean ± SE 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.

* Data were adapted from Chung and Buhler (1994).

**Effect of chemical inhibitors in DHP and N-oxide formation**

In order to examine the relative contribution of cytochrome P450, FMO and carboxylesterase in the metabolism of SN by sheep and hamster liver microsomes, several chemical inhibitors and anti-NADPH cytochrome P450 reductase IgG have been used with **in vitro** microsomal incubation. The data from Table 2.2 showed that there was an almost complete inhibition in DHP formation with SKF-525A in both species, whereas SN N-oxidation was only reduced by 7.6% in sheep and 34% in
hamster. Further evidence from anti-NADPH cytochrome P450 reductase IgG inhibition data (Table 2) showed that DHP formation was reduced by 68.3% in sheep and 82.2% in hamster; in contrast, SN N-oxide production was inhibited by only 8.1% in sheep and 54.7% in hamster. With two FMO inhibitors (methimazole and thiourea), the SN N-oxide formation was reduced 80 and 71% in hamster, respectively, whereas in sheep SN N-oxide production was reduced about 38.3 and 55.8%, respectively. The involvement of hepatic esterase hydrolysis in PA metabolism has also been examined. With TOCP, an esterase inhibitor, the DHP formation was reduced dramatically in sheep (90.8%), but with only 23% reduction in hamster. In contrast, with PMSF, another esterase inhibitor, the DHP production was decreased by 30.8% and 10.4% in sheep and hamster, respectively. There were no marked decreases in SN N-oxide formation with the two inhibitors in either species (Table 2.2).

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>DHP (as % of control) sheep</th>
<th>DHP (as % of control) hamster</th>
<th>N-oxide (as % of control) sheep</th>
<th>N-oxide (as % of control) hamster</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF-525A (0.5mM)</td>
<td>ND</td>
<td>1.8±0.2</td>
<td>92.4±3.5</td>
<td>65.9±3.0</td>
</tr>
<tr>
<td>Methimazole (0.25mM)</td>
<td>6.1±0.7</td>
<td>ND</td>
<td>62.3±4.9</td>
<td>20.1±1.9</td>
</tr>
<tr>
<td>Thiourea (0.25mM)</td>
<td>30.8±4.5</td>
<td>14.8±0.8</td>
<td>44.2±5.1</td>
<td>29.0±2.2</td>
</tr>
<tr>
<td>PMSF (1.0mM)</td>
<td>69.2±6.4</td>
<td>89.6±1.3</td>
<td>74.0±3.7</td>
<td>105.7±5.2</td>
</tr>
<tr>
<td>TOCP (0.1mM)</td>
<td>9.2±1.5</td>
<td>76.9±2.5</td>
<td>72.9±2.4</td>
<td>84.4±2.2</td>
</tr>
<tr>
<td>Anti-P450 reductase IgG*</td>
<td>32.7</td>
<td>17.8</td>
<td>91.9</td>
<td>45.3</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of control (no inhibitor) and are given as the mean ± SE of four animal liver microsomal incubations containing 0.5 mg microsomal protein. ND means the values are non-detectable.

*Each incubation was done in duplicate at a concentration of 30 mg/nmol P450 with preimmune IgG used as control.
Effect of anti-NADPH cytochrome P450 IgG in DHP and N-oxide formation

In order to further determine the contribution of cytochrome P450 and FMO in the metabolism of PA, immunoinhibition with rabbit anti-rat NADPH cytochrome P450 reductase IgG was carried out. The results from this experiment showed (Figure 2.1, 2.2) that there was a maximum inhibition in DHP production with 67.2% and 82.2% in sheep and hamster, respectively, whereas only 8.1% and 54.7% of SN N-oxide formation was eliminated in sheep and hamster, respectively.

Figure 2.1  Inhibition of SN metabolism in sheep liver microsomes by rabbit anti-rat NADPH cytochrome P450 reductase IgG
Effect of glutathione 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 (Figure 2.3, 2.4). 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 ($P > 0.05$).
Figure 2.3  The DHP and N-oxide formation from SN after incubation with sheep hepatic microsomes in presence of GSH, cytosol, and GSH plus cytosol
Figure 2.4 The DHP and N-oxide formation from SN after incubation with hamster hepatic microsomes in presence of GSH, cytosol, and GSH plus cytosol

2.5 Discussion

Species differences in the metabolism of PAs and other xenobiotic are well known. The previous studies (White et al., 1973; Chesney and Allen, 1973; Shull et al., 1976; Cheeke and Shull, 1985) have proposed that there was a correlation between the pyrrolic metabolites from PA metabolism and susceptibility among various species. Our data (Table 2.1) in this study showed that some susceptible species such as cattle and chickens have a low DHP rather than a high DHP production, while some of resistant animals such as hamster, rabbits and gerbil 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 developed by Kedzierski and Buhler (1986), between the pyrrole or N-oxide production and the resistance or susceptibility among tested species. It is interesting (Table 2.1) that resistant animals such as sheep, hamster, and rabbits catalyzed the highest proportion of parent compound, whereas those susceptible species such as cattle, chicken, and rat have a low capability toward metabolizing parent compounds. This indicates that the efficiency of 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 2.1, the hamster has the highest capability to catalyze the SN to yield the high rate of pyrrolic metabolites. The combination of these phenomena 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 detoxication 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 during the certain time period without causing the cytotoxic effects.

The use of chemicals or antibodies as inhibitors to identify the relative contribution of cytochrome P450 and FMO has been reported in several studies (Williams et al., 1989a, 1989b; 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 hamster, while SN N-oxide was only reduced by 7.6% in sheep and 34% in hamster. This result may suggest that cytochrome P450s mainly contribute to the SN metabolism. 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. Furthermore, the more specific inhibitor, an anti-rat NADPH cytochrome P450 reductase IgG, was used to further confirm the contribution of cytochrome P450s. The results (Table 2.2) showed that when anti-P450 reductase IgG was used at a concentration of 30 mg/nmol P450, DHP production was reduced 68.3% in sheep and 82.2% in hamster; in contrast, SN N-oxide formation was inhibited by 8.1% in sheep and 54.7% in hamster. 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 is 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 this study two chemical FMO inhibitors were used to determine the involvement of FMO in SN metabolism. The data from Table 2.2 show that at a concentration of 0.25 mM, methimazole reduced SN N-oxide production by 37.7% in sheep and 79.9% in hamster, while thiourea competitively inhibited 55.8% of N-oxidation from SN metabolism in sheep
and 71% in hamster. These results demonstrate that FMO play a major role in detoxication of PA in both species.

The hepatic esterase hydrolysis has been shown to play an important role in PA metabolism (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 \([^3H]\)-JB and \([^3H]\)-SN at rates of 4.5 and 11.5 nmol/min/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 hamster. The results from Table 2.2 show that DHP formation has been reduced dramatically (90.2%) in sheep and only 23% in the hamster 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 hamster, respectively, while there is no significant reduction in SN N-oxide formation in both species. PMSF has been demonstrated 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 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. administration of PAs was given (White et al., 1973).

Conjugation with cellular nucleophiles such as GSH has been shown to be an important detoxication process with respect to pyrrolic metabolites (Lame et al., 1990; Reed et al., 1992; Dueker et al., 1994). The results from this study (Figure 2.3; 2.4) show that when 2.0 mM GSH was added in hepatic microsomal incubation mixtures, the DHP formation was reduced 79% and 63% in sheep and hamster (P < 0.01), respectively, while SN N-oxide formation from SN was unaffected (P > 0.05). The presence of the cytosol fraction which is rich in glutathione-S-transferase (GST) without GSH in the incubation mixtures did significantly decrease the production of pyrrolic metabolites in sheep (P < 0.01) and hamster (P < 0.05). But the presence of cytosol fraction with GSH compared to the absence of the cytosol fraction with aditonal 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 guinea pig and rat hepatic glutathione-S-transferase, they found that there was no significant difference between the in vitro rate of reaction of GSH conjugation with jacobine in the presence of rat GST and the non-enzymatic rate. The guinea pig GST
catalyzed the formation of JB-GSH at approximately twice the non-enzymatic rate. 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 under physiological conditions, whereas 95-100% of dehydrosenecionine were conjugated with GSH in a very short time period under in vitro physiological conditions. 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 at Figure 2.3 and 2.4 suggest that GSH conjugation may play an important biological role in the detoxication of PA in sheep and hamsters.

In conclusion, our study demonstrates that there is no strong correlation between the pyrrolic metabolites and the susceptibility of animals to PA toxicity among the tested species. The species differences in susceptibility to PA toxicity are mainly 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 hamster. The involvement of esterase hydrolysis may have a significant impact on DHP formation in sheep with much less
effect in hamster. The indirect evidence shows that there is a high rate of GSH-DHP conjugation in both species.

2.6 References


3. The role of CYP3A and CYP2B in bioactivation and detoxication of pyrrolizidine (Senecionine) alkaloids in sheep and hamsters

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3.1 Abstract

Sheep and hamsters are resistant to pyrrolizidine alkaloid (PA) toxicity. Our results show that the rate of senecionine (SN) activation measured by (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) formation far exceeded the rate of SN N-oxide formation (detoxication) in hamsters. In contrast, SN N-oxide was the major metabolite in sheep with much lower DHP production. Using anti-sheep CYP3A and CYP2B IgG, the roles of CYP3A and CYP2B in bioactivation and detoxication of PA were investigated in vitro with sheep and hamster hepatic microsomes. Immunoinhibition data show that CYP3A plays the major role in the conversion of PA to pyrrolic metabolites in both species (over 90% in sheep; 68% in hamster). This enzyme also contributes 38.8% and 41.3% of SN N-oxidation in sheep and hamsters, respectively. In contrast, CYP2B has a limited capacity toward DHP formation in both species (47% in sheep; and 32% in hamster), while the enzyme catalyzed only 24.6% and 35.4% SN N-oxidation in sheep and hamster, respectively. These results suggest that CYP3A has an important role in bioactivation and detoxication of PA in both species, whereas CYP2B is less efficient in biotransformation of PA.

3.2 Introduction

The pyrrolizidine alkaloids (PA) are found in a wide variety of plants throughout the world (Cheeke and Shull, 1985). The hepatotoxicity of the alkaloids has been recognized in animals and humans (Mattocks, 1986). PAs are primarily metabolized by hepatic monooxygenases located in the endoplasmic reticulum to yield
the pyrrolic metabolites and PA N-oxides (Mattocks, 1986). 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-pyrroliazine (DHP). The DHP is less reactive than the dehydroalkaloids but is capable of alkylating cellular macromolecules (Mattocks and Bird, 1983). These pyrrole derivatives are thought to be responsible for the toxic and carcinogenic effects (Mattocks and Bird, 1983). N-oxidation of PAs and 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 thought to represent detoxication pathways (Mattocks, 1986).

Species differences in susceptibility of animals to PA toxicity have been reported in numerous studies (White et al., 1973; Chesney and Allen, 1973; Shull et al., 1976; Swick et al., 1982; Winter et al., 1988). Among animal species, sheep, guinea pigs, gerbils, rabbits, hamsters and Japanese quail are highly resistant to PA whereas rats, cattle, horses and chickens are highly susceptible (Cheeke and Shull, 1985). The species differences in resistance or susceptibility appear to be primarily a consequence of differences in hepatic PA metabolism. Previous studies showed that there are marked species differences in bioactivation and detoxication of PAs. Williams et al. (1989a) reported that DHP formation from the PA senecionine (SN) was primarily biotransformed by CYP3A2 in rats, while SN N-oxidation was catalyzed mainly by CYP2C11. Moreover, using an immunoinhibition approach with anti-human hepatic CYP3A4 antibodies, Miranda et al. (1991) showed that CYP3A4 was the
major enzyme involved in bioactivation and detoxication of SN in human liver. The orthologues of CYP2B1 and CYP3A have been suggested to play an important role in bioactivation of SN in guinea pigs (Miranda et al., 1992). A recent study has indicated that the CYP3A has an important biotransformation role in SN metabolism in vitro in rats but not in guinea pigs (Chung and Buhler, 1994). In contrast, CYP2B has been suggested to play the most important role in bioactivation of SN in guinea pigs, while CYP2C and CYP3A had little influence in bioactivation of SN (Chung and Buhler, 1995). Because of this marked species differences in PA metabolism, identification of the specific enzymes that may be involved in biotransformation of PAs to pyrrolic metabolites may be an important step in understanding the potential hazards of PAs in animals and humans.

Sheep are very resistant to PA toxicity, while hamsters are resistant to oral doses of PA (Cheeke and Pierson-Goeger, 1983), but susceptible to i.p. administration of PAs (White et al., 1973). Sheep have a low pyrrole production rate and relatively high N-oxide formation (Shull et al., 1976; Winter et al., 1988), while the hamsters have a relatively high pyrrole production and low N-oxide formation (White et al., 1973). In this study, we chose sheep and hamsters as animal models to characterize the roles of CYP3A and CYP2B in bioactivation and detoxication of SN. We present evidence that the CYP3A plays an important role in both bioactivation and detoxication of SN in sheep and hamsters, whereas the CYP2B is less efficient in this biotransformation.
3.3 Materials and Methods

Chemicals and antibodies

The SN (purified from extracts of *Senecio triangularis*) was obtained from 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\(^+\) and preimmune rabbit IgG were obtained from Sigma Chemical Co. (St. Louis, MO). Most chemicals and materials used in SDS-PAGE and Western blotting were purchased from Bio-Rad Laboratories (Richmond, CA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and ECL detection agents used in Western blotting were obtained from Amersham (Arlington Heights, IL).

Microsome preparation

Four adult male hamsters were killed by cervical dislocation. Sheep livers were obtained from wethers slaughtered by conventional techniques in a commercial slaughter plant. The livers were immediately removed and homogenized in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) containing 1.15% KCl, 1.0 mM EDTA in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 9,000g for 30 min at 4 °C, and the supernatant was then centrifuged at 105,000g at 4 °C for 60 min. The centrifuged supernatant was saved as cytosol fraction, and the microsomal pellet was washed once by resuspension in the above homogenizing buffer and recentrifuged at
105,000 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.

**In vitro metabolism of SN by microsomes**

Microsomal incubations were carried out in a mixture containing 0.25-0.50 mg microsomal protein, 0.1 M potassium phosphate buffer (pH 7.6), 1.0 mM EDTA, 0.5 mM senecionine and a NADPH-generating system (10.0 mM glucose-6-phosphate, 1.0 unit/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. After one hour incubation at 37 °C with shaking (100 cycle/min), the reaction was terminated by rapid cooling on ice water. The reaction mixture was then centrifuged at 46,000g for 45 min at 4 °C, and an aliquot of the supernatant was analyzed by HPLC with PRP-1 column at λ=220 nm wavelength as previously described (Kedzierski and Buhler, 1986).

**Immunoinhibition**

Antibodies for sheep hepatic CYP3A and CYP2B were purified from rabbits as described previously (Pineau et al., 1990; Kaddour et al., 1992). The concentrations of antibodies used in hepatic microsomes inhibition were from 0.5 to 25 mg/nmol P450. The concentration of hepatic microsomes used in incubations were 0.25 and 0.35 nmol of P450 from hamsters and sheep, respectively. The antibodies and microsomes were
preincubated for 20 min at room temperature, after which the buffer system and SN were added as described above.

**Western blot analysis**

Hepatic microsomes protein (40 µg/line) was subjected to 7.5% SDS-PAGE according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred to a nitrocellulose membrane at 4 °C overnight using a Bio-Rad Transfer Blot apparatus (30 mAmp) by the method of Towbin et al. (1992). Nonspecific sites were blocked by incubation of nitrocellulose membranes with 2% BSA in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 1 hour at room temperature. Anti-sheep CYP3A and CYP2B antibodies with dilution of 1:100 were incubated with the membranes for 2 hours at room temperature. After three washes (10 min) with TBST, secondary antibody (HRP-conjugated goat anti rabbit IgG, at 1:2500) was added and incubated for 1 hour at room temperature. Then, the membranes were washed (10 min) three times with TBST. Specific binding of anti-CYP3A and CYP2B antibodies was detected by Amersham ECL detection system.

**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).
3.4 Results

Metabolism of senecionine by hepatic microsomes

The rates of DHP and SN N-oxide formation from sheep and hamster hepatic microsomal incubations are presented in Table 3.1. The mean levels of DHP produced from hamster liver microsomes far exceeded the levels of SN N-oxide formation (DHP/N-oxide=2.29), while SN N-oxide was the major metabolite in sheep with much lower DHP production (DHP/N-oxide=0.26).

Table 3.1  In vitro metabolism of senecionine by hepatic microsomes to form DHP and N-oxide in sheep and hamsters.

<table>
<thead>
<tr>
<th>Species</th>
<th>DHP</th>
<th>N-oxide</th>
<th>Ratio of DHP/N-oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>0.45±0.07^a</td>
<td>1.76±0.08^a</td>
<td>0.26±0.05^a</td>
</tr>
<tr>
<td>Hamster</td>
<td>3.55±0.08^b</td>
<td>1.55±0.01^a</td>
<td>2.29±0.07^b</td>
</tr>
</tbody>
</table>

Mean ± SE in the same column followed by different superscripts are different (P<0.05). Hepatic microsomal protein was 0.5 mg in the incubation mixtures.

Immunochemical detection of CYP3A and CYP2B

Immunochemical detection was carried out to identify if there was cross-reaction with CYP3A and CYP2B from hepatic microsomes of hamster and sheep. Anti-sheep CYP3A and CYP2B IgG were hybridized to show there were two bands around MW 52-54 kd, indicating the antibody does cross-react with CYP3A and CYP2B isozymes in both species (Figure 3.1).
**Figure 3.1** Immunochemical detection of CYP3A and CYP2B in hepatic microsomes in sheep and hamsters. 40 μg hepatic microsomal protein per lane were loaded to SDS-PAGE, and detected by anti-sheep CYP3A and CYP2B antibodies as described in Materials and Methods.

**Immunoinhibition of microsomal senecionine metabolism**

The role of CYP3A and CYP2B in SN metabolism was assessed by incubating hepatic microsomes with increasing amounts of anti-sheep CYP3A and CYP2B IgG. At an antibody concentration of 5.0 mg/nmol of P450, DHP and SN N-oxide formation were inhibited 90.5% and 22.7% (Figure 3.2), respectively, by anti-sheep CYP3A antibody in sheep. When the concentration of anti-CYP3A IgG was increased to 20 mg/nmol P450, DHP production was almost completely inhibited (96.5%), while SN N-oxide was also inhibited 38.8% in sheep (Figure 3.3). In contrast, when the concentration of anti-sheep CYP2B IgG was gradually increased to 20 mg/nmol P450, DHP and SN N-oxide formation were inhibited 47.4% and 24.6% in sheep (Figure
3.4), respectively. Similar phenomena were also seen in hamsters; data from Table 3.1 show that at a concentration of 20 mg/nmol P450, DHP and SN N-oxide production are inhibited 69.5% and 41.3% (Figure 3.5), respectively, by anti-sheep CYP3A antibody, whereas at the same concentration, anti-sheep CYP2B inhibit 32.5% DHP and 35.4% SN N-oxide production in hamster (Figure 3.6). These results suggest that CYP3A is the major enzyme involved in PA bioactivation as well as being partially involved in PA detoxication. CYP2B has only a minor contribution to SN biotransformation.

Figure 3.2 Immunoinhibition of SN metabolism in sheep hepatic microsomes by anti-CYP3A IgG. Hepatic microsomes (0.35 nmol of P450) from four male adult sheep were preincubated with rabbit anti-sheep CYP3A IgG (0.5-5.0 mg/nmol P450), and DHP and SN N-oxide were measured as described in Materials and Methods. The data were expressed as % of controls (preimmune rabbit IgG was used as control).
Figure 3.3  Immunoinhibition of SN metabolism in sheep hepatic microsomes by anti-CYP3A IgG. Hepatic microsomes (0.35 nmol of P450) from four male adult sheep were preincubated with rabbit anti-sheep CYP3A IgG (2.5-20 mg/nmol P450), and DHP and SN N-oxide were measured as described in Materials and Methods. The data were expressed as % of controls (preimmune rabbit IgG was used as control).
Figure 3.4 Immunoinhibition of SN metabolism in sheep hepatic microsomes by anti-CYP2B IgG. Hepatic microsomes (0.35 nmol of P450) from four male adult sheep were preincubated with rabbit anti-sheep CYP2B IgG (2.5-20 mg/nmol P450), and DHP and SN N-oxide were measured as described in Materials and Methods. The data were expressed as % of controls (preimmune rabbit IgG was used as control).
Immunoinhibition of SN metabolism in hamster hepatic microsomes by anti-CYP3A IgG. Hepatic microsomes (0.25 nmol of P450) from four male adult hamster were preincubated with rabbit anti-sheep CYP3A IgG (2.5-20 mg/nmol P450), and DHP and SN N-oxide were measured as described in Materials and Methods. The data were expressed as % of controls (preimmune rabbit IgG was used as control).
3.5 Discussion

Species differences in PA metabolism are well known. Previous researches have shown that sheep and hamsters are both very resistant to PA toxicity (Shull et al., 1976; Cheeke and Pierson-Goeger, 1983). The differences between the two species are that sheep have a low pyrrole production with high SN N-oxidation while hamsters have a relatively high of pyrrole formation (Cheeke and Shull, 1985). Data from our in vitro hepatic microsome metabolism (Table 3.1) show that SN N-oxide is the major metabolite from SN metabolism in sheep (DHP/N-oxide=0.26), while the rate of DHP
formation far exceeded the rate of SN N-oxidation (DHP/N-oxide=2.29) in hamsters. Pyrrolic metabolites are responsible for PA toxicity (Mattocks, 1986). The low rate of pyrrole formation could be the primary determinant in resistance to PA toxicity in sheep. The explanation for resistance of hamsters to PA toxicity is not clear. Previous studies (Reed et al., 1992; Dueker et al., 1994) and data from our lab (data not published) showed that there appeared to be a high rate of GSH-DHP conjugation in various species. Thus, this may be one of the factors in resistance of hamsters to SN intoxication.

Previous studies showed that CYP3A played the major role in PA biotransformation in rats but not in guinea pigs (Chung and Buhler, 1994), while CYP2B contributed mainly to bioactivation of SN in guinea pigs but CYP3A had little influence in bioactivation of SN (Chung and Buhler, 1995). Immunoinhibition data from this study (Figure 3.3; 3.5) show that CYP3A contribute 96.5% and 69.5% bioactivation capability in DHP formation in sheep and hamster, respectively. These results are in agreement with the suggestion from other studies (Williams et al., 1989a; Miranda et al., 1991) in which CYP3A has been demonstrated to be the major enzyme involved in bioactivation of SN in the rat and human. CYP3As have been shown to be responsible for the metabolism of a wide array of clinically and toxicologically important agents, and to be inducible by steroids, macrolide antibiotics, and phenobarbital (Gonzalez, 1988). The results from this study and previous research suggest that CYP3A also play the major role in PA metabolism in some animal species. With this P450 subfamily there are also sex differences in the expression of the enzyme
among species. CYP3A are normally dominant in males. Previous studies showed that when female rats were challenged with spironolactone, a known inducer of CYP3A, there was a 500% increase in both DHP and SN N-oxide formation (Chung and Buhler, 1994), suggesting that CYP3A could be the major PA metabolizing enzyme in induced animals. Thus, it will be interesting to know, in further studies, if sheep and hamsters that are exposed in vivo to inducers of CYP3A will become more or less susceptible to PA toxicity. Our previous study demonstrated that the formation of SN N-oxide was carried out largely by FMO (55-71%) in sheep and hamster. The data from this study showed that CYP3A isoyme accounts for 38.8% and 41.3% SN N-oxide formation in sheep and hamsters, respectively. This suggests that CYP3A is involved in both reactions, with less efficiency in N-oxidation of SN.

CYP2B are a phenobarbital (PB)-induced subfamily. The expression of CYP2Bs in animals is highly related to the presence of inducers. These enzymes show loose substrate specificity compared with all other cytochrome P450 forms and can catalyze the oxidation of a wide variety of foreign substrates (Omura, 1993). Miranda et al. (1992) reported that PB treatment increased DHP formation from senecionine by 274% in male guinea pigs. Moreover, Chung and Buhler (1995) showed that CYP2B was responsible for over 70% of DHP production from SN in both untreated and PB-treated guinea pigs. This enzyme also contributed along with FMO to SN N-oxidation in both untreated (34%) and PB-treated (40%) guinea pigs. Our data from this study (Figure3.4; 3.6) show that CYP2B is less efficient in PA metabolism in sheep and hamsters. With anti-sheep CYP2B antibody, DHP formation was inhibited 47.4% and
32.5% in sheep and hamsters, respectively, whereas SN N-oxidation was reduced 24.6% and 35.4% in sheep and hamsters, respectively. It seems that CYP2B is more efficient at DHP formation in sheep, whereas this enzyme is more active in SN N-oxidation in hamsters. Because CYP2Bs are PB-inducible enzymes, this subfamily has a major role in PA metabolism in induced animals.

In this study, we conclude that CYP3A plays the major role in bioactivation and detoxication of SN in sheep and hamsters. This isozyme seems more efficient in bioactivation rather than detoxication of SN. CYP2B is also involved in biotransformation of SN with less efficient capability in sheep and hamsters.

3.6 References


4. Conclusions

In this study, we provide evidence for differences in pyrrolic metabolites and SN N-oxide formation among eight animal species, the relative contributions of cytochrome P450s, FMO and hepatic esterases in SN metabolism, the roles of cytochrome P450 isozymes 3A and 2B in SN metabolism, and the possible detoxication pathway of GSH conjugation. Our results demonstrate that there is no strong relationship between the pyrrolic metabolites production and the susceptibility of animals to PA toxicity. Cytochrome P450s play the major role in DHP formation from SN metabolism in both sheep and hamsters, whereas the SN N-oxidation is carried out largely by FMO. Hepatic esterases may have a significant impact in DHP formation in sheep but not in hamsters. CYP3A play the major role in the bioactivation of SN in sheep and hamsters; in contrast, CYP2B has a limited capacity in biotransformation of SN. There is a high rate of GSH-DHP conjugation in hepatic microsomal incubation mixtures from both species. Therefore, low rates of pyrrolic metabolite production coupled with glutathione conjugation may explain the resistance of sheep to SN, whereas the high rates of GSH-DHP conjugation may be one of the factors responsible for the resistance of hamsters to SN intoxication.

The major metabolic routes for PA in animals are dehydrogenation to pyrrolic derivatives, conversion to N-oxides and hydrolysis (Mattocks, 1986). Our results from this study and previous research suggest that the resistance of species to PA intoxication is the balance of these pathways with other secondary detoxication pathways such as
glutathione conjugation. In consequence, the specific and efficient involvement of distinct enzyme systems together with unique substrate specificities may explain the species differences in PA metabolism in animals.
5. Bibliography


