AN ABSTRACT OF THE THESIS OF

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Title: Comparative Metabolism of the Pyrrolizidine Alkaloid Senecionine in Rat and Guinea pig

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The species/strain differences in bioactivation and detoxication of the pyrrolizidine alkaloids (PAs) in rats and guinea pigs have been investigated in terms of the roles of cytochrome P450s, flavin-containing monooxygenases (FMOs) and carboxylesterases. There was no difference in (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP; activation of the PAs) formation from the PA senecionine (SN) by liver microsomes from both sexes of Sprague-Dawley (SD) and Fischer 344 (F344) rats. However, hepatic microsomes from both male and female F344 rats produced higher SN N-oxidation (a detoxification pathway) by 88% and 180%, respectively, compared to that of SD rats. SN N-oxide was primarily produced by FMO in both sexes of F344 rats, while P450s provided the major N-oxidation route in SD rats. This result showed a significant strain difference in PAs metabolism. Treatment with spironolactone (SPL), an inducer of cytochrome P4503A, increased DHP and N-oxide formation from SN by 500% in female SD rats

while causing a 50% increase and a 50% decrease in DHP and N-oxide production, respectively, in male SD rats. By contrast, SPL dosing of guinea pigs resulted in a 50% increase in both DHP and N-oxide formation with no apparent differences between sexes. This result indicated that SPL had a greater effect in rats than in guinea pigs.

Cytochrome P4502B played the most important role (over 70%) in the bioactivation of SN in the guinea pig. However, purified guinea pig P4502B (M_r=57,512 by mass spectroscopy) had a minimal specific activity toward SN. This phenomenon reveals that metabolism by a purified P450 in reconstitution system may be quite different from that of the same P450 in microsomes where a mixture of P450s are present. By contrast, guinea pig cytochromes P4502C (M_r=56,496 by mass spectroscopy) and P4503A (M_r=54-56,000 by SDS-PAGE) had limited capacity for DHP formation in microsomes. Also cytochrome P4502B is responsible along with FMO for the detoxication of SN in the guinea pig. Purified guinea pig carboxylesterase (GPH1) hydrolyzed the PA jacobine (JB), which is unusually toxic to guinea pigs, at a slower rate than that for SN. Also JB yielded much less JB N-oxide compared to that seen with SN upon incubation with guinea pig liver microsomes. Therefore, the combination of high pyrrole, lower N-oxide formation and little hydrolysis appeared to be the major factors in the susceptibility of guinea pigs to JB intoxication.

Comparative Metabolism of the Pyrrolizidine Alkaloid Senecionine in Rat and Guinea pig

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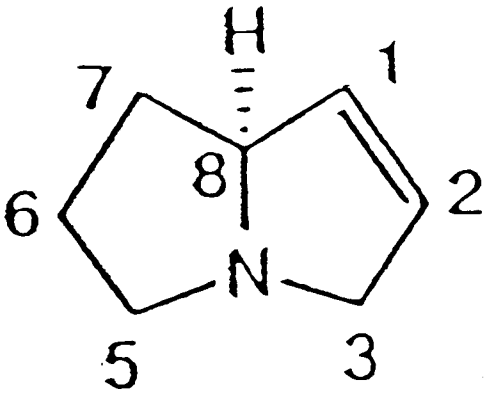
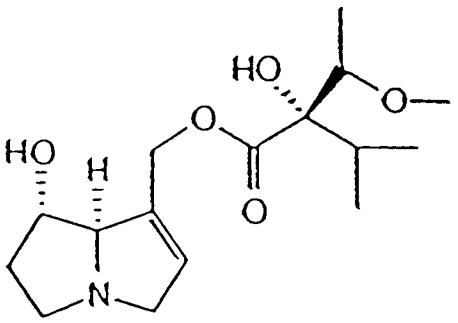
I. INTRODUCTION

Distribution of Pyrrolizidine Alkaloids

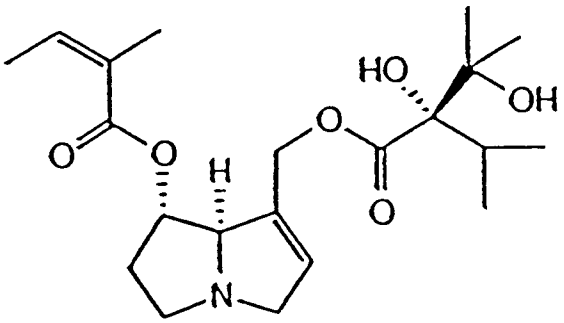
Pyrrolizidine alkaloids (PAs) are found in a large number of plant species such as *Compositae*, *Leguminosae* and *Boraginaceae*. All climates and areas of the world have representatives of these plants (Bull et al., 1968). Of special interest in Oregon are *Senecio jacobaea* (tansy ragwort) and *Senecio vulgaris* (common groundsel) because of their prevalence in animal grazing areas. *Senecio jacobaea* is a biennial plant with a green leafy stem and yellow clustered flower head (Kingsbury, 1964). *Senecio vulgaris* is a low annual herb with a green leafy stem and yellow flowers. *Senecio jacobaea* contains six major pyrrolizidine alkaloids: jacobine, jacoline, jaconine, jacozine, senecionine and seneciphylline (Bull et al., 1968). Many of these alkaloids are cytotoxic and are often responsible for poisoning livestock and humans (Mattocks, 1986). Many of the toxic PAs are allylic esters of the bases retronecine or its stereoisomer heliotrioline with toxicities varying according to the nature of the esterified necic acid (Fig. I-1). PAs can be monoesters, open diesters or closed diesters, the latter being the most hepatotoxic (Smith and Culvenor, 1981).



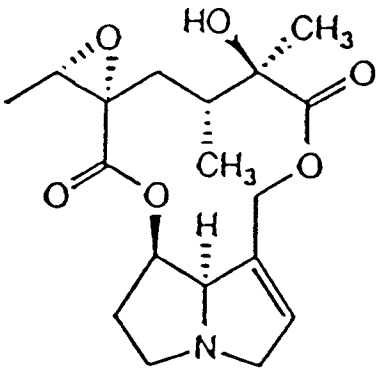
Pyrrolizidine Base



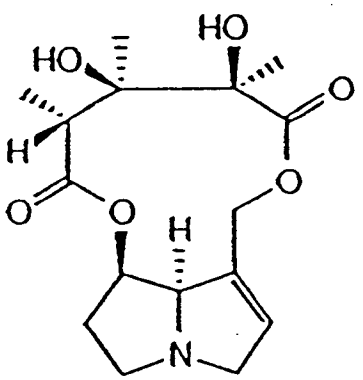
Heliotrine



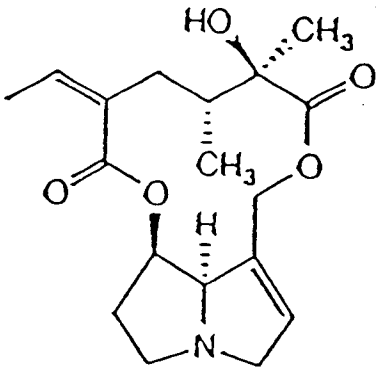
Heliosupine



Jacobine



Monocrotaline



Senecionine

Fig. I-1. Structures of pyrrolizidine alkaloids.

The tissue distributions of PAs in animals after the administration of radiolabelled PAs has been studied. Eastman and Segall (1982) investigated the distribution of [^{14}C]-labelled 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 CO_2 (0.21%) and the milk (0.04%). A relatively high level in liver (1.92%) reflects the potential for hepatotoxicity by this PA compared to small portion 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 distribution of [^{3}H]-dehydroretronecine (DHR), a pyrrolic degradation product of PAs, has been measured in male rats given an s.c. dose of 65 mg/kg (Hsu et al., 1974). There was a rapid general distribution of radioactivity throughout the body tissues. Unlike PAs, DHR was not preferentially bound in liver. However, there was a very large amount in the glandular part of the stomach. Excretion of DHR occurred via the urine (25% in 24 hr) and the bile.

Metabolism of PAs

PAs are rather chemically inert. It is, therefore, unlikely that they would be able to react with cell constituents under physiological conditions. On the other hand, metabolites known to be formed from PAs in the liver are highly reactive and considerably more cytotoxic than their parent alkaloids.

Once ingested by an animal, the PA can follow various pathways of distribution, metabolism and elimination, and the eventual biological effects will depend on the balance of these various processes. In laboratory animals, the principal known routes of PA metabolism are 1) ester hydrolysis to form the corresponding necine bases (retronecine or heliotrioline); 2) conversion to N-oxides; and 3) dehydrogenation to form pyrrolic derivatives (Fig. I-2). The first two types of metabolites appear to be detoxication pathways and only the last class of metabolites cause cytotoxicity.

Esterase hydrolysis of a toxic PA leads to the necine and necic acid moieties, neither of which are hepatotoxic. There are three kinds of evidence relating to the importance of PA hydrolysis for detoxification by mammalian enzymes: (1) metabolic activation of PAs is enhanced in animals whose esterase activity is inhibited; (2) direct *in vitro* measurements of PA hydrolysis by esterase preparations show relatively high activities in some animal species; and (3) identification of hydrolysis products formed *in vivo* have demonstrated the formation of relatively nontoxic necine bases such as retronecines (Bull et al., 1968). Steric hindrance around the ester groups is a major factor controlling hydrolysis of individual PAs (Mattocks, 1982).

Two major kinds of metabolites are formed by the enzymatic oxidation of PAs in animals: pyrrolic derivatives and N-oxides. The PAs are metabolized, via cytochrome P450s, to PA pyrroles through dehydrogenation of the pyrrolizidine ring (Fig. I-3). These highly reactive pyrrole metabolites

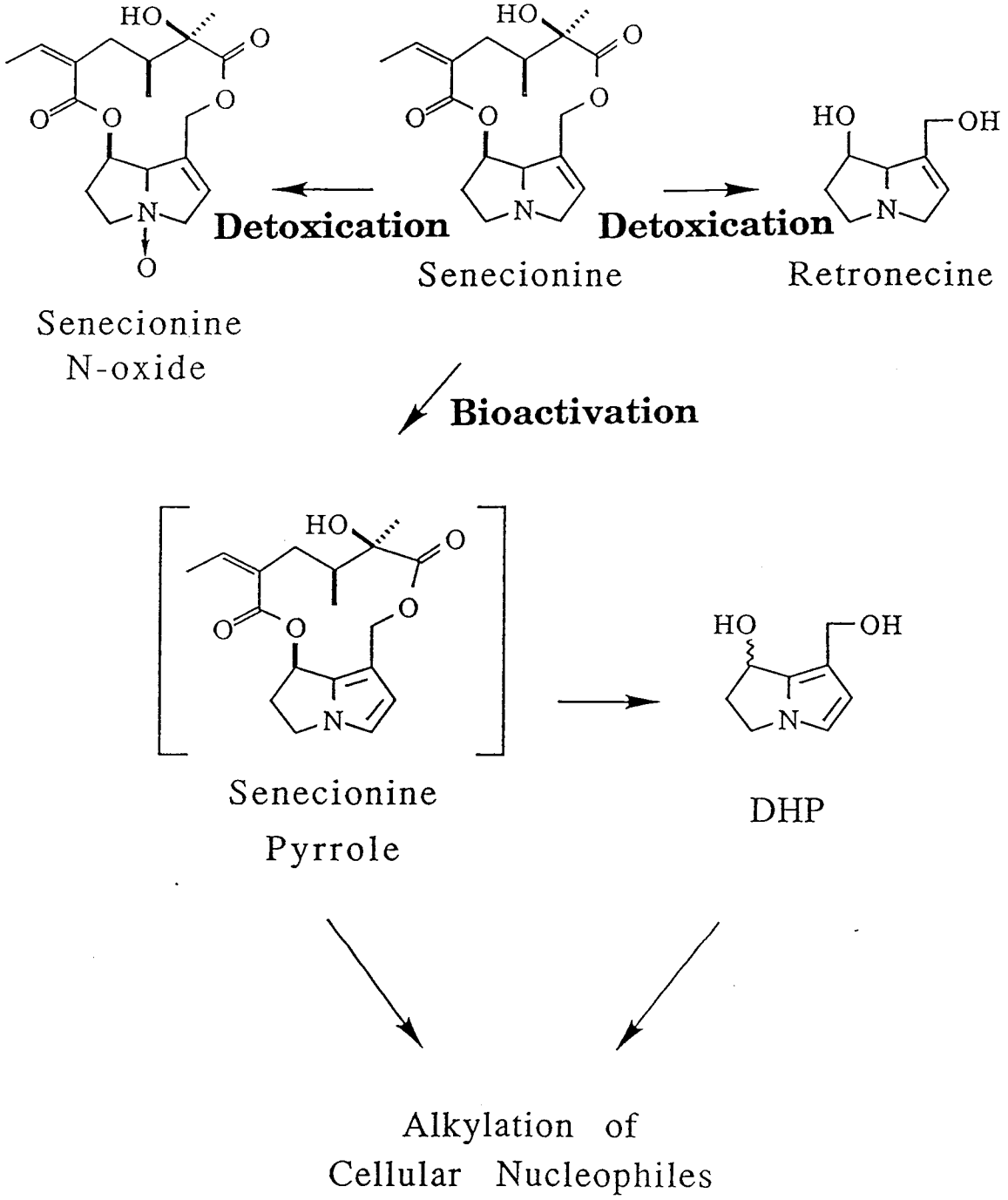


Fig. I-2. Major metabolic pathways for pyrrolizidine alkaloids.

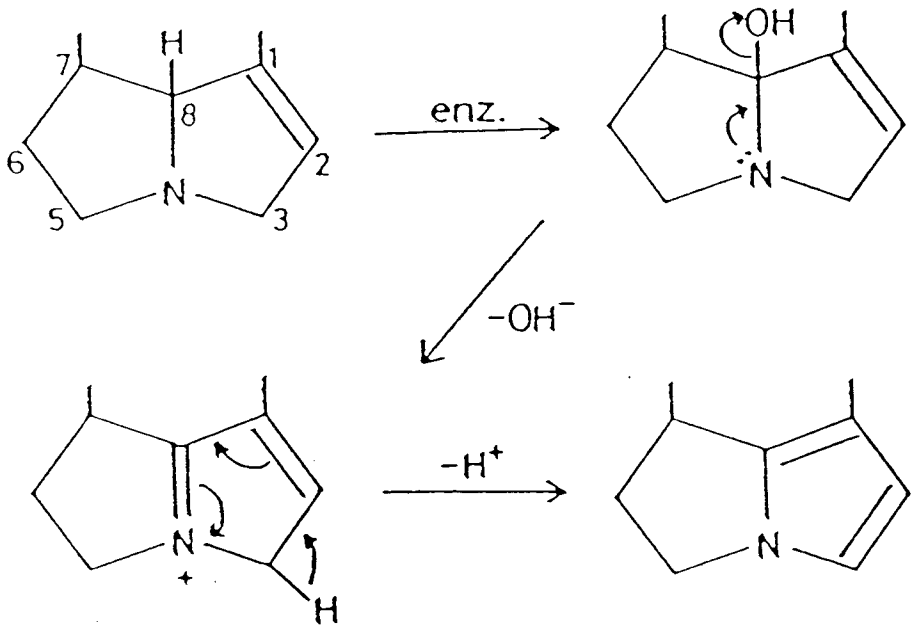


Fig. I-3. Mechanism of pyrrole formation.

are unstable and either undergo rapid hydrolysis to yield the more 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) attempts 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 pyrroles are readily detectable by the strong magenta color with a modified Ehrlich reagent. This color reaction is positive in the urine, liver and other tissues of rats shortly after they have been given various PAs (Mattocks, 1968).

A second oxidative pathway results in formation of PA N-oxides. This reaction is catalyzed both by cytochrome P450s and flavin-containing monooxygenases (FMOs) that are localized in the endoplasmic reticulum of primarily the liver. The N-oxides, once formed, are more hydrophilic than their parent alkaloids and readily excreted via the urine. In addition, the N-oxides of PAs are not converted to pyrrolic metabolites by microsomal enzymes after oral administration of N-oxides (Mattocks, 1971). However, substantial reduction of PA N-oxides back to the parent PAs can occur in the gut (Mattocks, 1971). Evidence suggests that the latter reaction may be brought about by enzymes in the intestinal flora, rather than from the gut itself.

Antibiotic treatment, which considerably lowers the bacterial content of the gut, causes a large decrease in the amount of N-oxide reduced.

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 most 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.

The actions of other mammalian enzymes on PAs may lead to the production of other metabolites besides pyrroles and N-oxides. Liver microsomes from female mice produce 19-hydroxysenecionine (6.8%) from senecionine (Eastman and Segall, 1982). The acid moiety of heliotrine is demethylated to heliotridine trachelanthate in sheep (Jago, 1969). In ovine rumen fluid, heliotrine is converted to the non-toxic base (Lanigan and Smith, 1970). The 1-aldehyde and the 7-methyl ether derivative of DHR also are detectable after the *in vitro* metabolism of senecionine by mouse liver microsomes (Segall et al., 1984).

Toxicity of PAs

There are many different PAs and this group of compounds can have a variety of toxic actions in various species of laboratory and farm animals. All the hepatotoxic alkaloids are esters of unsaturated necines. Different unsaturated PAs can produce similar hepatotoxic effects, but their degree of toxicity may vary widely (Table I-1).

Sufficiently large doses of most PAs can cause rapid death. This type of acute toxicity is associated with pharmacological actions of the compounds rather than cytotoxic actions. Death may be preceded by convulsions or by coma. The more lipophilic PAs are especially liable to cause acute toxicity and death.

While PAs produce a diversity of biological effects, the principal pathology is irreversible liver cirrhosis with pronounced fibrosis and biliary hyperplasia. The sequence of events in acute pyrrolizidine hepatotoxicity is as follows (Barnes et al., 1964). The PA is 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 hepatic veins leading to cell proliferation and venoocclusion. Some of the reactive metabolite may proceed further by way of the bloodstream to damage other organs such as lung. Most of the other tissue lesions and signs of toxicity, including mortality, can be related to impaired liver function. In chronic hepatotoxic disease produced by PAs, the liver becomes small and its surface has a granular,

Table I-1. Acute toxicity data of pyrrolizidine alkaloids*.

PAs	Animal	Sex#	Route§	LD_{50} (mg/kg)
Heliosupine	rat	M	i.p.	60
Heliotrine	rat	M	i.p.	296
Heliotrine N-oxide	rat	M	i.p.	5000
Jacobine	rat	F	i.p.	138
	guinea pig	M	i.p.	100†
Monocrotaline	rat	M	i.p.	109
		F	i.p.	230
	guinea pig	M	i.p.	>1000
Retrorsine	rat	M	i.p.	34
		F	i.p.	153
	guinea pig	M	i.p.	>800
Senecionine	rat	M	i.p.	50
	mouse	U	i.v.	64
Seneciphylline	rat	M	i.p.	77
		F	i.p.	83
	guinea pig	U	i.v.	50-80

*Adapted from Chen et al. (1940), Mattocks (1986) and Swick et al. (1982).
#M, male; F, Female; U, unspecified.
§i.p., Intraperitoneal; i.v., intravenous; †LD_{100} value.

mottled and nodular appearance. There may be some post-necrotic fibrosis and thus the liver continues to undergo cell division cycle but bypasses mitosis. The resulting giant hepatocytes (megalocytosis) often show varying degrees of degeneration. Other common chronic features of PA hepatotoxicity include a proliferation of the bile ducts (Schoental and Magee, 1959).

Similar toxic actions of PAs have been demonstrated in many different animals, but there may be large quantitative differences between animal species. The LD_{50} of retrorsine varies from 34 mg/kg in male rats to over 800 mg/kg in guinea pigs (White et al., 1973). This difference is mainly due to the ability of the animal to convert the PAs to their pyrrolic and/or N-oxide metabolites.

Some animal species show a distinct sex difference in their susceptibility to PA hepatotoxicity. This again is probably related to tissue differences in PA metabolism. Male rats are over four times as susceptible as are females to the acute toxicity of retrorsine and with monocrotaline a two-fold difference in LD_{50}s exists between males and females (Mattocks, 1972). In contrast, no significant sex difference was found for retrorsine in mice (White et al., 1973).

A higher toxicity of retrorsine in 14-day-old rats compared to adults has been attributed to three factors (Mattocks and White, 1973). Young animals have a relatively lower liver weight (2.8% of body weight compared with 4.3% in older rats), which makes the dose of PAs to the liver higher in younger rats.

Secondly, there is a higher portion of pyrrolic metabolites bound to liver tissue in young rats. Finally, there is a greater susceptibility to PA-induced cytotoxicity in tissues where the cells are rapidly dividing.

The effects of PAs in animals can be influenced by food intake and nutritional status. Rats fed a low protein diet are more susceptible to the toxicity of *Senecio jacobaea* PAs whereas high protein diets afford some protection (Cheeke and Garman, 1974). The acute hepatotoxicity of retrorsine is 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 copper^{+2} (50 ppm) in the diet of rats increases the hepatotoxicity of *Senecio jacobaea* (Miranda et al., 1981).

Pretreatment of rats with phenobarbital causes an increase in susceptibility to PAs under conditions where the rate of metabolism in rats is normally low, but phenobarbital is protective where metabolism is normally fast, even though pyrrole production is increased (Mattocks, 1972). Pretreatment with SKF 525A, an inhibitor of cytochrome P450, reduces the susceptibility of male rats to retrorsine toxicity. Pretreatment with zinc^{+2} 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 animals with tri-orthocresyl phosphate, an inhibitor of esterase activity, can result in a large proportion of PAs being converted to pyrrolic metabolites *in vivo* (Mattocks, 1981). Cysteine pretreatment doubles

the liver glutathione (GSH) level and halves the toxicity of retrorsine whereas 2-chloroethanol reduces the liver GSH concentrations to one-fourth of control values and doubles the alkaloid's toxicity (White, 1976).

The liver, where many PAs are converted to toxic metabolites, is the commonest site of injury by these alkaloids. There is no evidence that substantial amounts of toxic metabolites are formed from PAs in other tissues. Thus, the extent to which an extrahepatic tissue is damaged will depend not only on its intrinsic susceptibility to damage but also on the amounts of reactive PA metabolites that are able to reach the tissue in active form from liver. The more reactive the metabolite, the smaller the amount that would survive the journey from liver to an extrahepatic tissue in order to cause damage. The lung is the most common extrahepatic site for toxic actions of PAs. Macrocyclic diesters with 11-members such as monocrotaline and fulvine are particularly active in causing pneumotoxicity (Barnes et al., 1964). Monocrotaline pyrrole is less reactive than retrorsine pyrrole (Mattocks, 1969). Monocrotaline pyrrole also is more stable than other pyrroles. This may be the reason why monocrotaline is more effective in damaging the lung. Early lung changes caused by PAs include alveolar edema and hemorrhage. The initial injury leads to a progressive thickening of medial walls of small pulmonary arteries and veins. This then results in increased pulmonary pressure, endothelial damage and the development of right ventricular hypertrophy (Ghodsi and Will, 1981). A normal function of pulmonary endothelial cells is

to remove and metabolize circulating vasoactive substances and drugs. This activity may be impaired in animals suffering from PA-induced lung injury.

Chronic heart damage often occurs as a secondary result of PA-induced lung damage. Thus, right ventricular hypertrophy develops in rats fed monocrotaline in the diet (Turner and Lalich, 1965). Cor pulmonale has been similarly induced with seneciphylline (Ohtsubo et al., 1977).

There are reports of kidney damage in animals poisoned with PAs. Fulvine damages the epithelium of proximal convoluted tubules in rats (Persaud et al., 1970). Retrorsine causes a severe toxic nephritis with damaged glomeruli and hemorrhage in the adrenal glands in monkeys (Van der Watt and Purchase, 1970).

Infrequent enlarged islet cells have been seen in pigs fed *Crotalaria retusa* (Hooper and Scanlan, 1977). Islet cell tumors of the pancreas have been reported in rats fed monocrotaline (Hayashi et al., 1977).

Spongy degeneration in brains of calves and sheep fed with PAs was observed due to increased blood ammonia, secondary to the severe liver damage: a similar lesion was produced in sheep given ammonium acetate intravenously (Hooper, 1972).

The International Agency for Research in Cancer (IARC) concluded that some PAs such as lasiocarpine, monocrotaline, retrorsine, riddelliine, senecionine and seneciphylline are hepatocarcinogens (IARC, 1976 and 1983).

A mixture of intermedine and lycopsamine extracted from seeds of *Amsinckia intermedia* produced tumors of the pancreas in rats (Schoental et al., 1970).

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 in human lymphocytes (Kraus et al., 1985). Lasiocarpine and its pyrrolic metabolites can exert their antimitotic activities resulting in megalocytosis (Samuel and Jago, 1975).

In summary, many of the PAs produce acute and chronic hepatotoxicity with cirrhosis and megalocytosis leading to irreversible liver damage. Some PAs are pneumotoxic, producing pulmonary arterial hypertension and right ventricular hypertrophy, or carcinogenic (McLean, 1970) and show mutagenicity in various test systems (White et al., 1983).

Species differences in response to PAs

There is a marked variation in the sensitivity of animal species to the toxic effects of PAs (Table I-2). There are several factors that may account for species differences in susceptibility and resistance of some animals to PA toxicity. These include: 1) lack of PA absorption; 2) degradation of PAs in the rumen; 3) lack of hepatic formation of pyrroles; and 4) conjugation and excretion of PAs and pyrroles.

Table I-2. Susceptibility of animals to PA intoxication in comparison with their rates of *in vitro* hepatic pyrrole production*.

Animal	Susceptibility	Pyrrole	LD (% of body weight)#
Chicken	High	Low	39
Cow	High	High	4
Guinea pig	Low	Low	119
Horse	High	High	8
Japanese Quail	Low	Low	2450
Rabbit	Low	High	113
Rat	High	High	21
Sheep	Low	Low	302

*Adapted from Cheeke and Shull (1985).

#Chronic lethal dose of *Senecio jacobaea*

(1). Mouse

The mouse is susceptible to PA poisoning. After feeding tansy ragwort which contains the six major PAs such as jacobine, jacoline, jaconine, jacozine, senecionine and seneciphylline, young male mice developed edema, megalocytosis and cytoplasmic invaginations of hepatocytes (Hooper, 1974). In addition to the liver lesions, lung and kidney also were affected. Mice injected i.p. with the alkaloid lasiocarpine exhibited lesions of the gastrointestinal tract (Hooper, 1975).

(2). Rat

Rats are susceptible to both injected and ingested PAs. Livers from rats fed *Senecio jacobaea* developed megalocytosis, vascular congestion and hemorrhage (Bull et al., 1968). After consuming a diet containing *Crotalaria spectabilis* seed, rats developed lung lesions including hydrothorax and edema (Allen and Carstens, 1970). Kidneys and livers were mildly damaged. The LD_{50} in male rats injected i.p. with retrorsine was 34 mg/kg; senecionine 50 mg/kg; monocrotaline 109 mg/kg (Mattocks, 1972); and seneciphylline 80 mg/kg (Anon, 1949). Tissue damage associated with acute death included hemorrhagic necrosis and congestion of the liver, ascites, and pulmonary edema.

(3) Hamsters

Hamsters were more susceptible to intoxication by injected alkaloids than rats and mice (Rose et al., 1959). Pyrrole production rates were several

times greater in male hamsters than in male rats when both monocrotaline and tansy ragwort mixed alkaloids were fed (Shull et al., 1976).

(4) Guinea pig

Liver tumors due to pyrrolizidine alkaloid poisoning were not induced in guinea pigs (Schoental, 1969; McLean, 1970). Guinea pigs, injected with four times the monocrotaline LD_{50} for rats, remained normal for 6 weeks and no pathologic changes were seen at necropsy (Chesney and Allen, 1973). A dose of 800 mg retrorsine/kg was not sufficient to kill guinea pigs (White et al., 1973) and guinea pigs were resistant to the acute hepatotoxic effects of senecionine (McLean, 1970). However, Chen et al. (1940) observed that guinea pigs injected intravenously with a 2% solution of seneciphylline in doses of 50-80 mg/kg died after convulsions. Jacobine is also very toxic to the guinea pig (Swick et al., 1982) giving an LD_{50} similar to that seen in the rat (Bull et al., 1968). Pyrrole formation in guinea pigs was 30 times slower than in injected rats, while N-oxide production was greater. It thus appears that guinea pigs are very resistant to certain PAs but are intoxicated by other PAs, notably seneciphylline and jacobine.

(5) Rabbit

Rabbits are well recognized to be very resistant to PA-induced toxicity. However, a rabbit fed *Crotalaria crispata* which contained monocrotaline, fulvine and crispatine, died after 136 days (Bull et al., 1968). The liver showed sign of megalocytosis. Kidney was affected with enlarged proximal convoluted

tubule epithelial cells. Lungs showed patchy emphysema and enlarged alveolar cells. Among the resistant species, rabbits produce the highest amounts of pyrroles in *in vitro* preparations. The resistance is due to the high conjugation and excretion of pyrroles produced.

(6) Avian species

Turkeys are very susceptible to poisoning by seed from *Crotalaria spectabilis* which contains monocrotaline (Allen, 1963). Typical histological changes occur in the liver with necrosis, bile duct proliferation and fibrosis. Chickens also are readily intoxicated by *Crotalaria* seed and tansy ragwort (Campbell, 1956). However, quail differ in their susceptibility to PAs toxicity (Buckmaster et al., 1977). Japanese quail allowed to eat 10% tansy ragwort in a standard layer mash consumed the mixture for 365 days with no ill effects.

(7) Sheep

Sheep can consume 300% of their body weight of *Senecio jacobaea* but still survive. The specific organism, named *Peptococcus heliotrinreducans*, responsible for reductive breakdown of heliotrine in sheep rumen has been isolated (Lanigan, 1976). The organism is in competition with rumen methanogenic bacteria for molecular hydrogen.

(8) Cattle

After consumption of alfalfa hay contaminated with *Senecio vulgaris*, twelve dairy heifers suffered ataxia, prolapsed rectum and congested mucus

membranes (Fowler, 1968). The very firm livers showed megalocytosis and excess fibrosis. PAs were found in the milk of cows that had consumed 10 g of dried tansy ragwort, but the calves were not affected (Dickinson et al., 1976).

(9) Horse

In horses, neurological disturbances are seen, after consuming PAs, including head pressing against solid objects and walking in a straight line (Van Es et al., 1929). As a result, drowning is common. Neurological signs in horses are due to elevated blood ammonia caused by decreased ability of the liver to convert ammonia to urea (Rose et al., 1957).

(10) Human

Humans are also subject to pyrrolizidine alkaloid poisoning. Clinical reports on *Senecio* contamination of bread and tea have been issued from Africa and India (McLean, 1970). Symptoms include abdominal pain, distended abdomen, ascites and death. Ingestion of herbal teas prepared from *Senecio longilobus*, which contains riddelliine, retrorsine, seneciphylline and senecionine, has been fatal to children in the United States (Huxtable et al., 1977). Successive intake caused ascites and liver enlargement.

Objectives

Rats are highly susceptible, whereas guinea pigs are very resistant to PA intoxication (McLean, 1970). In guinea pigs, where PA pyrrole formation is particularly low, a dose of 800 mg/kg retrorsine is insufficient to kill the animals. Pretreatment with phenobarbital which induces cytochrome P450s increases the hepatic metabolic activity and the LD_{50} is lowered to 210 mg/kg (White et al., 1973). Guinea pigs are similarly resistant to monocrotaline (Chesney and Allen, 1973) and senecionine (McLean, 1970), but not to jacobine or to mixed alkaloids from *Senecio jacobaea* which are highly toxic at 100-150 mg/kg (Swick et al., 1982).

In Sprague-Dawley rats (Williams et al., 1989a) and humans (Miranda et al., 1991b), liver microsomal conversion of the PA SN to DHP is catalyzed primarily by enzymes belonging to the cytochrome P4503A subfamily. In guinea pigs, however, the orthologs of cytochrome P4502B and P4503A both participate in DHP formation by hepatic microsomes (Miranda et al., 1992). Cytochrome P4502C11 is responsible for SN N-oxidation in Sprague-Dawley rats (Williams et al., 1989a). Flavin-containing monooxygenase (FMO) accounts for no more than 20 % of the PA N-oxidase activity of Sprague-Dawley rat liver microsomes (Williams et al., 1989b), but the major catalyst for the N-oxidation of SN in guinea pigs is the FMO system (Miranda et al., 1991a).

We chose for further study the rat and guinea pig as animal models for susceptible and resistant species, respectively. We have investigated the species/strain difference in terms of the role of cytochrome P450s, flavin-containing monooxygenase and carboxylesterases in bioactivation and detoxication of the pyrrolizidine alkaloids. The PA senecionine was chosen because it has high toxicity in rats but low toxicity in guinea pigs and it is a good substrate for the enzymes examined. Jacobine also was studied since it is unusually toxic to the guinea pig which is a species resistant to other PAs.

SPECIFIC OBJECTIVES:

1. To investigate the influence of strain on the metabolism of the PA senecionine (SN) using Sprague-Dawley and Fischer 344 rats (strain differences between rats).
2. To determine the effect of spironolactone (SPL), a known inducer of cytochrome P4503A, on the metabolism of the SN in rats and guinea pigs (species differences between rats and guinea pigs).
3. To determine which P450 isozyme is responsible for the bioactivation and detoxication of SN in guinea pigs (guinea pigs as resistant animals).
4. To determine which factor is responsible for susceptibility of guinea pigs to the PA jacobine (guinea pigs as susceptible animals).

II. METABOLISM OF THE PYRROLIZIDINE ALKALOID SENECIONINE IN FISCHER 344 RATS*

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Abstract

Wide variations of metabolism of the pyrrolizidine alkaloids (PAs), such as senecionine (SN), between animal species are known to exist but little work has been done to investigate differences in animal strain. The metabolism of SN in Fischer 344 (F344) rats has been studied in order to compare to that of the previously studied Sprague-Dawley (SD) rats (*Drug Metab. Dispos.* **17**, 387, 1989). There was no difference in DHP (activation) formation by hepatic microsomes from either sex of between SD and F344 rats. However, hepatic microsomes from male and female F344 rats had greater activity in the N-oxidation (detoxication) of SN by 88% and 180%, respectively, compared to that of male and female SD rats. In a test for optimum pH, the maximum for SN N-oxidation by hepatic microsomes in females was pH 8.5 whereas in males SN N-oxide formation showed a bimodal pattern with peaks at pH 7.6 and 8.5. Use of specific inhibitors showed that SN N-oxide was primarily produced by flavin-containing monooxygenase (FMO) in both sexes of Fischer 344 rats. In contrast, SN N-oxide formation is known to be catalyzed mainly by P450 rather than FMO in Sprague-Dawley rats. This study, therefore, demonstrates that substantial differences in PA metabolism can exist between animal strains.

Pyrrolizidine alkaloids (PAs) are constituents of certain plant species (Mattocks, 1986). Human exposures can occur through the ingestion of foodstuffs such as herbal teas, milk, honey, etc. Some of these alkaloids are toxic to animals and humans. PAs are metabolized by liver monooxygenases yielding PA pyrroles and N-oxides (Mattocks and Bird, 1983a). PA pyrroles are considered the ultimate toxic metabolites whereas N-oxides are believed to be non-toxic. The PA pyrroles either react with cellular macromolecules or are hydrolyzed to form a secondary pyrrole known as (±) 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP). DHP also is a strong electrophile capable of alkylating cellular nucleophiles including DNA (White and Mattocks, 1972).

A marked variation exists in the sensitivity of animal species to the toxic effects of PAs. In Sprague-Dawley (SD) rats (Williams et al., 1989a) and humans (Miranda et al., 1991b), the microsomal formation of DHP and N-oxide from the PA, senecionine (SN), is catalyzed primarily by enzymes belonging to the cytochrome P4503A subfamily. Flavin-containing monooxygenase (FMO) accounts for no more than 20% of the PA N-oxidase activity of SD rat liver microsomes (Williams et al., 1989b). However, FMO was the major catalyst of the N-oxidation of SN in guinea pig (Miranda et al., 1991a).

Recently, evidence was presented that rat strains are highly polymorphic in electrophoretic recognizable isoforms of cytochrome P450 (Rampersaud and Walz, 1987) and esterases (Simon et al., 1985). Microsomal drug metabolism

in a large number of inbred rat strains varied over a 1.3- to 7.3-fold range depending on the enzyme activity being measured (Koster et al., 1989). Fischer 344 (F344) rats were found to be significantly different from SD rats in terms of the effect of cyclosporine on ethylmorphine N-demethylase and aniline hydroxylase activities (Augustine and Zemaitis, 1989). F344 rats also produced substantial liver damage associated with the oxidative stress of diquat while SD rats displayed only minimal liver necrosis (Tsokos-Kuhn, 1988).

Differences in terms of species susceptibility are well documented relative to PAs poisoning. However, little work has been done investigating strain differences in PAs metabolism. We hypothesized that F344 rats would have a different metabolism of PAs compared to SD rats. In this study, we present evidence that F344 rats in both sexes had the greater *in vitro* SN N-oxidation compared to that found with SD rats. Another hepatic enzyme system (FMO but not P450) in F344 strain is also mainly responsible for the N-oxidation of the PA SN in contrast to SD rats.

MATERIALS AND METHODS

Chemicals. SN was purified from extracts of *Senecio jacobaea* and the DHP and N-oxide metabolite standard were prepared as previously described (Kedzierski and Buhler, 1986b). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, 1-benzylimidazole, methimazole, SKF 525A and thiobenzamide were obtained from the Sigma Chemical Co. (St Louis, MO). Testosterone and its metabolites were purchased from Steroid Reference Collection (London, UK).

Microsome Preparation. F344 and SD rats (male and female, 8 weeks old from Simonson Inc. Gilroy, CA) were starved 24 hr prior to killing. Livers were homogenized with 0.1 mM potassium phosphate buffer, pH 7.4, containing 0.15 M KCl and 1 mM EDTA. Microsomes were prepared by standard differential centrifugation and stored in 0.1 M potassium phosphate buffer, pH 7.4, containing 20 % glycerol and 0.1 mM EDTA at -80°C.

Enzyme Assays. The *in vitro* metabolism of SN by microsomes from rat liver was carried out using the procedure described by Miranda et al. (1991a). A typical incubation consisted of 0.3 mg microsomal protein, 0.1 M potassium phosphate buffer, pH 7.6 or 8.6, 0.5 mM SN, 1 mM EDTA and a NADPH-generating system (10 mM glucose-6-phosphate, 1.0 units/mg glucose-6-phosphate dehydrogenase and 1 mM $NADP^+$) in a total volume of 0.5 ml. After a 1 hr incubation (formation of DHP and N-oxide was linear up to 1 hr under

the assay conditions used) at 37°C, the reaction was terminated by rapid cooling on ice. The mixture was centrifuged at 46,000 g for 45 min at 4°C and an aliquot of the supernatant was analyzed by HPLC (Kedzierski and Buhler, 1986a). When inhibitors (1-benzylimidazole, methimazole or SKF 525A) were added, the incubation mixture was first preincubated with the inhibitors at 37°C for 20 min before the addition of SN. Microsomal protein contents were determined by the method of Lowry et al. (1951). Total cytochrome P450 content was estimated using a spectral method (Omura and Sato, 1964). Testosterone hydroxylase assays were carried out by the method of Wood et al (1983). Thiobenzamide S-oxide formation was measured at 370 nm by the procedure of Cashman and Hanzlik (1981) at 37 °C in Tricine buffer (pH 8.4), with 2 mM NADPH, in the absence of n-octylamine. Statistical analysis was carried out using student t-test.

RESULTS

SN metabolism by microsomes

In both sexes, there was no significant difference in DHP formation from the PA SN by hepatic microsomes from SD and F344 rats (Fig. II-1). However, hepatic microsomes from male F344 had a greater SN N-oxide formation of 88% compared to that from male SD rats. Liver microsomes from female F344 also exhibited a 180% enhancement of female SD levels of SN N-oxide formation. These results may imply that different enzyme systems and/or specificities between SD and F344 strains are responsible for SN N-oxidation, or that more enzymes active in SN N-oxidation exist in F344 rats.

Inhibition studies

In order to examine whether different enzyme systems existing in these two strains were responsible for SN N-oxide formation, we employed specific chemical inhibitors for cytochrome P450s and FMOs in SN metabolism by F344 hepatic microsomes. SKF 525A and 1-benzylimidazole, specific inhibitors for P450s, decreased DHP formation by 70-95% in both sexes of F344, however, inhibited SN N-oxidation by only 0-30% in male and 40-45% in female F344 (Table II-1). Williams et al. (1989a) reported that SKF 525A and metyrapone, another inhibitor of P450s, inhibited DHP formation 95% and SN N-oxidation 56-87% in SN metabolism by liver microsomes from male SD rats. Methimazole, a competitive inhibitor for FMO, had no inhibitory activity

against DHP production by hepatic microsomes from both sexes of F344. However, methimazole decreased SN N-oxidation by 85-96% in both sexes of F344. We conclude that DHP formation is catalyzed by the P450 system while SN N-oxide is primarily produced by FMO in both sexes of F344 rats. In SD rats, however, both DHP and SN N-oxide formations are catalyzed by P450 systems (Williams et al., 1989a).

Optimum pH for DHP and SN N-oxide formation

We tried optimum pH tests since P450 and FMO were known to have different optimum pH for maximum activities. The optimum pH for P450 appears to be between 7.2 and 7.6 (Sato and Omura, 1978). This contrasts with FMO which possesses a pH optimum usually around 8.4-9.2 depending on the species and tissue (Ziegler, 1988). DHP was metabolized maximally at pH 7.6-7.8 in both sexes of F344 rats (Fig. II-2 and II-3). However, the maximum for SN N-oxide production in female F344 was pH 8.5 whereas SN N-oxide showed a bimodal pattern with peaks at pH 7.6 and 8.5 in male F344. These results also supported that DHP was catalyzed by P450 while SN N-oxide was primarily produced by FMO in both sexes of F344.

Thiobenzamide S-oxidation

We assayed thiobenzamide S-oxidation for further evaluation of FMO activities in SD and F344 rats. Thiobenzamide is a specific substrate used to

assay for FMO activity (Cashman and Hanzlik, 1981). The specific activities of hepatic microsomes from male F344 thiobenzamide S-oxide was 56% higher than male SD rats while there was no difference between female rats of the two strains (Fig. II-4).

Testosterone hydroxylase activity

We measured testosterone hydroxylase as an indicator substrate for estimation of the relative contributions of different cytochrome P450s. Testosterone 6β-hydroxylase activity, which is mostly catalyzed by cytochrome P4503A2 (Guengerich et al., 1982), is 50% higher in male SD than in male F344 rats (Fig. II-5). On the other hand, testosterone 16α- and 16β-hydroxylase activities, in which P4502C11 and P4502B1 participate (Nebert et al., 1987), are 120% and 84% higher in male F344 than that in male SD rats, respectively. These results indicate that the activity of P4503A2, which is an important enzyme for DHP formation in the SD rats (Williams et al., 1989a), is higher in male SD rats than in male F344 rats. Furthermore, the activity of P4502C11, which is responsible for SN N-oxidation in SD rats, is higher in F344 strain than in SD strain.

In female rats, testosterone 7α- and 6β-hydroxylase activities were 80% and 1500% higher in F344 than those in SD, respectively, whereas 16β-hydroxylase was 75% higher in SD than that in F344. Testosterone 16α-hydroxylase activity was not observed in female SD whereas some activities

were present in female F344. These results denote that the male dominant monooxygenase activities, such as P4503A2 and P4502C11, is much higher in female F344 while the female dominant monooxygenase activity like P4502A1 is higher in female SD than in female F344.

DISCUSSION

There are several possibilities to account for species differences in susceptibility and resistance of some animals to PA toxicity. These include differences in absorption, degradation, hepatic formation of pyrroles or PA N-oxides, or conjugation and excretion of PAs and pyrroles (Mattocks, 1986). Strain differences may be explained in a similar fashion.

We have assayed microsomal activities toward SN in order to verify the hypothesis that there are strain differences in PAs metabolism between F344 and SD rats. While there were no differences in DHP formation by hepatic microsomes in both sexes of SD and F344 rats, both sexes of F344 rats showed the greater N-oxidation of the PA SN compared to that of SD rats. Recently, Pan et al. (1993) demonstrated that F344 rats were resistant to the cardiovascular toxicity of monocrotaline pyrrole at doses that cause severe damage in SD rats, perhaps due to the presence of more refractory pulmonary endothelial cells in F344 rats. It is, therefore, possible that F344 rats may be unusually resistant to the toxicities of PAs, both because of the faster detoxication that we have observed, but also due to having more refractory tissues.

Evidence based on enzyme inhibition data and optimal pH suggests that hepatic microsomal DHP formation was catalyzed primarily by P450 systems in both sexes of F344 rats as previously demonstrated in SD rats (Williams et

al., 1989a) and in guinea pigs (Miranda et al., 1991a). However, SN N-oxidation was primarily catalyzed by FMO in both sexes of F344 rats. This contrasts with SD rats where SN N-oxidation is carried out primarily by P4502C11 (Williams et al., 1989a). This is very similar to the situation in guinea pigs which are well known to be very resistant to PA intoxication (Chesney and Allen, 1973; McLean, 1970; White et al., 1973).

Lawton and Philpot (1993a) suggest that all five FMO genes (FMO1, 2, 3, 4 and 5) present in the rabbit are also present in humans, guinea pig, hamsters, rats and mice based on blot analysis of genomic DNA. With respective to substrate specificities of FMOs, the overall size of the nucleophile appears to be a major factor limiting access to the 4a-hydroperoxyflavin in different FMO isoforms (Nagata et al, 1992; Ziegler, 1993). From the study of the substrate specificities of four FMO isoforms in the SN metabolism using FMO expressed microsomes (FMO1, 2, 3 and 4), which showed distinct substrate specificities against methimazole and n-octylamine (Lawton and Philpot, 1993b), only FMO1 expressed microsome exhibited a minimal activity of SN N-oxidation (Chung et al., 1993d).

Thiobenzamide S-oxidation assays showed that male F344 rats had greater FMO activity compared to that of male SD rats. However, the interpretation of thiobenzamide S-oxidase activity is rather equivocal since there was no difference in FMO activity between females of these two strains, even though FMO appeared to be responsible for SN N-oxidation in female

F344 rats. Therefore, we suggest that F344 rats, at least the females, may have distinct FMO isoforms from SD rats.

The results of the testosterone hydroxylase assays were relatively ambiguous and difficult to interpret. According to the observed testosterone hydroxylase activities, more DHP formation should be occurring with microsomes from male SD rats than with microsomes from male F344 rats. However, SN conversion to DHP was higher in male F344 rats. If P4503A2 and P4502C11 are as important to PA metabolism in F344 rats as they are in SD rats, differences in the levels of these P450s in F344 rats may explain better than FMO the differences in PA metabolism between these rat strains. This equivocal phenomenon may reinforce the need for purification of FMOs and confirmation of substrate specificity toward PAs in both strains.

In conclusion, FMO was the major catalyst for SN N-oxidation whereas P450 systems catalyzed formation of pyrrolic metabolites in Fischer 344 rats. Unlike Sprague-Dawley rats, Fischer 344 rats exhibit SN oxidation that is similar to that of guinea pigs, a species that is resistant to PA toxicity. This study, therefore, demonstrates that substantial differences in PA metabolism can exist between animal strains.

Table II-1. Effect of inhibitors on the senecionine metabolism by hepatic microsomes from Fischer 344 rats.

	% of Control activity*			
	Male		Female	
Inhibitor	DHP	SN NO	DHP	SN NO
SKF 525A	19	124	31	60
1-Benzylimidazole	5	71	18	55
Methimazole	117	15	100	4

*Control activities for DHP are 2.13 and 0.50 nmole/min/mg protein in male and female, respectively. Control activities for SN N-oxide are 5.91 and 0.64 nmole/min/mg protein in male and female, respectively. Reactions carried out at pH 7.6 with SKF 525A (0.5 mM), 1-benzylimidazole (0.1 mM) or methimazole (0.25 mM).

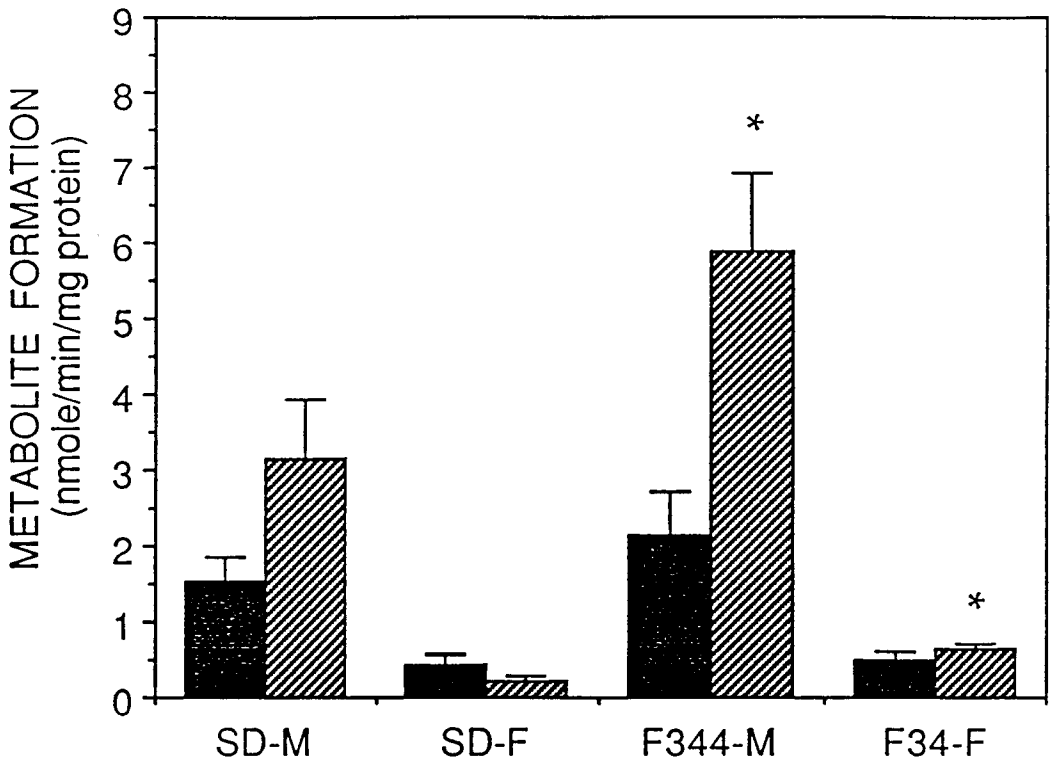

Fig. II-1. Comparison of senecionine metabolism by hepatic microsomes from Sprague-Dawley and Fischer 344 rats (SD, Sprague-Dawley; F344, Fischer 344; M, Male; F, Female; ■, DHP; ▨,SN N-oxide). *Significantly different from the corresponding sex of SD rats ($p<0.05$).

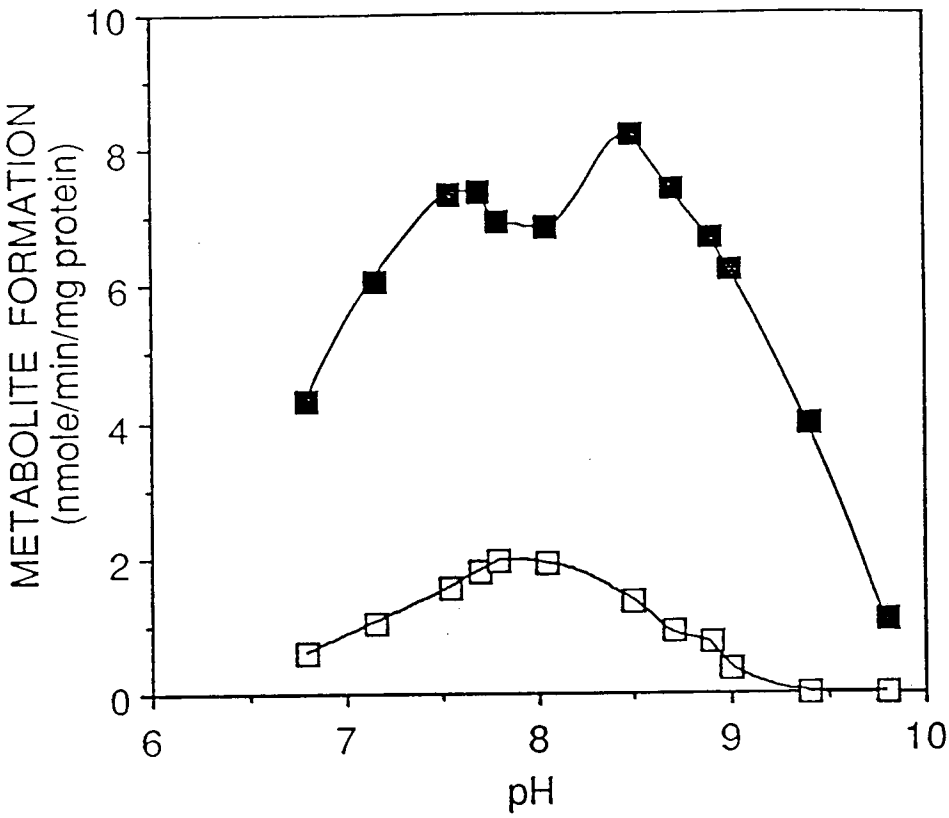

Fig. II-2. Effect of pH on the DHP (□) and senecionine N-oxide (■) formation by liver microsomes from male Fischer 344 rats upon incubation with SN. Reactions were carried out in potassium phosphate (pH 6.8 to 8.2), tricine (pH 8.4 to 8.8) or glycine (pH 0.0 to 9.8) buffer.

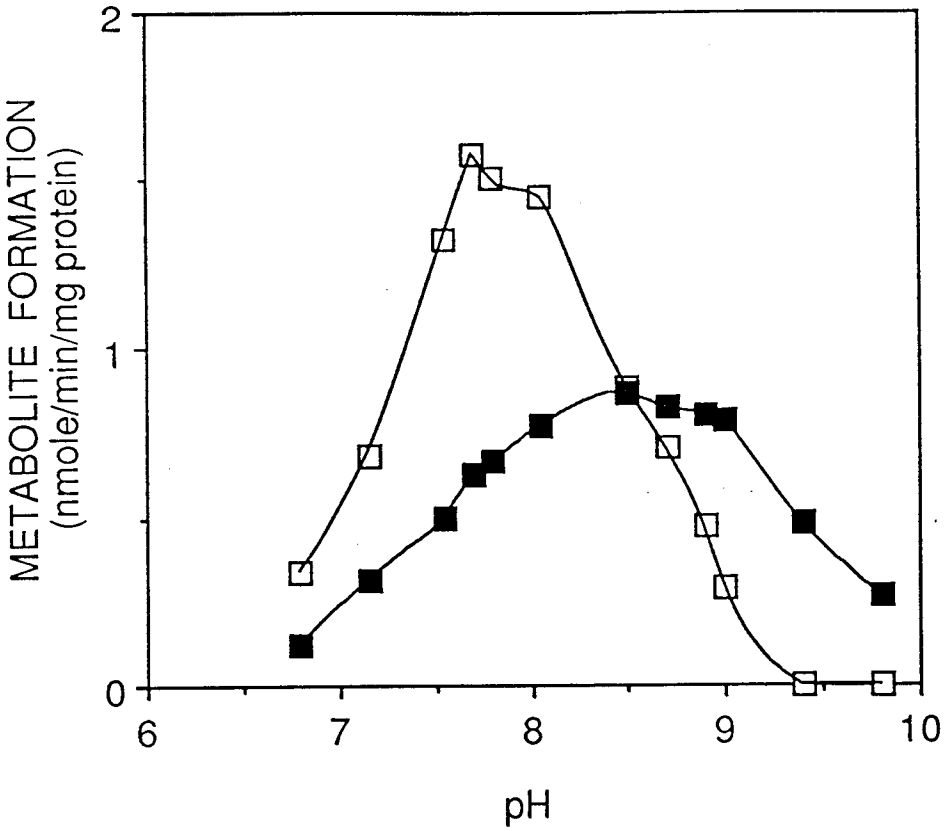

Fig. II-3. Effect of pH on the DHP (□) and senecionine N-oxide (■) formation by liver microsomes from female Fischer 344 rats upon incubation with SN. Reactions were carried out in potassium phosphate (pH 6.8 to 8.2), tricine (pH 8.4 to 8.8) or glycine (pH 0.0 to 9.8) buffer.

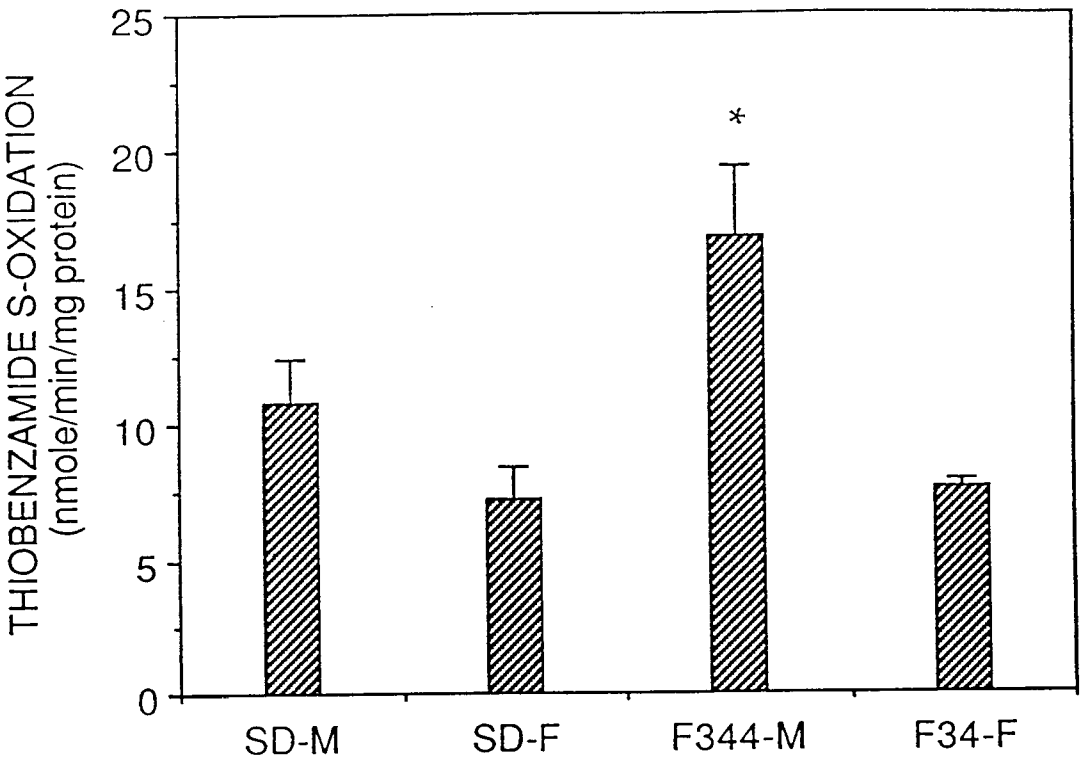

Fig. II-4. Comparison of thiobenzamide S-oxidation by hepatic microsomes from Sprague-Dawley and Fischer 344 rats (SD, Sprague-Dawley; F344, Fischer 344; M, Male; F, Female). *Significantly different from the corresponding sex of SD rats ($p<0.05$).

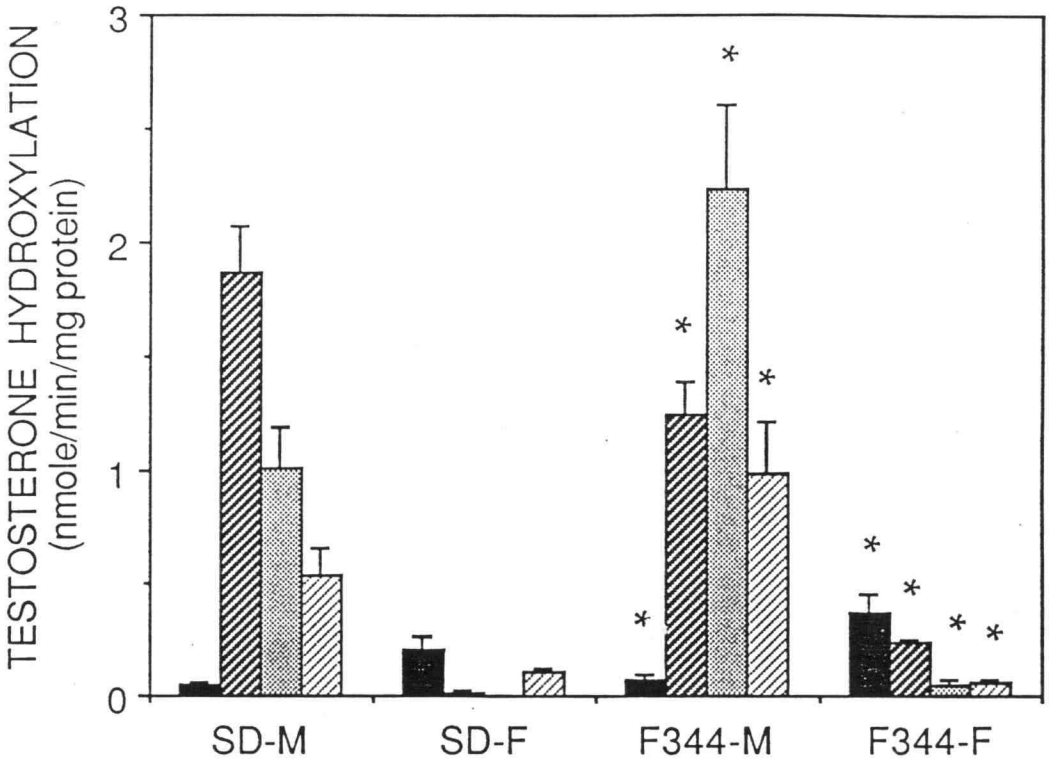

Fig. II-5. Comparison of testosterone metabolism by hepatic microsomes from Sprague-Dawley and Fischer 344 rats [SD;Sprague-Dawley, F344;Fischer 344, M;Male, F;Female, 7α- (■), 6β- (▨), 16α- (▩) and 16β-hydroxytestosterone (▨)]. *Significantly different from the corresponding sex of SD rats ($p<0.05$).

III. METABOLISM OF THE PYRROLIZIDINE ALKALOID SENECIONINE BY HEPATIC MICROSOMES FROM SPIRONOLACTONE TREATED RATS AND GUINEA PIGS[1]

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[1]Preliminary results of this study were presented at the 1992 meeting of the Society of Toxicology, Seattle, WA. This study was supported by NIH Grants CA22524 and ES00210. This manuscript was assigned Oregon Agricultural Experiment Station No. 10,042.

Abstract

Spironolactone (SPL), a known inducer of cytochrome P4503A1, was injected into rats and guinea pigs in order to investigate species differences in pyrrolizidine alkaloid (PA) metabolism. Liver microsomes from treated male rats showed an increased (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) formation of 47 % from the PA senecionine (SN) coincident with the induction of P4503A1, whereas SN N-oxidation was decreased by 49 %, probably due to a reduction in hepatic P4502C11 concentrations. By contrast, liver microsomes from SPL-treated female rats exhibited almost a 500 % increase in both DHP and SN N-oxide production coincident with the marked induction of P4503A1. In guinea pigs of both sexes, oral treatment with SPL caused an approximately 50 % increased formation of both DHP and SN N-oxide by liver microsomes. Only a slight increase in hepatic concentrations of P4503A1 occurred in the treated guinea pigs. Use of the P4503A1 specific inhibitor, triacetyloleandomycin, showed that P4503A1 played the important role in PA metabolism in untreated or SPL treated rats but not in both untreated and SPL treated guinea pigs. SPL treatment increased testosterone 16β-hydroxylase activity by 100% in both sexes of guinea pigs. This study demonstrated that P4503A was not the major enzyme for PA metabolism in guinea pigs.

Pyrrolizidine alkaloids (PAs) occur in plants from the families: Boraginaceae, Compositae and Leguminosae and some of these alkaloids are toxic to animals and humans (Mattocks, 1986). While PAs produce a diversity of biological effects, the principal pathology is irreversible liver cirrhosis with pronounced fibrosis and biliary hyperplasia. Most of the other tissue lesions and signs of toxicity, including mortality, can be related to impaired liver function.

There is, however, a marked variation in the sensitivity of animal species to the toxic effects of PAs. Rats are highly susceptible, whereas guinea pigs are very resistant to PA intoxication (McLean, 1970; Chesney and Allen, 1973; White et al., 1973). PAs are metabolized by hepatic monooxygenases, located in the endoplasmic reticulum, yielding PA pyrroles and N-oxides (Mattocks and White, 1971). PA pyrroles are considered the ultimate toxic metabolites, whereas PA N-oxides are more water soluble and relatively non-toxic (Mattocks, 1971). 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 (dehydroretronecine; DHP).

In rats (Williams et al., 1989a) and humans (Miranda et al., 1991a), the liver microsomal formation of the PA senecionine (SN) to DHP and SN N-oxide is catalyzed primarily by enzymes belonging to the cytochromes P4503A and P4502C11, respectively. In guinea pigs, however, the orthologues of P4502B1 and P4503A participate in DHP formation by hepatic microsomes (Miranda et

al., 1992). Flavin-containing monooxygenase (FMO) accounts for no more than 20 % of the PA N-oxidase activity of Sprague-Dawley rat liver microsomes (Williams et al., 1989b), but this enzyme is the major catalyst for N-oxidation of SN in guinea pigs (Miranda et al., 1991b).

Liver microsomes from dexamethasone (an inducer of cytochrome P4503A) treated guinea pigs increased the conversion of SN to DHP only by 37% (Miranda et al., 1992). We have studied the effect of spironolactone (SPL), another P4503A inducer (Heuman et al., 1982), on the metabolism of SN in guinea pigs and rats in order to evaluate the role of P4503A and investigate further species differences.

Materials and Methods

Chemicals.

SN was purified from extracts of *Senecio jacobaea* and dehydroretronecine (DHR) and SN N-oxide metabolite standards were prepared as described previously (Kedzierski and Buhler, 1986). Spironolactone (SPL) and triacetyloleandomycin (TAO) were obtained from Sigma Chemical Co. (St. Louis, MO). The chemicals and materials used in Western blotting and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA) and [^{125}I]-Protein A was obtained from ICN Radiochemicals (Irvine, CA).

Treatments of animals.

SPL was injected i.p. into male and female Sprague-Dawley rats (200-220 g, Simonson Inc., Gilroy, CA) and administered i.p. or orally to Hartley guinea pigs (400-450 g, Simonson Inc.) at 100 mg/kg body weight in propylene glycol (50 mg/ml) for i.p. or in saline (10 mg/ml) for oral administration, twice daily, for 3 days. Control animals received only vehicle. Animals were starved 24 hr prior to killing.

Microsome preparation.

Animals were anesthetized with carbon dioxide and killed by cervical dislocation. The livers were immediately removed and homogenized in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 0.15 M KCl and 1 mM EDTA. Microsomes were prepared by an initial centrifugation at 9,000 g for 30 min at 4 °C, with subsequent centrifugation of the supernatant at 105,000 g for 60 min at 4 °C. The microsomal pellet was recentrifuged in the homogenization buffer, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20 % glycerol (v/v) and 0.1 mM EDTA and then stored at - 80°C until used.

Metabolism of SN by microsomes.

The typical incubation with microsomes consisted of 0.3 - 0.5 mg microsomal protein, 0.1 M potassium phosphate buffer, pH 7.6, 0.5 mM SN, 1 mM EDTA, and a NADPH-generating system (10 mM glucose-6-phosphate, 1.0 unit/ml glucose-6-phosphate dehydrogenase and 1 mM $NADP^+$) in a total volume of 0.5 ml. After 1 hr incubation at 37°C with shaking (100 cycle/min), the reaction was terminated by rapid cooling on ice. The mixture was centrifuged at 46,000 g for 45 min at 4°C and an aliquot of the supernatant was analyzed by HPLC (Kedzierski and Buhler, 1986a). When

triacetyloleandomycin (TAO, 0.02 mM) was used to inhibit the reaction, the mixture was first preincubated with the inhibitor at 37°C for 20 min before the addition of SN.

Other assays.

Testosterone metabolism by microsomes was carried out by modification of the procedure described by Wood et al. (1983). Protein was determined as described by Lowry et al. (1951) with bovine serum albumin as standard. The method of Omura and Sato (1964) was used for the determination of total P450 content. Immunoblotting of microsomal proteins was performed using anti-rat P4503A IgG (Oxygene, Dallas, TX), anti-rat P4502B1 IgG, anti-rat P4502C11 IgG and anti-pig FMO IgG with detection of immunoreactive proteins by [^{125}I]-protein A as previously described (Miranda et al., 1991b). Statistical analysis was carried out using student's t-test.

Results

Effect of SPL on cytochrome P450 isozymes.

We observed no significant SPL-induced changes in the P450 content of liver microsomes in rats or guinea pigs of either sex (Fig. III-1). However, SPL treatment of male rats caused a 274% increase of 7α-testosterone hydroxylase activity [for which P4502A1 is presumed responsible (Waxman, 1988)] (Fig. III-2). 6β-Testosterone hydroxylase activity (in which P4503A1 participates) was not significantly increased by SPL treatment in male rats. SPL dosing of male rats also decreased 16α- and 16β-hydroxylation of testosterone (for which P4502C11 and P4502B1 are responsible, respectively) by 88% and 50%, respectively. In contrast, SPL treatment of female rats resulted in a 89%, 9900% and 247% increase of 7α-, 6β- and 16β-hydroxylation of testosterone, respectively, whereas 16α-testosterone hydroxylase was not detectable in either untreated or SPL treated animals (Fig. III-2).

In contrast, i.p. injection of SPL increased 16β-testosterone hydroxylation by 108% while 7α-, 6β- and 16α-hydroxylase activities were unchanged in male guinea pigs (Fig. III-3). SPL treatment (i.p.) did not affect testosterone metabolism we detected in female guinea pigs. Oral treatment of SPL increased 16β-testosterone hydroxylase activity by 121% and 103% in

male and female guinea pigs, respectively, whereas 7α-, 6β- and 16α-hydroxylase activities were unchanged (Fig. III-4).

Immunochemical detection of cytochrome P450s using anti-rat P4503A IgG showed that SPL treatment caused a large increase in the levels of P4503A in both sexes of rats and a relatively small induction of P4503A in both sexes of guinea pigs, dosed either orally or i.p. (Fig. III-5). Anti-rat P4503A IgG cross-reacted with two bands while the upper band increased due to SPL treatment in guinea pigs. The concentration of P4502B decreased by about 12% in SPL-treated (i.p.) male rats and i.p. injected female guinea pigs, while it increased in female rats (99%), i.p. injected male guinea pigs (24%), oral dosed male guinea pigs (23%) and oral dosed female guinea pigs (17%) as estimated by densitometric analysis of western blots using anti-rat P4502B1 IgG (Fig. III-5). A 26% reduction of cytochrome P4502C11 concentrations, a male dominant isoform in rats, was seen in SPL treated male rats as estimated by densitometric analysis of western blots using anti-rat P4502C11 IgG (Fig. III-5). Western blot using anti-pig FMO IgG showed that SPL treatment would not affect the level of FMO in both sexes of guinea pigs (data not shown).

Effect of SPL on DHP formation.

DHP formation from SN by SPL treated liver microsomes from male rats increased by 47% (Fig. III-6). By contrast, SPL treatment resulted in a 475%

increase ($p < 0.01$) in DHP formation by hepatic microsomes from female rats, effectively eliminating the sex differences in conversion of SN to DHP seen in untreated animals. Inhibition studies with TAO, which is an specific inhibitor of P4503A1 (Wrighton et al., 1985; Delaforge et al., 1983), also showed that the induced P4503A1 contributed to enhanced DHP production in SPL treated rats of both sexes (Table III-1).

In guinea pigs, i.p. injection of SPL increased DHP formation by 66% in males but not significantly in female animals (Fig. III-6). No differences in DHP formation by male and female guinea pig liver microsomes were observed upon oral administration of SPL. Inhibition of DHP formation with TAO was only 11% to 28% in untreated male and female guinea pigs (Table III-2). Inhibition with TAO also showed that P4503A contributed to only 20% to 27% of the DHP production by microsomes from SPL treated guinea pigs, by either oral or i.p.

Effect of SPL on SN N-oxidation.

SN N-oxide formation was decreased by 49 % after SPL treatment of male Sprague-Dawley rats (Fig. III-7), probably due to a reduction of P4502C11 (Fig. III-5). Inhibition studies with TAO showed that absolute SN N-oxide production (the difference in N-oxide formation in the presence and absence of TAO) by P4503A1 in treated male rats was almost the same as that observed

in untreated male rats (Table III-1). This was quite interesting since it imply that the increased levels of P4503A1 induced by SPL did not contribute to SN N-oxidation in treated male rats. Unlike in males, SPL treatment of female Sprague-Dawley rats increased SN N-oxidation by 525 % with hepatic microsomes (Fig. III-7), probably as a result of an apparent induction of P4503A1 (Table III-1 and Fig. III-5). Inhibition studies also showed that the relative importance of P4503A1 in SN N-oxidation increased after SPL treatment in both sexes of rat, whereas the relative role of P4503A1 in DHP formation remained unchanged after SPL treatment (Table III-1).

In guinea pigs, oral administration of SPL resulted in 85 % and 55 % increases in hepatic microsomal SN N-oxidation, respectively, whereas i.p. injection caused no significant increase in male and female animals, respectively (Fig. III-7). Inhibition studies with TAO showed that P4503A did not contribute significantly to SN N-oxidation in untreated and SPL treated guinea pigs of either sex (Table III-2).

Discussion

There are some conflicting reports in the literature on the effects of SPL on the P450 content in rat liver microsomes. Oral administration of SPL at 100 mg/kg, twice daily for 4 days, decreased the hepatic P450 content of female rats but not of male rats (Fujita et al., 1982). Another study showed oral dosing with SPL at 100 mg/kg daily for 4 days to female rats produced no significant increases in total hepatic P450 (Heuman et al., 1982). A single i.p. administration of SPL at 150 mg/kg to male rats led to a 21 % loss of hepatic microsomal P450 after 3 hr (Decker et al., 1986). SPL has been shown to act initially as a suicide substrate and subsequently as an inducer of the P4503A subfamily being more potent in adrenal and testicular tissue than liver (Sherry et al., 1986). In this study, however, we observed no significant changes in liver microsomal P450 content in either sex of rats or guinea pigs (Fig. III-1). Therefore, we agree with the conclusion of the Sherry et al. (1986) study.

In Sprague-Dawley rats, the conversion of SN to DHP is catalyzed primarily by the male dominant P4503A subfamily (Williams et al., 1989a). DHP formation in untreated female rats is quite low due to the limited presence of this isozyme. However, pretreatment with SPL eliminated the sex difference in DHP formation causing a 10-fold greater increase in female rats than in males. Inhibition studies showed that P4503A in treated females was

responsible for 82 % of the DHP production. The induced level of P4503A paralleled the increased DHP production in female rats.

The orthologues of cytochromes P4502B1 and P4503A have been shown to participate in DHP formation in guinea pig liver microsomes (Miranda et al., 1992). In guinea pigs, only male liver microsomes from i.p. injection of SPL increased DHP formation significantly. Inhibition studies with TAO showed that P4503A played a minimal role in DHP formation in both untreated and SPL treated guinea pigs. Phenobarbital treatment increased DHP formation by 220% in male guinea pigs (Chung and Buhler, 1993c). These results may imply the possibility that in the guinea pig P4502B1 is more important than P4503A in the bioactivation of SN.

SN N-oxide formation was decreased following SPL treatment of male rats. This phenomenon is quite interesting because treatment with dexamethasone, another P4503A inducer, increased both DHP and SN N-oxide formation (Williams et al., 1989a). The N-oxidation of SN in male Sprague-Dawley rats is catalyzed primarily by P4502C11 and P4503A2. Cytochrome P4503A1 induced by SPL apparently did not contribute to SN N-oxidation. Instead, the decrease of SN N-oxidation may have been primarily due to the loss (69 %) of P4502C11 activity in male rats. A decrease in rat P4502C11 concentrations following dosing with 3,4,5,3',4',5'-hexachlorobiphenyl or 3-methylcholanthrene was reported to be due to decreased hepatic P4502C11 mRNA concentrations (Yeowell et al., 1987). Thus, there is a possibility that

SPL may repress the transcription of P4502C11 although SPL produces less striking effects than the compounds that interact with the Ah receptor.

Unlike the situation in male rats, induced P4503A in female rats played an equally important role in both the activation and detoxication of SN, as reflected by the 5-fold increase of both DHP and SN N-oxide production. SPL treatment also eliminated the sex difference of rats in SN N-oxidation, however, in this case, female rats increased but male rats decreased.

Unlike DHP formation, only oral administration of SPL resulted in significant increase of SN N-oxidation in guinea pigs. However, inhibition studies with TAO showed that P4503A did not contribute to SN N-oxidation in guinea pigs. FMO, a major enzyme for SN N-oxidation in the guinea pig, was not induced by SPL treatment. SPL treatment increased P4502B activity by over 100% upon oral administration with SPL in both sexes of guinea pigs. P4502B may be responsible for the increased SN N-oxidation. However, why the increased testosterone 16β-hydroxylase activity in male guinea pigs with i.p. injection of SPL is not reflected by enhanced SN N-oxidation still remains to be answered.

These results showed that the metabolism of SN in guinea pigs could be affected by inducing P450 isoforms even though FMO, which is generally not inducible by xenobiotics, is a major enzyme for N-oxide formation in this species (Miranda et al., 1991b).

In conclusion, our studies showed that SPL caused a marked induction of P4503A in rats but only a slight effect in guinea pigs. Furthermore, the induced P4503A played an important role in bioactivation and detoxification of the PA SN in rats but not in guinea pigs. In addition, SPL treatment also reduced the level of P4502C11, another important enzyme in the detoxification of SN by male Sprague-Dawley rats. SPL treatment was shown to eliminate the sex differences in SN metabolism found in rats. Oral treatment with SPL increased testosterone 16β-hydroxylase activity in guinea pigs. This study demonstrated that the cytochrome P4503A subfamily, an important enzyme for bioactivation of the PA SN in Sprague-Dawley rats, was not the major enzyme responsible for PA metabolism in guinea pigs.

Table III-1. Effect of triacetyloleandomycin, an inhibitor of cytochrome P4503A1, on senecionine metabolism by rat liver microsomes.

	Male			Female		
	-TAO	+TAO	%Inhibition	-TAO	+TAO	%Inhibition
DHP						
-SPL	1.55	0.36	77%	0.44	0.15	66%
+SPL	2.28	0.68	70%	2.51	0.45	82%
SN N-oxide						
-SPL	3.15	2.08	34%	0.23	0.21	9%
+SPL	1.62	0.65	60%	1.47	0.28	81%

Activities are expressed as nmole/min/mg protein. The values represent the means of duplicate determinations [-TAO, without triacetyloleandomycin; +TAO, with triacetyloleandomycin (0.02 mM); -SPL, untreated; +SPL, SPL pretreated].

Table III-2. Effect of triacetyloleandomycin, an inhibitor of cytochrome P4503A1, on senecionine metabolism by guinea pig liver microsomes.

	Male			Female		
	-TAO	+TAO	%Inhibition	-TAO	+TAO	%Inhibition
DHP						
I.P.						
-SPL	0.70	0.61	13%	0.74	0.65	12%
+SPL	1.15	0.86	23%	0.93	0.74	20%
Oral						
-SPL	0.43	0.38	11%	0.70	0.50	28%
+SPL	0.65	0.47	27%	1.10	0.80	27%
SN N-oxide						
I.P.						
-SPL	2.39	2.51	-5%	2.52	2.39	5%
+SPL	2.86	2.95	-3%	2.80	2.99	-7%
Oral						
-SPL	2.02	1.80	11%	2.23	2.43	-9%
+SPL	3.73	3.51	6%	3.44	3.47	-1%

Activities are expressed as nmole/min/mg protein. The values represent the means of duplicate determinations [-TAO, without triacetyloleandomycin; +TAO, with triacetyloleandomycin (0.02 mM); -SPL, untreated; +SPL, SPL pretreated].

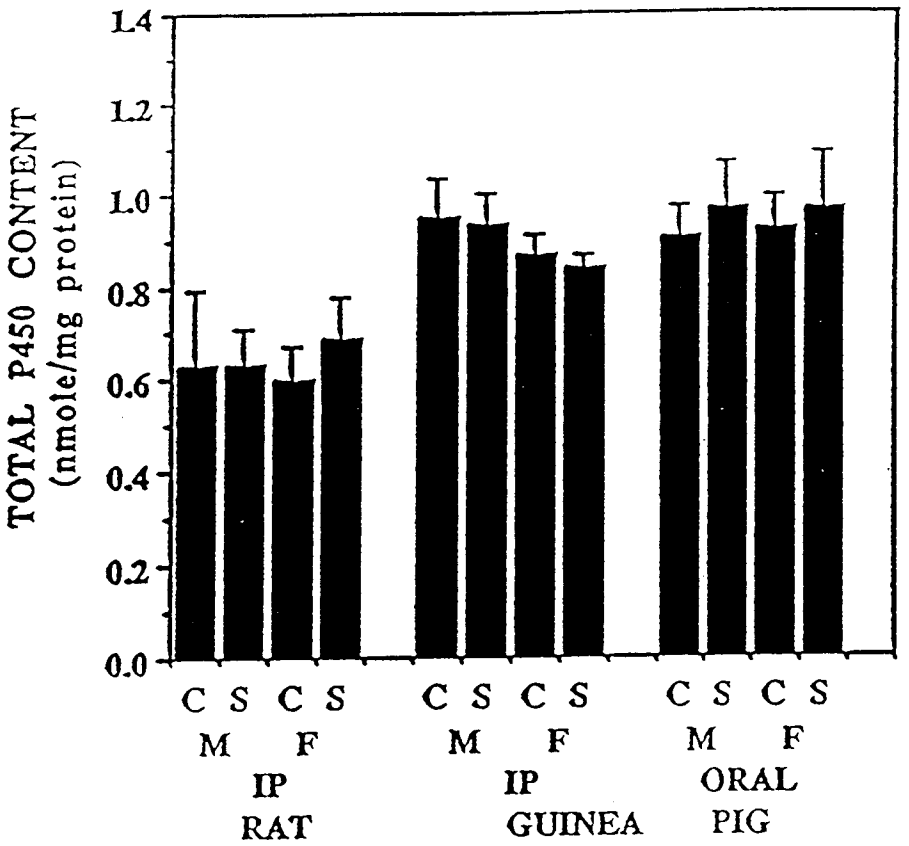

Fig. III-1. The total cytochrome P450 content of liver microsomes from rats and guinea pigs. Values represent means of four animals ± SE (C, control; S, SPL treated; M, male; F, female)

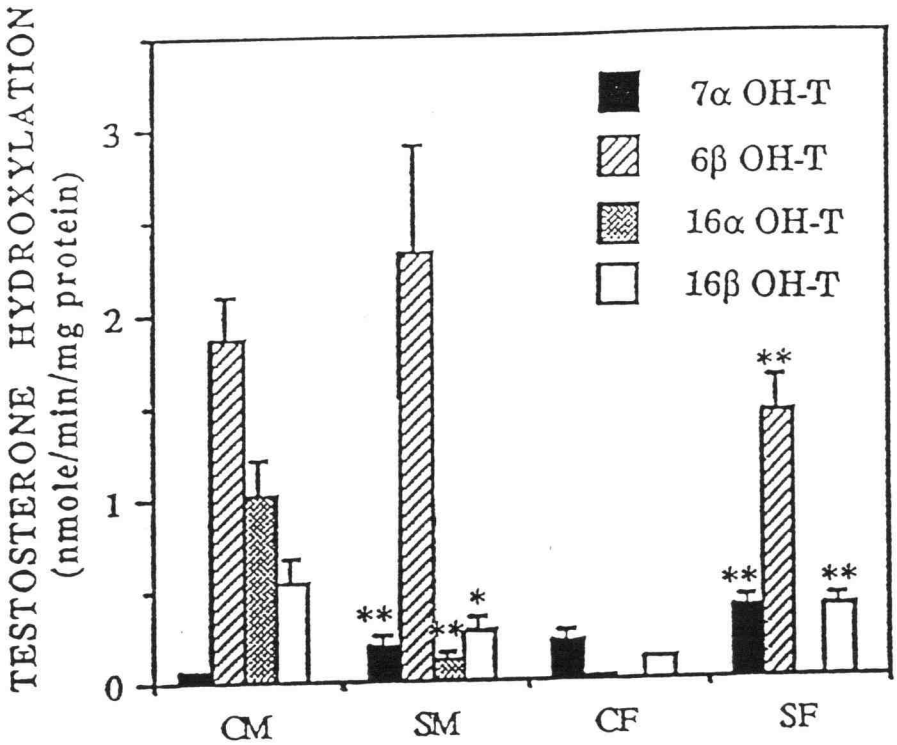

Fig. III-2. Testosterone metabolism by rat liver microsomes. Values represent means of four animals ± SE (CM, control male; SM, SPL treated male; CF, control female; SF, SPL treated female; 7α, 6β, 16α and 16β represent the position of hydroxylation of testosterone, i.e. 7α OH-T means 7α-hydroxytestosterone). Asterisk indicates significant difference from controls (*$p < 0.05$; **$p < 0.01$, Student's t-test).

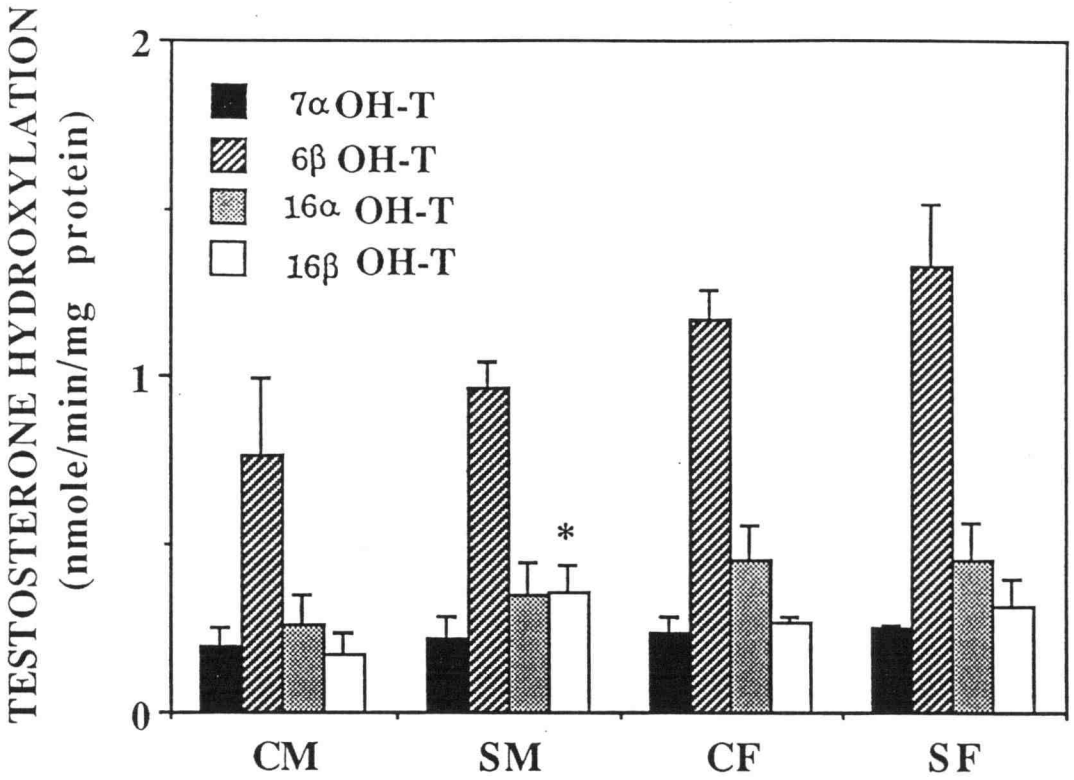

Fig. III-3. Testosterone metabolism by liver microsomes from SPL treated, i.p., guinea pigs. Values represent means of four animals ± SE (CM, control male; SM, SPL treated male; CF, control female; SF, SPL treated female; 7α, 6β, 16α and 16β represent the position of hydroxylation of testosterone). Asterisk indicates significant difference from controls (*$p < 0.05$, Student's t-test).

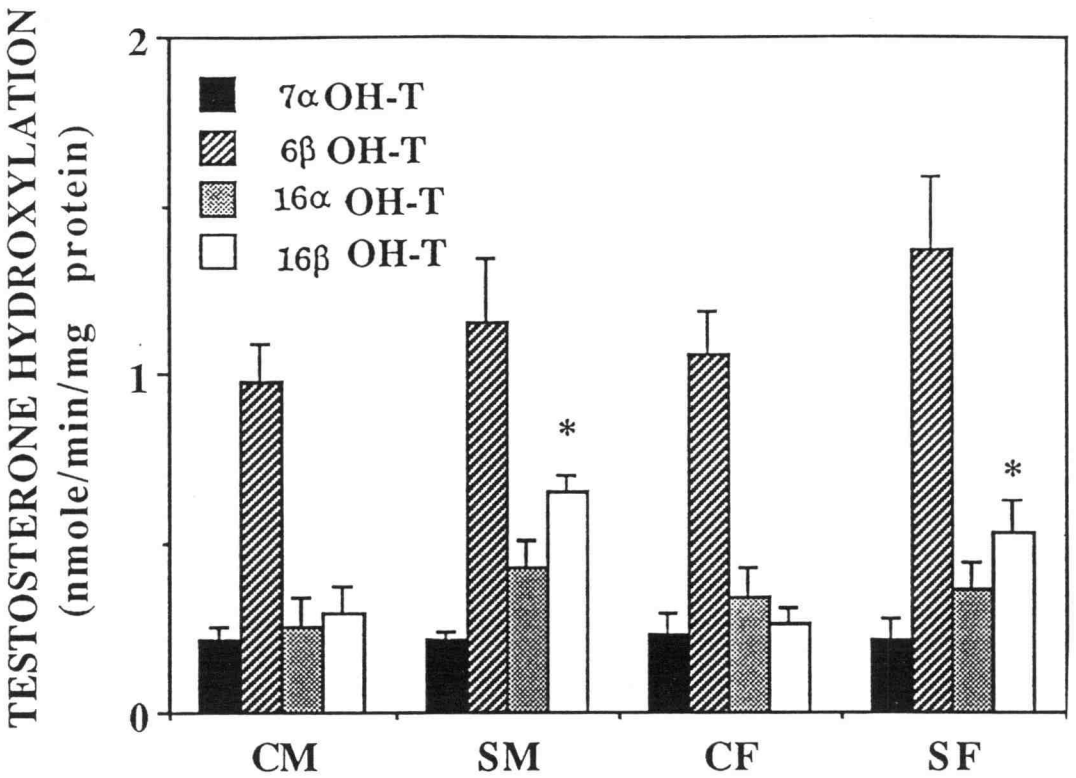

Fig. III-4. Testosterone metabolism by liver microsomes from SPL treated, oral dosing, guinea pigs. Values represent means of four animals ± SE (CM, control male; SM, SPL treated male; CF, control female; SF, SPL treated female; 7α, 6β, 16α and 16β represent the position of hydroxylation of testosterone). Asterisk indicates significant difference from controls (*$p < 0.05$, Student's t-test).

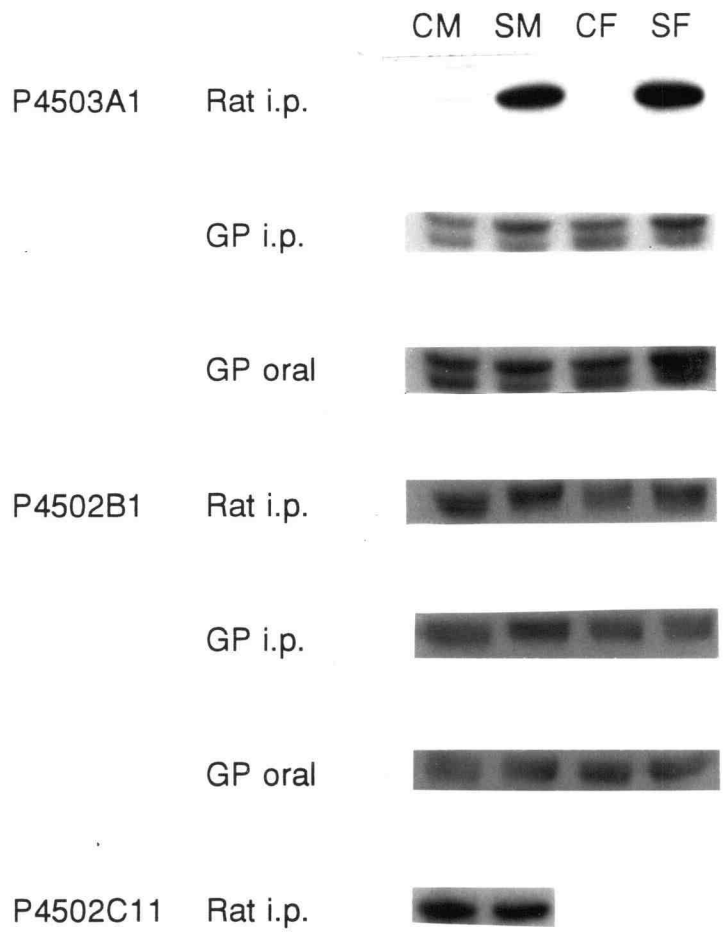

Fig. III-5. Immunochemical detection of cytochrome P4503A1, P4502B1 and P4502C11 in liver microsomes from rats and guinea pigs. Each lane contains 20 μg of pooled microsomes from each treated group (CM, control male; SM. SPL treated male; CF, control female; SF, SPL treated female).

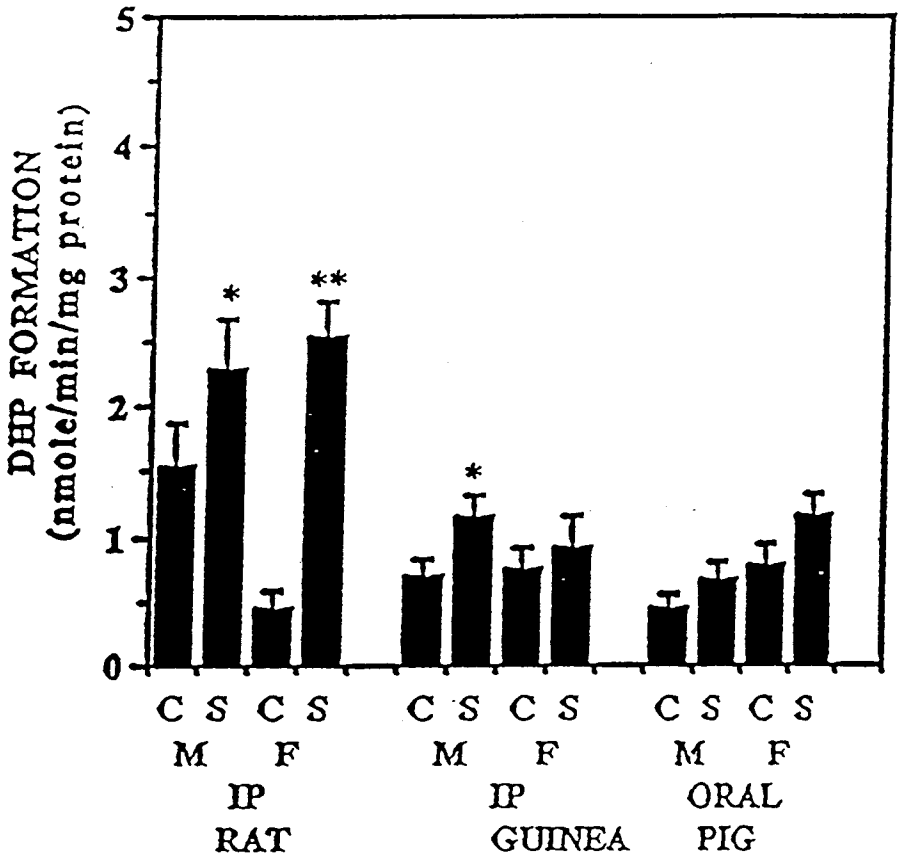

Fig. III-6. DHP formation from senecionine by liver microsomes from rats and guinea pigs. Values represent means of four animals ± SE (C, control; S, SPL treated; M, male; F, female). Asterisk indicates significant difference from controls (*$p < 0.05$; **$p < 0.01$, Student's t-test).

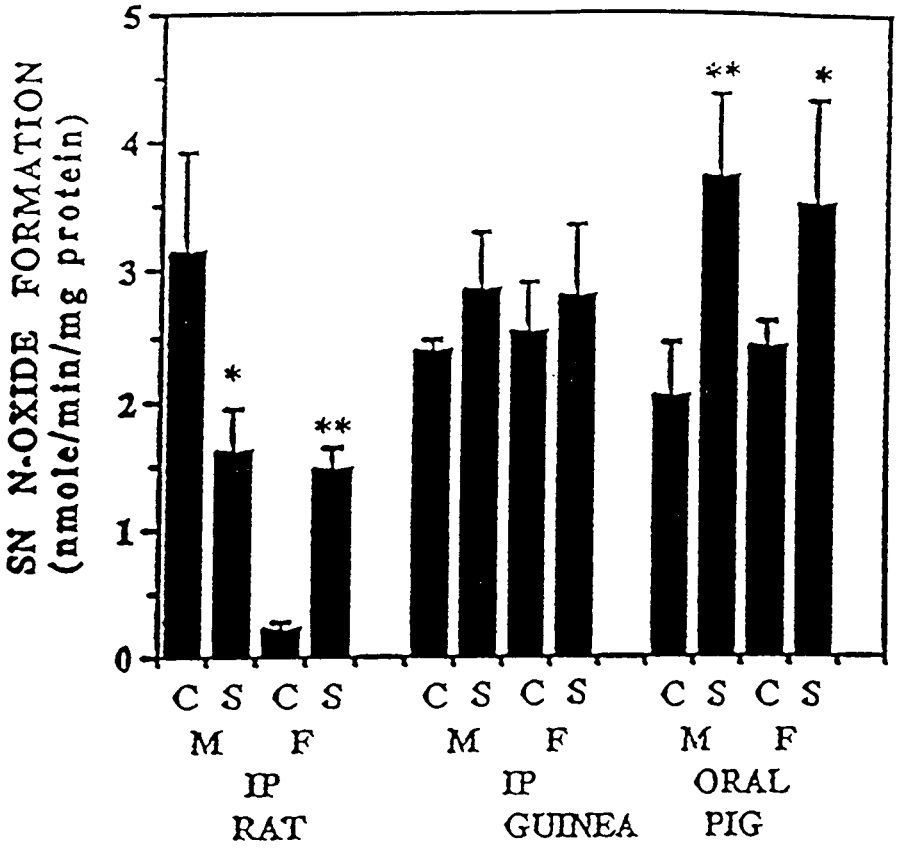

Fig. III-7. N-oxide formation from senecionine by liver microsomes from rats and guinea pigs. Values represent means of four animals ± SE (C, control; S, SPL treated; M, male; F, female). Asterisk indicates significant difference from controls (*$p < 0.05$; **$p < 0.01$, Student's t-test).

IV. CYTOCHROME P4502B IS A MAJOR BIOACTIVATION ENZYME FOR THE PYRROLIZIDINE ALKALOID SENECIONINE IN GUINEA PIG

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Abstract

1. Guinea pigs are well documented to be resistant to the toxicity of pyrrolizidine alkaloids (PAs). We have purified three cytochrome P450s from the liver of phenobarbital (PB) treated guinea pig in order to evaluate the role of these enzymes in PAs metabolism.

2. Reconstituted guinea pig cytochrome P4502B (M_r=57,512 by mass spectrometry; PB inducible) catalyzed the conversion of the PA senecionine (SN) to the pyrrolic metabolite (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP, activation) and SN N-oxide (detoxication) at a minimal rate even though hepatic microsomes from PB treated guinea pigs increased DHP formation by 224% and SN N-oxidation by 70%.

3. A cytochrome P4503A form (M_r=54-56,000 by SDS-PAGE) had lost its activity during the final purification processes. However, examination of the catalytic activity of semipurified guinea pig P4503A showed substantial conversion of SN to DHP. A purified cytochrome P4502C type (M_r=56,496 by mass spectrometry) isoform produced SN N-oxide from SN at the rate of 13.3 nmole/min/mg protein but little DHP formation was detectable.

4. Immunoinhibition of SN metabolism by rabbit anti-guinea pig cytochrome P4502B, P4502C and P4503A IgG indicated that P4502B played

the most important role (over 70%) in bioactivation of SN in both untreated or PB-treated guinea pig while the P4502C and P4503A isoforms seem to have little influence (around 13%). P4502B also contributed along with flavin-containing monooxygenase to detoxification of SN in both untreated (34%) and PB treated (40%) guinea pig.

5. This study suggests that cytochrome P4502B plays the most important role in SN bioactivation in the guinea pig.

Pyrrolizidine alkaloids (PAs) are found in a large number of plant species occurring throughout the world (McLean, 1970). Many of these alkaloids are cytotoxic and carcinogenic and these are often responsible for poisoning livestock and people (Mattocks, 1986). PAs are metabolized by liver monooxygenases yielding PA pyrroles and N-oxides (Mattocks and Bird, 1983a). PA pyrroles are considered the ultimate toxic metabolites whereas PA N-oxides are believed to be non-toxic. The PA pyrroles either react with cellular macromolecules or are hydrolyzed to form a secondary pyrrole known as (±) 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP). DHP also is a strong electrophile capable of alkylating cellular nucleophiles including DNA (White and Mattocks, 1972).

Similar toxic actions of PAs have been demonstrated in mary animal species, but there may be large quantitative differences (White et al., 1973). Guinea pigs are very resistant to the toxicity of PAs (Chesney and Allen, 1973; White et al., 1973), but curiously not to that of jacobine (Swick et al., 1982). Miranda et al. (1991a) reported that the rate of N-oxidation from the PA senecionine (SN) by hepatic flavin-containing monooxygenase (FMO) was much higher in the guinea pig than in the rat. Dueker et al. (1992a) demonstrated that the guinea pig hepatic microsomal carboxylesterases had the capability of hydrolysis of PAs while the rat exhibited no such hydrolytic capability. We have previously shown that the combination of high pyrrole and a low N-oxide

formation together with little hydrolysis were major factors in the susceptibility of guinea pigs to intoxication by the PA jacobine (Chung and Buhler, 1993a).

In Sprague-Dawley (SD) rats (Williams et al., 1989) and humans (Miranda et al., 1991b), the microsomal formation of pyrrole from SN is catalyzed primarily by enzymes belonging to the cytochrome P4503A subfamily. FMO accounts for no more than 20% of the PA N-oxidase activity of SD rat liver microsomes (Williams et al., 1989b). However, DHP formation from SN increased by only 47% in liver microsomes from dexamethasone (DEX) treated (P4503A inducible) guinea pigs while phenobarbital (PB) treatment (P4502B inducible) increased DHP production from SN by 270% (Miranda et al., 1992). We have previously demonstrated that spironolactone treatment (another inducer of P4503A) also increased pyrrole formation by only 50% from SN in hepatic guinea pig microsomes (Chung and Buhler, 1993b).

Therefore, based on these findings we hypothesized that cytochrome P4502B isozyme was a more important enzyme in the guinea pig than P4503A in the bioactivation of PAs. To verify this conclusions, we have purified liver P450s from PB treated guinea pigs and carried out *in vitro* metabolism with SN in order to assess their importance in bioactivation and detoxification of this PA. We now present evidence that cytochrome P4502B plays the most important role in SN bioactivation in the guinea pig.

Materials and Methods

Chemicals

SN was purified from extracts of *Senecio jacobaea* and the DHP and N-oxide metabolite standard were prepared as previously described (Kedzierski and Buhler 1986b). Cholic acid from Sigma Chemical Co. (St. Louis, MO) was recrystallized from ethanol. Dilauroyl-L-phosphatidylcholine (DLPC), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, Lubrol PX, $NADP^+$, NADPH, phenobarbital (PB), Triton N-101 and *w*-aminooctylagarose (AOA) were obtained from the Sigma Chemical Co. (St Louis, MO). DEAE Sephacel was purchased from Pharmacia (Sweden). Hydroxylapatite (HAP) was obtained from Bio-Rad Laboratory (Richmond, CA).

Microsomes Preparation

Hartley guinea pigs (425 g, male and 8 weeks old from Simonson Inc. Gilroy, CA) were treated with 80 mg/kg of PB (solubilized with 0.07 N NaOH in saline) for 4 days. Animals were starved for 24 hr prior to killing. Liver was homogenized with 0.1 mM potassium phosphate buffer, pH 7.4, containing 0.15 M KCl and 1 mM EDTA. Microsomes were prepared by standard differential centrifugation and stored in 0.1 M potassium phosphate buffer, pH 7.4, containing 20 % glycerol and 0.1 mM EDTA at -80°C.

Cytochrome P450 Purification

Guinea pig cytochrome P450s were purified by the method of Guengerich and Martin (1980) and Oguri et al. (1991) with the following modifications (Fig. IV-1). Briefly, the solubilized microsomes (0.6% cholate) were applied onto an *w*-aminooctylagarose (AOA) column (2.6 x 21.5 cm). The P450s were eluted with 0.1 M KP*i* buffer (pH 7.25) containing 0.08% Triton N-101 and 0.33% sodium cholate. The eluent produced two peaks (AOA 1 and AOA 2) detected by absorbance at 417 nm (Fig. IV-2). Peak AOA 1 and AOA 2 fractions were collected, pooled, concentrated and then loaded onto a DEAE Sephacel (DEAE) column (2.6 x 37 cm), respectively, and eluted with a linear gradient of 0 to 0.2 M NaCl in 0.01 M KP*i* buffer (pH 7.7) containing 0.1% Lubrol PX and 0.2% sodium cholate. The AOA 1 produced three peaks (DEAE 1, 2 and 3) as reflected by absorbance at 417 nm (Fig. IV-3), however, the P450 in the DEAE 3 peak had degraded to P420. The pooled AOA 2 fraction, upon subsequent DEAE Sephacel column chromatography, gave a single major peak (DEAE 4) and several tiny peaks with absorbance at 417 nm. DEAE fractions 1, 2 and 4 were further purified on a hydroxylapatite (HAP) column (1 x 18 cm). The proteins were eluted with a linear gradient of 0.01 to 0.125 M KP*i* buffer (pH 7.7) containing 0.1% Lubrol PX and 0.2% sodium cholate. Chromatography of DEAE 1,2 and 4 fractions on hydroxylapatite yield three purified P450 fraction (HAP 1, 2 and 3). All steps were carried out at 4°C.

Preparation of antibodies against purified guinea pig P450s

Adult female New Zealand white rabbits (2.3 kg) were immunized intradermally at 20 sites along the flanks with 50 µg of the purified cytochrome P450s mixed with complete Freund's adjuvant (1:1 ratio). One week later, rabbits were boosted intradermally with 50 µg of the immunogen in incomplete Freund's. After 3 weeks, animals were again boosted with a subcutaneous injection of 10 µg of the immunogen with incomplete Freund's. Two weeks later, blood was drawn and sera collected from each animal. Every 3 weeks thereafter, rabbits were boosted with 10 µg of the purified P450s. The specificity of the resulting antibodies was confirmed with Ouchterlony double diffusion analysis of sera as described by Thomas et al. (1976).

Enzyme assays

The *in vitro* metabolism of SN by rat liver microsome was carried out using the procedure described by Miranda et al. (1991a). A typical incubation consists of 0.3 mg of microsomal protein, 0.1 M KP*i* buffer, pH 7.6 or 8.6, 0.5 mM SN, 1 mM EDTA and a NADPH-generating system (10 mM glucose-6-phosphate, 1.0 units/mg glucose-6-phosphate dehydrogenase and 1 mM $NADP^+$) in a total volume of 0.5 ml. After a 1 hr incubation (formation of DHP and N-oxide was linear up to 1 hr under the assay conditions used) at 37°C, the reaction was terminated by rapid cooling on ice. The mixture was centrifuged at 46,000 g for 45 min at 4°C and an aliquot of the supernatant was analyzed

by HPLC (Kedzierski and Buhler 1986). The above protocol was slightly modified when anti-guinea pig P450 IgG serum was used to titrate the microsomal enzyme activity. The IgG serum (0 to 30 mg/nmole P450) and 0.3 mg of microsomes were preincubated for 20 min at room temperature prior to the addition of other components.

In reconstitutive experiments with purified guinea pig P450s, 0.4 nmole rat liver NADPH-cytochrome P450 reductase, 0.1 nmole P450 and 10 μg of L-α-DLPC (sonicated prior to addition) were preincubated for 10 min at room temperature. Subsequently, 0.1 M KP*i* buffer (pH 7.6) and 0.5 mM SN were added to yield a total volume of 0.5 ml and preincubated at 37 °C for 3 min prior to initiation of the reaction with 1.5 mM NADPH. After a 1 hr incubation, metabolites were analyzed using HPLC as described above. Benzphetamine N-demethylase assays were accomplished as described by Guengerich (1982).

Other methods

The amino terminal sequence of the purified guinea pig cytochrome P4502B was determined by an Applied Biosystems 475A gas-phase protein sequencer coupled with Edman degradation by the Central Analytical Laboratory of Oregon State University Center for Gene Research and Biotechnology. Mass spectrometry of the purified P450s was run as described by Lewis et al. (1993) using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic

acid) as the matrix and bovine serum albumin as the internal standard. The analyses were generously carried out by Ms. Elizabeth Barofsky in the Mass Spectrometry Core Facility of Oregon State University Environmental Health Sciences Center. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970). Microsomal and purified cytochrome P450 protein contents were determined by the method of Lowry et al. (1951). Total cytochrome P450 content was estimated using a spectral method (Omura and Sato 1964).

Results

Purification of P450s from guinea pig liver.

Three P450 isozymes from liver microsome of PB treated guinea pigs were purified (Table IV-1). While the guinea pig P450s HAP 1 and 2 were catalytically active, HAP 3 which had been derived from DEAE 4 had lost its catalytic activity. The specific contents of the isolated HAP 1 and 2 were 10.2 and 7.2 nmole/mg protein, respectively. Each P450 was examined by immunochemical detection using anti-rat P4502B1, P4502C11 and P4503A2 IgG (data not shown). Thus, HAP 1 was tentatively identified as a P4502B type; HAP 2 as a P4502C form; and HAP 3 as a P4503A ortholog.

Characterization of purified P450s

To help identify the P450 subfamilies of the isolated guinea pig isozymes, the molecular weights of the purified enzymes have been determined using a matrix-assisted laser desorption ionization on a time-of-flight (MALDI-TOF) mass spectrometer with sinapinic acid as the matrix (Lewis et al., 1993). The molecular weight of guinea pig P4502B was determined to be 57,512 Da with BSA (66,431 Da) as the internal standard (Fig. IV-4). Similarly, the molecular weight of guinea pig P4502C was 56,496 Da (Fig. IV-5). However,

the mass spectrometry for P4503A isoform could not be attempted because this degraded isoform had shown two bands of P450 range on SDS-PAGE (data not shown).

An NH_2-terminal amino acid sequence of 39 residues for purified guinea pig P4502B was obtained (Fig. IV-6). The first 33 amino acids of our sequence was identical to that of $P450_{GP\text{-}1}$ sequence of 33 residues reported by Oguri et al. (1991). This sequence had been shown to have a 79%, 71% and 69% homology with rabbit P4502B4 (Gasser et al., 1988), human P4502B1 (Yamano et al., 1989) and rat P4502B2 (Frey et al., 1985), respectively.

Catalytic activity of purified P450s

The catalytic activities of purified guinea pig liver P450s and microsomes are summarized in Table IV-2. In reconstitution experiments, purified guinea pig P4502B converted SN to DHP and SN N-oxide at rates of 0.65 and 1.50 nmole/min/nmole P450, respectively. In contrast, hepatic microsomes from PB treated guinea pigs increased DHP formation by 224% and SN N-oxide by 70%. In reconstitution experiments, the P4502C isoform produced SN N-oxide from SN at the rate of 13.3 nmole/min/nmole P450 whereas DHP formation was minimal. The P4503A isoform had lost its activity during the final purification processes and, consequently, its enzymatic activity could not be determined.

Immunoinhibition of microsomal SN metabolism by anti-guinea pig P450s IgG

Titration of SN metabolism by rabbit anti-guinea pig cytochrome P4502B IgG inhibited 69% of DHP formation in hepatic microsomes from untreated guinea pigs while only 43% and 14% of DHP formation were inhibited by P4502C and P4503A IgG, respectively (Fig. IV-7). Using microsomes from PB treated guinea pigs, immunoinhibition of DHP formation from SN by anti-guinea pig P4502B IgG was 70% compared to only 11% and 13% by antibody against purified guinea pig P4502C and P4503A IgG, respectively (Fig. IV-8). Differences in the absolute inhibition of microsomal DHP formation between untreated and PB treated guinea pigs by P4503A IgG confirmed that PB also induced P4503A isozyme in this species. The decreased percentage of contribution to DHP formation by P4502C in PB treated guinea pigs suggests that the P4502C isoform was refractory to PB induction. The results of the immunoinhibition studies on microsomal DHP formation using anti-cytochrome P450 IgGs indicated that in guinea pigs P4502B was the most important enzyme (up to 70%) for activation of SN in both untreated and PB-treated animals. P4503A isoform, however, had only a small (about 13%) contribution to DHP formation in both untreated and PB-treated guinea pigs. P4502C was estimated to be responsible for 43% of the SN converted to DHP in untreated guinea pigs while only 11% in PB-treated animals.

Titration of microsomal SN metabolism by rabbit anti-guinea pig cytochrome P4502B IgG inhibited 34-40% of SN N-oxidation in untreated and PB treated guinea pigs while less than 20% of SN N-oxide formation was inhibited by P4502C and P4503A IgG, respectively (Fig. IV-9 and IV-10). Therefore, it seems that P4502B also played some role (up to 40%) in detoxification of SN and P4502C and P4503A had only a minimal influence on SN detoxification in both untreated and PB treated guinea pigs.

Discussion

Three P450 isoforms were purified from liver microsomes of PB treated guinea pigs in order to assess the role of these enzymes in PA metabolism. After tentative identification of these isozymes with western blots, we employed MALDI-TOF mass spectrometry which has been recently developed for determination of mass of large molecules such as biopolymers. This MALDI-TOF mass spectrometry has been used to provide an accurate molecular weight determination of rat P4502B1/2 within 0.075% error (Lewis et al., 1993). Therefore, we are able to report the very precise molecular weights of guinea pig cytochrome P4502B and P4502C to be 57,512 and 56,496, respectively, under the experimental conditions used.

By contrast, Oguri et al. (1991) reported the molecular weight of $P450_{GP\text{-}1}$, which was identical to P4502B based on the amino terminal sequence, as 52,000 based on SDS-PAGE estimation. SDS-PAGE has been the traditional method for assessment of the molecular weight of P450s. This method, however, was not always consistent with the cDNA-derived amino acid sequence values (Levin et al., 1984) probably due to the influence, such as glycosylation, other post-translation modifications, or P450s' high hydrophobicities which interact with sodium dodecyl sulfate, on their migration in SDS-PAGE. Also the actual molecular weight range of P4502B family in

other species, estimated by cDNA-derived amino acid sequence, is reported to be 55,000 to 56,000 (Frey et al., 1985; Gasser et al., 1988; Yamano et al., 1989).

We next evaluated the catalytic activities of the purified guinea pig P450 isoforms in reconstituted systems for PA metabolism using SN as the substrate. We also determined the relative roles of these P450s through the use of immunoinhibition experiments in the microsomal metabolism of SN. Unfortunately, the results of the reconstitutive experiments did not always agree with those inferred from the immunoinhibition studies. In reconstitution experiments, purified P4502B converted SN to DHP and SN N-oxide at rates of 0.65 and 1.50 nmole/min/nmole P450, respectively. Pretreatment with PB (an inducer of cytochrome P4502B1) also significantly increased conversion of SN to both DHP and SN N-oxide in microsomes. However, titration of microsomal SN metabolism by anti-guinea pig P4502B IgG inhibited of DHP formation about 70% in both untreated and PB treated guinea pigs. This suggests that the rabbit anti-guinea pig P4502B IgG also may have been inhibiting other P450 isoforms involved in DHP production.

Reconstituted cytochrome P4502C produced SN N-oxide from SN at the rate of 13.3 nmole/min/nmole P450 whereas DHP formation was at a minimal rates. However, the P4502C IgG inhibited 43% of microsomal DHP formation from SN but only 19% of SN N-oxidation in untreated guinea pigs.

Turnover numbers in a reconstituted enzyme system do not always reflect contribution of the enzyme in intact microsomes. Sundheimer et al. (1983) also showed that rat $P448_{MC}$ (P4501A1) catalyzed acetanilide 4-hydroxylation in a constituted system, but did not contribute in 3-methylcholanthrene induced rat liver microsomes.

The microsomal formation of pyrrole from SN is catalyzed primarily by enzymes belonging to the cytochrome P4503A subfamily in rats (Williams et al., 1989a) and humans (Miranda et al., 1991b). However, we observed that cytochrome P4502B played the most important role (over 70% of the SN bioactivation) in the guinea pig, even though P4502B had a minimal specific activity toward the PA SN. This phenomenon reveals that metabolism by one P450 by itself in reconstitutive systems is quite different from how that P450 behaves in a mixture of P450s. Especially it is noteworthy that the amounts of enzymes involved in SN bioactivation overcomed the low specific activity of P4502B for pyrrole formation from the PA SN in the guinea pig.

Recently, Ohmori et al. (1993) demonstrated by immunoquantification that P4502B isoform comprised 47% of total P450 in liver microsomes of untreated guinea pig while P4502B1 was less than 0.1% of total P450 in untreated rat. However, P4502B2 is constitutively expressed in the liver of rats while P4502B1 is absent in liver until PB treatment and is constitutively expressed in lung and testis (Traber et al., 1988). By contrast, P4502C6,

P4502C11 and P4503A2 are major constitutive isoforms found in the rat (Imaoka et al. 1991). In guinea pigs, P4501A1, P4503A and $P450_{17\alpha}$ are known to be other constitutive forms (Nelson et al., 1993). Therefore, difference in the major constitutive P450s present in the livers of rats and guinea pigs may help explain the apparent disparity in the major enzymes for bioactivation of PA between these two species.

Miranda et al. (1991a) reported that hepatic FMO was a major enzyme for N-oxidation of SN in the guinea pig. In the present study, cytochrome P4502B seemed to play some role (up to 30% of the N-oxidation). This was the greatest contribution among the P450 isozymes tested, suggesting that P4502B was involved in detoxication of the PAs by the guinea pig. Hence, next to FMO, P4502B is the second most important enzyme for the detoxication of SN in the guinea pig.

In conclusion, cytochrome P4502B isoform was the most important enzyme for the bioactivation of the PA SN in the guinea pig while P4503A2 was the most important in the rat. By contrast, FMO was a major enzyme for SN N-oxidation while P4502B isoform played some role in SN N-oxidation in the guinea pig.

Table IV-1. Purification of cytochrome P450s from liver microsome of phenobarbital treated guinea pigs.

	Protein (mg)	Specific activity (nmole/mg)	Total P450 (nmole)	Recovery (%)
Microsomes	905	1.01	1068	100
Solubilized Mic.	886	0.86	758	71
AOA 1	93	3.93	365	34
DEAE 1	59	4.79	283	26
HAP 1	14	10.21	143	13
DEAE 2	8	3.63	29	2.7
HAP 2	0.7	7.24	5	0.5
DEAE 3	3	0	0	0
AOA 2	95	1.38	132	12
DEAE 4	4.7	3.81	18	1.7
HAP 3	0.96	1.40	1.34	0.1

Table IV-2. The catalytic activities of guinea pig hepatic microsomes and purified guinea pig liver cytochrome P450s.

Protein	Substrate		Specific activity*
Control microsome	Benzphetamine		8.54 ± 1.2
	SN	to DHP	0.69 ± 0.1
		to N-oxide	2.43 ± 0.2
PB microsome	Benzphetamine		24.9 ± 2.7
	SN	to DHP	2.2 ± 0.7
		to N-oxide	4.1 ± 0.9
HAP 1 (P4502B)	Benzphetamine		53.2
	SN	to DHP	0.65
		to N-oxide	1.52
HAP 2 (P4502C)	SN	to DHP	0.08
		to N-oxide	13.3
HAP 3 (P4503A)	SN	to DHP	--#
		to N-oxide	--

*Activities were expressed as the mean ± SD for microsomes and HCHO nmole/min/mg microsomal protein for benzphetamine d-methylation and DHP or SN N-oxide nmole/min/mg protein for SN. Activities were the mean of duplicated determination for purified P450s and HCHO, DHP or SN N-oxide nmole/min/nmole P450.

#The catalytic activity of P4503A isoform have been lost during purification steps.

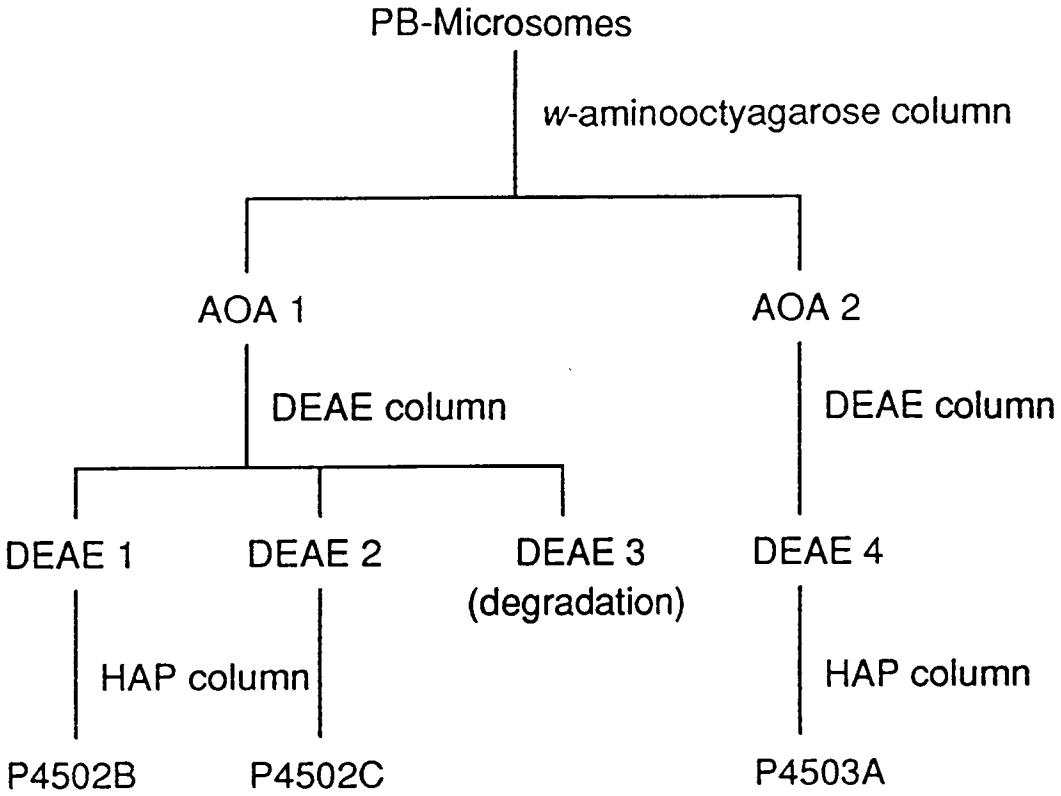

Fig. IV-1. An outline of the purification of guinea pig liver cytochrome P450s.

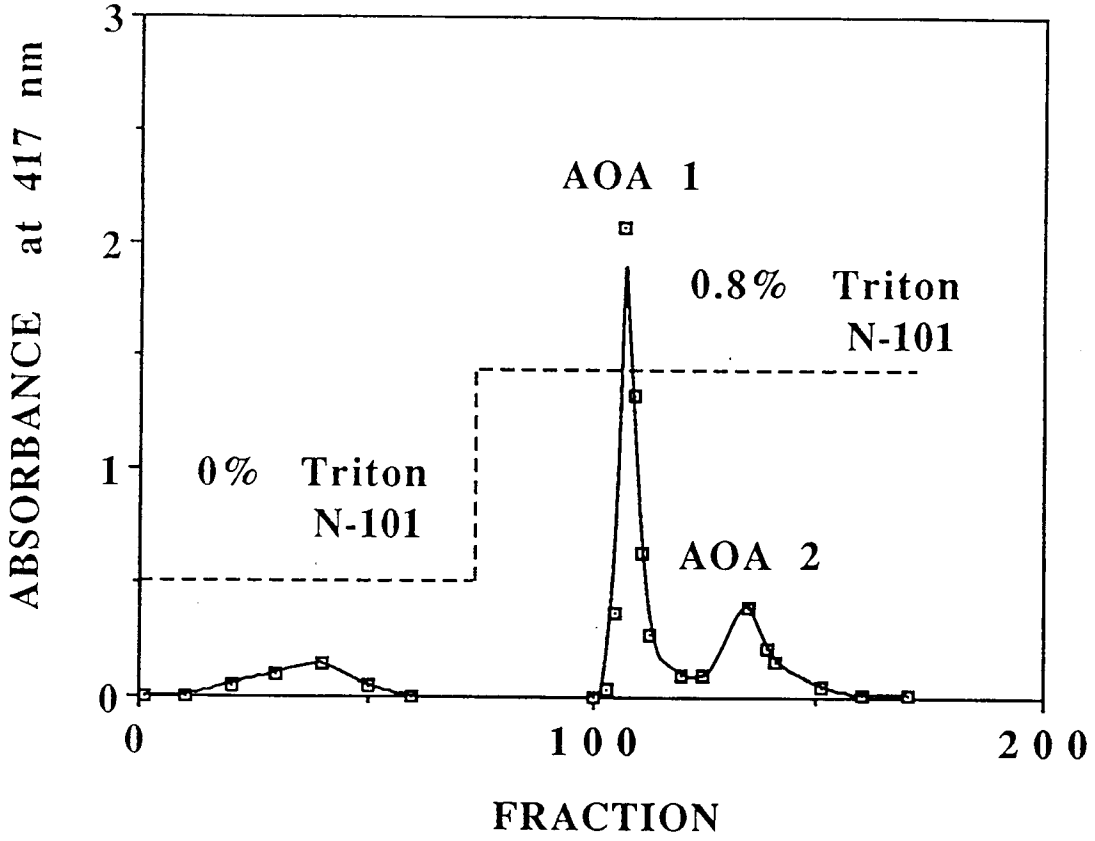

Fig. IV-2. Elution profiles of *w*-aminooctylagarose (AOA) column chromatography for the purification of guinea pig liver P450s.

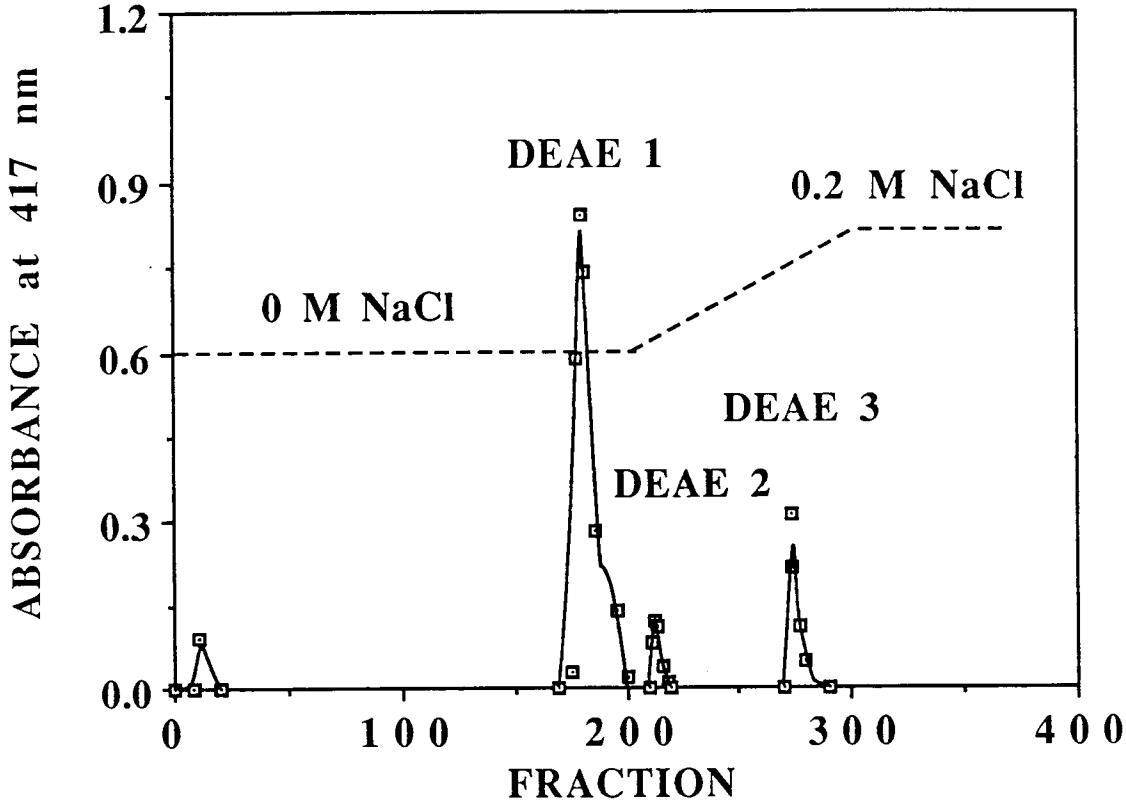

Fig. IV-3. Elution profiles of DEAE Sephacel (DEAE) column chromatography for the purification of guinea pig liver P450s (AOA 1 peak).

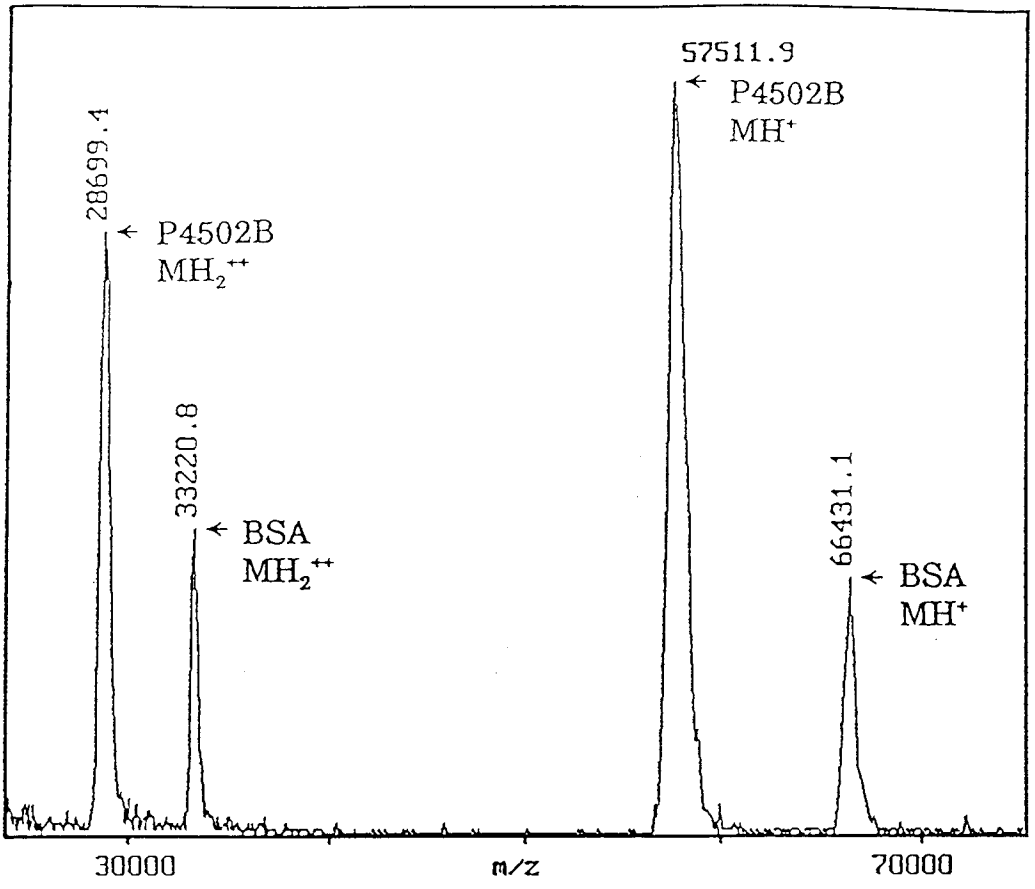

Fig. IV-4. Matrix-assisted laser desorption ionization on a time-of-flight (MALDI-TOF) mass spectrum of guinea pig liver P4502B. 0.45 µg of P4502B was loaded with BSA (66,431 Da) as the internal standard. Sinapinic acid was used as the matrix.

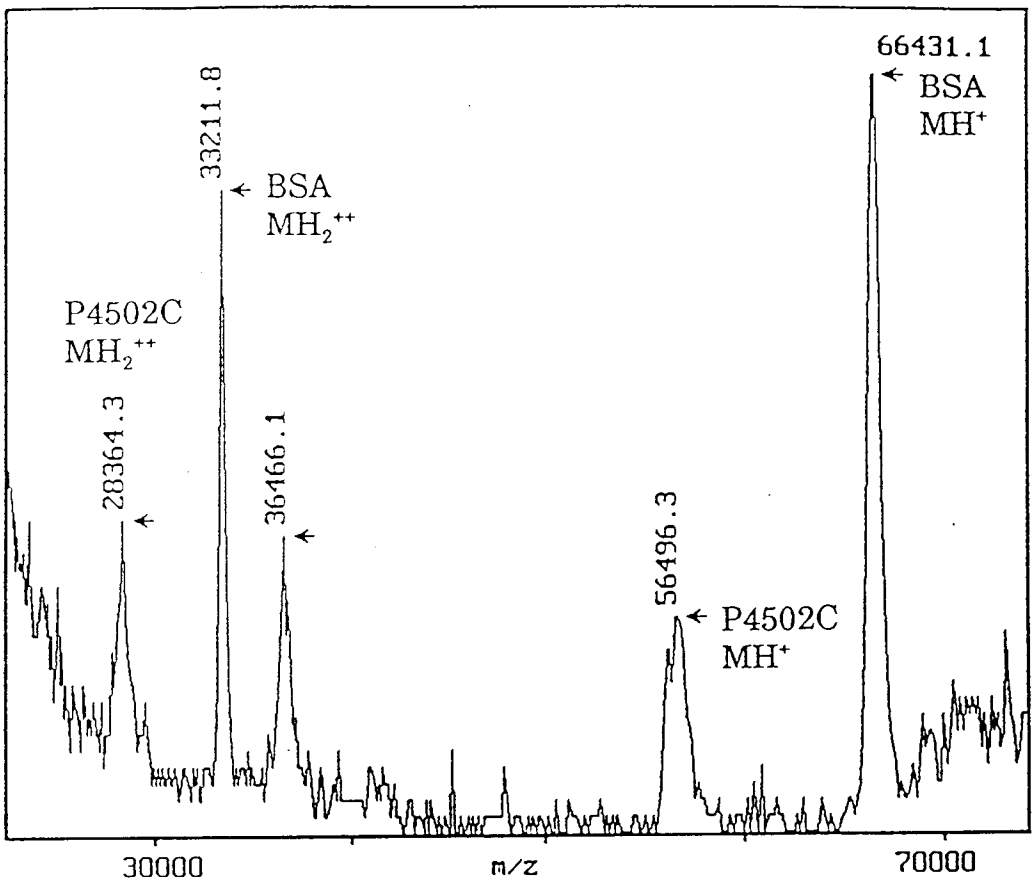

Fig. IV-5. Matrix-assisted laser desorption ionization on a time-of-flight (MALDI-TOF) mass spectrum of guinea pig liver P4502C type. 0.26 μg of P4502C was loaded with BSA (66,431 Da) as the internal standard. Sinapinic acid was used as the matrix.

```
 1                    5                   10
MET-GLU-LEU-SER-LEU-LEU-LEU-PHE-LEU-ALA-

 11                   15                  20
LEU-LEU-LEU-GLY-LEU-LEU-LEU-LEU-LEU-PHE-

 21                   25                  30
LYS-GLY-HIS-PRO-LYS-ALA-HIS-GLY-ASN-LEU-

 31                   35                  40
PRO-PRO-GLY-PRO-HIS-PRO-LEU-PRO-PHE-(LEU)-(GLY)-
```

Fig. IV-6. The amino terminal sequence of guinea pig cytochrome P4502B.

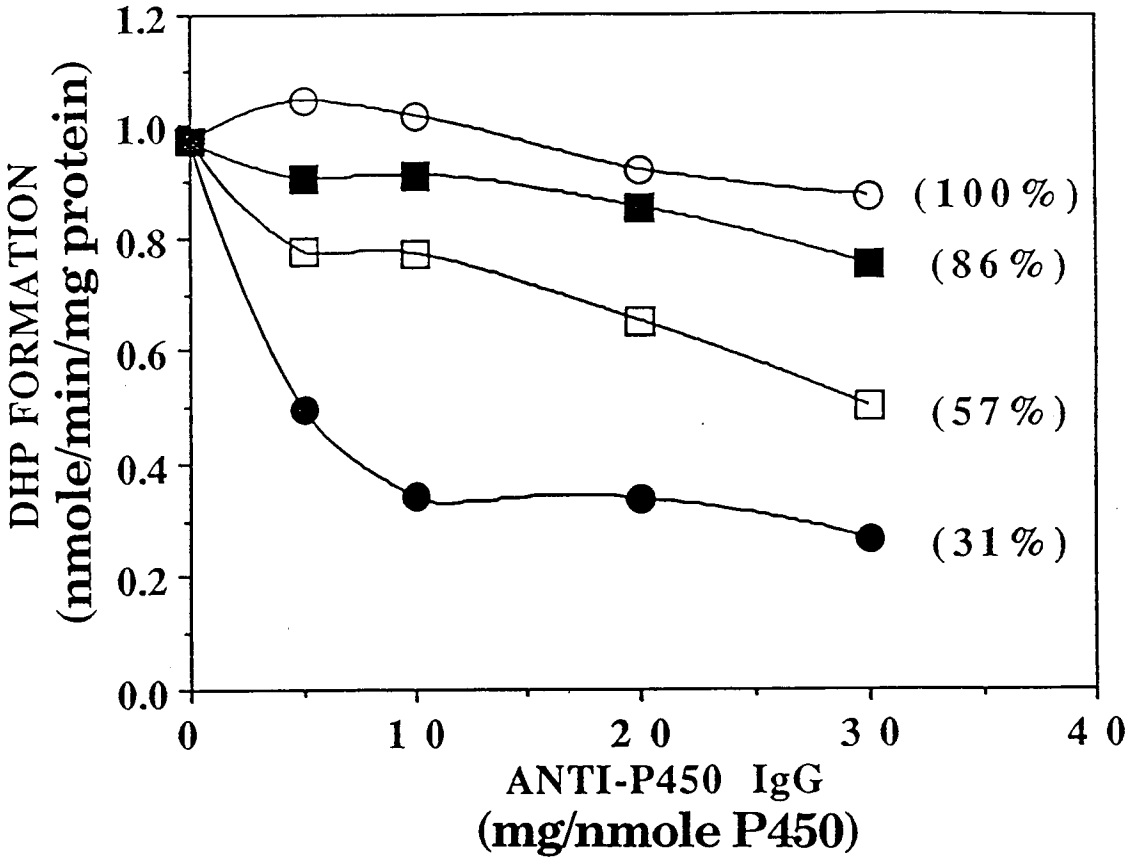

Fig. IV-7. Immunoinhibition of DHP formation in untreated guinea pig liver microsomes by anti-P450 IgGs [Preimmun IgG (○); Anti-P4503A IgG (■); Anti-P4502C IgG (□); Anti-P4502B IgG (●)].

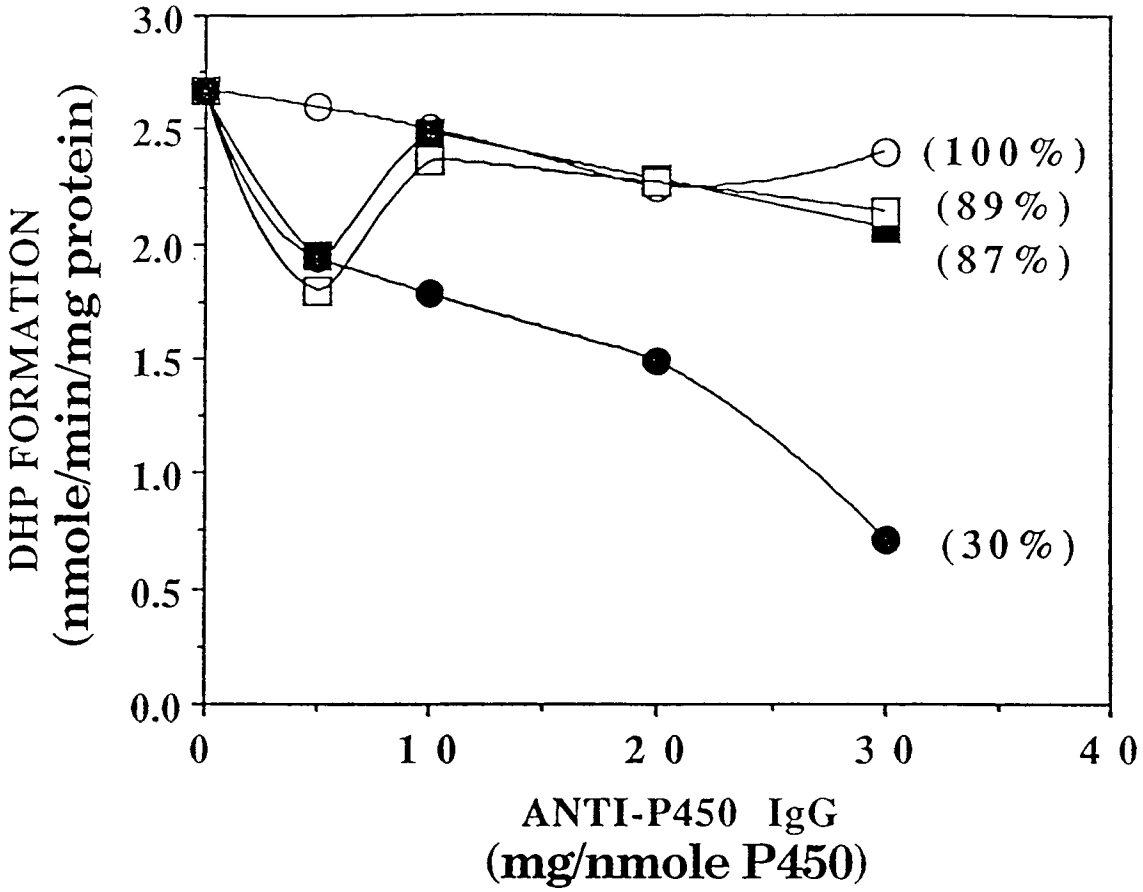

Fig. IV-8. Immunoinhibition of DHP formation in phenobarbital-treated guinea pig liver microsomes by anti-P450 IgGs [Preimmun IgG (○); Anti-P4503A IgG (■); Anti-P4502C IgG (□); Anti-P4502B IgG (●)].

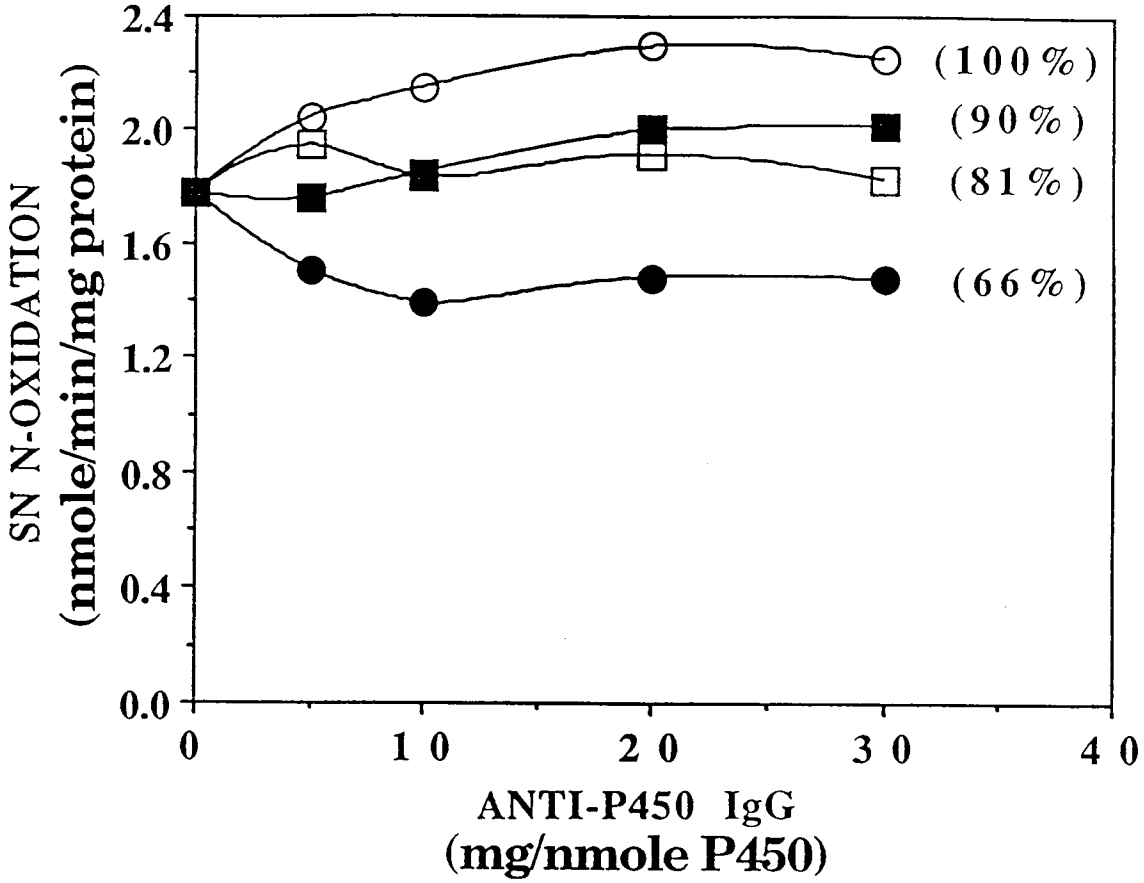

Fig. IV-9. Immunoinhibition of SN N-oxide formation in untreated guinea pig liver microsomes by anti-P450 IgGs [Preimmun IgG (○); Anti-P4503A IgG (■); Anti-P4502C IgG (□); Anti-P4502B IgG (●)].

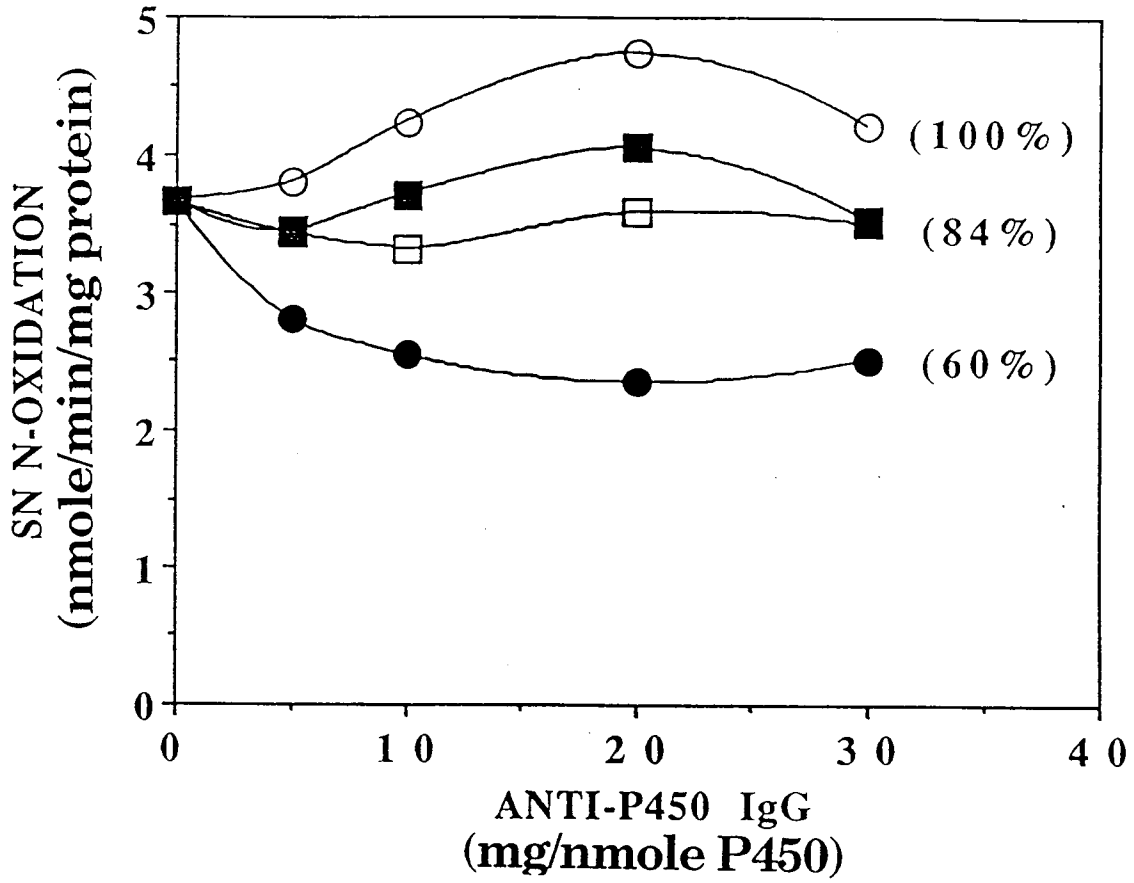

Fig. IV-10. Immunoinhibition of SN N-oxide formation in phenobarbital-guinea pig liver microsomes by anti-P450 IgGs [Preimmun IgG (○); Anti-P4503A IgG (■); Anti-P4502C IgG (□); Anti-P4502B IgG (●)].

V. HIGH CAPACITY FOR PYRROLE FORMATION TOGETHER WITH LOW RATES OF N-OXIDATION AND LITTLE HYDROLYSIS ARE THE MAJOR FACTORS FOR THE SUSCEPTIBILITY OF GUINEA PIG TO THE PYRROLIZIDINE ALKALOID JACOBINE*

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Abstract

Guinea pigs are resistant to the toxicities of pyrrolizidine alkaloids (PAs). However, the PA jacobine (JB) is unusually toxic to this species. To investigate the potential for esterlytic cleavage of PAs, we have initially purified two forms of hepatic carboxylesterase (GPL1 and GPH1) from guinea pigs. The major form GPL1 was purified to a specific activity of 486 μmole/min/mg protein in hydrolysis of p-nitrophenyl acetate (NPA) while the minor form GPH1 yielded p-nitrophenol with a specific activity of 86 μmole/min/mg protein from NPA. The metabolism of the highly toxic PA [^{3}H]-JB and PA [^{3}H]-senecionine (SN) which has lower toxicity then were studied using the purified guinea pig carboxylesterases and guinea pig liver microsomes. Purified carboxylesterase (GPH1) hydrolyzed [^{3}H]-JB and [^{3}H]-SN at rates of 4.5 and 11.5 nmole/min/mg protein, respectively. Carboxylesterase GPL1, however, had no activity toward PAs. Liver microsomes converted [^{3}H]-JB to the pyrrolic metabolite (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) and JB N-oxide at rates of 0.329 and 0.104 nmole/min/mg protein (DHP/N-oxide ratio: 3.16). Conversely, liver microsomes produced DHP and SN N-oxide from [^{3}H]-SN at rates of 0.460 and 0.865 nmole/min/mg protein (DHP/N-oxide ratio: 0.53). Therefore, we concluded that the combination of high pyrroles and low N-oxide formation and little hydrolysis are the major factors responsible for intoxication by guinea pigs by the PA JB.

Abbreviation: Con-A, concanavalin A-Sepharose 4B; DE 52, diethylaminoethyl cellulose 52; DHP, (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine; FMO, flavin-containing monooxygenase; HAP, hydroxylapatite; HPLC, high performance liquid chromatography; JB, jacobine; MN, monocrotaline; NPA, p-Nitrophenyl Acetate; PAs, Pyrrolizidine alkaloids; PMSF, phenylmethylsulfonyl fluoride; RT, retrorsine; SN, senecionine; SP, seneciphylline.

Pyrrolizidine alkaloids (PAs) occur in several species of plants. The widespread geographical distribution of PA-containing plants results in hepatotoxicity and livestock losses throughout the world (Fowler, 1968). PAs are metabolized by liver monooxygenases yielding PA pyrroles and N-oxides. PA pyrroles are considered the ultimate toxic metabolites whereas PA N-oxides are believed to be non-toxic. The PA pyrroles either react with cellular macromolecules or are hydrolyzed to form a secondary pyrrole known as (±) 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP).

Hydrolysis of a toxic pyrrolizidine ester leads to the necine and necic acid moieties, neither of which are hepatotoxic; thus hydrolysis amounts to detoxication (Mattocks, 1982). Treatment of rats with an esterase inhibitor, triorthocresylphosphate, increased the hepatotoxicities of PAs due to the larger portion of the dose available for conversion to toxic metabolites (Mattocks, 1970). Hosokawa et al. (1990) purified two isozymes of carboxylesterase, with nomenclature of GPL1 and GPH1, from guinea pig liver. Purified guinea pig GPH1 hydrolyzed SP, MN and SN, however, only minimal activity was seen with SN (Dueker et al., 1992b). The hydrolytic activity for PAs by purified guinea pig carboxylesterase varies appreciably depending on the structure of PAs (Dueker et al., 1992b).

Guinea pigs are known to be very resistant to the toxicities of the PAs monocrotaline (MN) (Chesney and Allen, 1973) and retrorsine (RT) (White et al., 1973), but very susceptible to seneciphylline (SP) (Chen et al., 1940) and

jacobine (JB) (Swick et al., 1982). The objective of this study was to determine which factors were responsible for the high toxicity of JB in guinea pigs. We have purified carboxylesterase from guinea pig liver and then assayed the enzymes for hydrolytic activity against JB and SN. We present evidence that the combination of high rates of activation (pyrrole production), low N-oxidation (detoxication) and little hydrolysis to the nontoxic retronecine were responsible for the high toxicity of JB to the guinea pig.

Materials and Methods

Chemicals- Tritiated jacobine and senecionine (99.7% of tritium resided in the necine base) were made biosynthetically in *Senecio jacobaea* as described previously (Reed et al, 1985; Reed and Buhler, 1988). p-Nitrophenyl acetate (NPA), methyl α-D-mannopyranoside and concanavalin A-sepharose 4B (Con A-sepharose) were purchased from Sigma Chemical Co. (St. Louis, MO). DE-52 anion exchange resin was purchased from Whatman (Maidstone, KY). Hydroxylapatite (HAP) was obtained from Bio-Rad Lab. (Richmond, CA). Optifluor scintillation cocktail was from Packard Canberra Company (Downers Grove, IL).

Preparation of microsomes-Hartley guinea pigs (400-450 g and 8 weeks old males) were purchased from Simonson Inc. (Gilroy, CA). Five animals were anesthetized with an overdose of CO_2 and killed by decapitation. The livers were quickly removed and homogenized with 0.1 M Tris-acetate buffer, pH 7.5, containing 0.1 M KCl and 1 mM EDTA. Microsomes were prepared by standard differential centrifugation at 9000 g for 30 min and at 105,000 g for 60 min at 4 °C. The microsomes were stored in 10 mM Tris-acetate buffer, pH 7.4, containing 1 mM EDTA, 1 mM PMSF and 20 % glycerol at -80 °C until used.

Purification of Carboxylesterases-Microsomal carboxylesterases were purified by the methods of Harano et al. (1988) and Dueker et al. (1992b) with the following modifications (Fig. V-1). All steps were carried out at 4 °C. Microsomes (672 mg) were solubilized in buffer A (50 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$) containing 1 % Triton X-100 at a final protein concentration of 3 mg/ml. The solution was stirred for 90 min and centrifuged at 105,000 g for 90 min. The supernatant was applied to a Con A-sepharose column (1 x 12 cm) preequilibrated with the filtered buffer A containing 1 % Triton X-100 in a flow rate of 30 ml/hr. The column then was washed with 100 ml of buffer A containing 1 % Triton X-100 and 0.5 M NaCl and then washed with 100 ml of buffer A. The column was eluted with 200 ml of buffer A containing 0.5 M methyl α-D-mannopyranoside. The eluent was concentrated to 10 ml on an Amicon 8200 membrane concentrator with a PM 30 Diaflo filter and concentrated again after adding 100 ml of 10 mM Tris-HCl buffer (pH 8.0) and dialyzed with 12000-14000 MW cut Spectrapor molecularporous membrane against 1 liter of 10 mM Tris-HCl buffer (pH 8.0) at 4 °C for 22 hr. The dialyzed fraction was applied to a Whatman DE-52 column (1 x 12 cm) preequilibrated with the dialyzed buffer. The column was eluted with a linear gradient of 0 to 200 mM NaCl in 10 mM Tris-HCl buffer with a flow rate of 20 ml/hr. Absorbance was monitored at 254 nm and esterase activity (2 μl aliquats of each fraction) was determined at 405 nm using p-nitrophenyl acetate as described by Krisch (1966). DE-52 fractions

produced three peaks of esterase activity. Tubes making up the these active fractions were combined, concentrated, dialyzed against 500 ml of 10 mM potassium phosphate buffer (pH 7.0) for 16 hr and each was then applied to a hydroxylapatite column (1 x 20 cm) equilibrated with the dialyzed buffer. Carboxylesterases were purified by elution with a linear gradient of 10 to 400 mM of potassium phosphate buffer (pH 7.0) after washing with 50 ml of 10 mM buffer. Esterase activity (2 - 10 µl) was monitored. Active fractions were combined and concentrated to 10 mM Tris-HCl buffer (pH 8.0) and stored at -80 °C until assayed. Protein was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard. Purity was determined by SDS-PAGE as described by Laemmli (1970) with 8 % acrylamide gel.

Determination of hydrolysis rates-The hydrolysis of p-nitrophenyl acetate (1.6 mM) was determined colorimetically in 50 mM Tris-HCl buffer (pH 8.0) at 25 °C by measuring the release of p-nitrophenol at 405 nm according to the method of Kirsch (1966).

The hydrolysis of PAs was carried out using 0.1 M potassium phosphate buffer, pH 7.4, 0.5 mM [^{3}H]-SN (0.58 µCi/µmole) or [^{3}H]-JB (0.44 µCi/µmole), 0.4 µg (14 nM) of carboxylesterase in a total volume of 0.5 ml for 1 hr at 37 °C. The reaction was terminated by rapid cooling on ice. An 100 to 300 µl aliquot was injected onto the PRP-1 column (Hamilton, Reno, NV) and eluted with 5%

acetonitrile and 0.01 N NH_4OH with a flow rate of 1.5 ml/min. The eluent was added to optifluor cocktail and counted using a Tri-Carb liquid scintillation analyzer (Downers Grove, IL).

The *in vitro* metabolism of JB and SN by microsome from guinea pig liver was carried out using the procedure described by Miranda et al. (1991a). A typical incubation consisted of 0.3 mg microsomal protein, 0.1 M potassium phosphate buffer, pH 7.6, 0.5 mM [^{3}H]-SN or [^{3}H]-JB, 1 mM EDTA and 0.1 mM NADPH in a total volume of 0.5 ml. After a 1 hr incubation at 37°C, the reaction was terminated by rapid cooling on ice. The mixture was centrifuged at 46,000 g for 45 min at 4°C and an aliquot of the supernatant was analyzed by HPLC (Kedzierski and Buhler, 1986a). The eluent was analyzed as described above.

Results

Purification of carboxylesterases from guinea pig hepatic microsomes- Carboxylesterases were purified from guinea pig liver in order to assess their roles in PA detoxication. Two isozymes, GPL1 and GPH1, were purified as described by Hosokawa et al. (1990). At each step, the hydrolytic activity toward NPA was monitored. Con A chromatography produced an eight-fold increase in specific activity (Table V-1). DE 52 fractions produced three peaks of esterase activity. Each pooled fraction was applied onto hydroxylapatite column. DE 52-1 and DE 52-2 fractions produced only one peak (HAP 1 and HAP 2, respectively), but DE 52-3 fraction formed four peaks of esterase activity (HAP 31, 32, 33 and 34) (Fig. V-2). HAP 1, 2, 31 and 32 had mobilities with a molecular weight of 60,000 in SDS-PAGE, which is similar to that of GPL1 (Hosokawa et al., 1990) (Fig. V-3). However, HAP 33 and 34 exhibited mobilities with a molecular weight of 57,000, similar to GPH1 (Hosokawa et al., 1990), with slight contamination of GPL1. Six different fractions showed different specific activities toward NPA (Table V-1).

*Hydrolysis of PAs by carboxylesterases-*The guinea pig carboxylesterases hydrolyzed PAs to a necic acid and a necine base (retronecine). We incubated [^{3}H]-SN and [^{3}H]-JB with the six fractions of carboxylesterases in order to verify their roles in PAs detoxification. The GPL1-type fractions, HAP 1, 2, 31 and 32, had no hydrolytic activities toward SN or JB (Table V-2). These

results agreed with Dueker et al. (1992b). HAP 33 and 34, a GPH1 type fractions, hydrolyzed SN at low rates. Only HAP 34 hydrolyzed JB at a lesser rate than that of SN.

Tritiated PAs metabolism by guinea pig liver microsomes-Liver microsomes converted JB to the pyrrolic metabolite (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizidine (DHP) and JB N-oxide at rates of 0.329 and 0.104 nmole/min/mg protein giving a DHP/N-oxide ratio of 3.16 (Table V-3). In contrast, liver microsomes produced DHP and SN N-oxide from SN at rates of 0.460 and 0.865 nmole/min/mg protein yielding a DHP/N-oxide ratio of 0.53. Therefore, the metabolism of SN by guinea pig liver microsomes was 3-fold higher than that of JB. Furthermore, the JB metabolism by guinea pig liver microsomes showed a tendency forward the increased toxicity in this animals.

Discussion

One of the most curious things to toxicologists is why some animals are resistant to the toxicity of a chemical but others are not, and why some chemicals produce toxicity in an animal but others do not. Guinea pigs have been studied as the resistant species against the toxicity of PAs (Chesney and Allen, 1973), however, guinea pigs have been known to be unusually susceptible to JB intoxication (Swick et al., 1982). Once ingested by an animal, PAs may be excreted, be activated by conversion to toxic metabolites or be detoxified to harmless metabolites which can then be eliminated from the body. The biological effects of ingested PAs will depend on the balance of these three pathways. The major known routes of metabolism of PAs are hydrolysis, N-oxidation and dehydrogenation to pyrroles derivatives. The last is the only one known to be associated with cytotoxicity. We have attempted to answer why JB was so toxic to guinea pigs.

Dueker et al. (1992a) reported that guinea pig hepatic microsomes hydrolyzed MN, while the rat exhibited no such hydrolytic capabilities (Lame et al., 1991). We purified carboxylesterase GPL1 and GPH1 from guinea pig liver and assayed for hydrolytic activity against SN and JB. As observed by Dueker et al. (1992a), GPL1 had no activity toward PAs (Table V-2). SN was a better substrate for the GPH1 carboxylesterase than was JB (Table V-2).

This means that JB ingested by an animal is more resistant to degradation by esterlytic cleavage than is SN. Consequently, greater quantities of JB remain available for conversion to toxic pyrroles.

Mattocks (1982) illustrated that steric hindrance around the ester groups was a major factor inhibiting hydrolysis. The only structural difference between JB and SN is an epoxide between C-15 and C-20 position in JB (Fig. V-4). There is thus, a possibility that the epoxide of JB may interfere with hydrolysis at O-17 near to the C-15 position, an effect was not possible with SN.

However, differences in the hydrolytic capability toward PAs by an animal can not fully explain the resistance of the animal to PA intoxication. It is necessary, therefore, to also consider the capability of the animal to convert PAs to reactive pyrroles and relatively harmless N-oxides since guinea pigs were very susceptible to the toxicity of the PAs such as seneciphylline (SP) (Chen et al., 1940) even though purified guinea pig carboxylesterase GPH1 had the highest activity toward SP among PAs studied (Dueker et al., 1992a). We have now examined the capacity of these other metabolic pathways of PAs in guinea pig liver microsomes to help explain these differences.

Little consideration for pyrrole and N-oxide formation from JB has been attempted. We measured JB N-oxide during HPLC by its absorbance at 210 nm. However, due to the low absorbency of JB N-oxide its limit of detection is much larger (2.5 nmole) compared to that of SN N-oxide (0.3 nmole). We

have used [^{3}H] labeled JB and SN to determine pyrrole and N-oxide formation by guinea pig liver microsomes. JB and SN produced similar amounts of DHP in microsomal incubations but JB yielded much less N-oxide compared to SN. The ratio of DHP/N-oxide formed from JB was 6 times higher than that from SN, which contributes to the greater availability of JB pyrrole for bioactivation. Therefore, we concluded that the limited hydrolysis as well as the high pyrrole and low N-oxide formation rates were primarily responsible for unusual toxicity of JB to guinea pigs.

Miranda et al. (1991a) demonstrated that FMO was a major enzyme for N-oxidation of SN and cytochrome P450 mainly was responsible for DHP formation. SN had a high partition coefficient like 6.3 (Mattocks and Bird, 1983a). There was no report about partition coefficient of JB. However, we can presume that the partition coefficient of JB is lower than that of SN because the solubility of JB in water is higher. The most lipophilic PAs are metabolized at the fastest rates (Mattocks and Bird, 1983a). Therefore the low lipophilicity of JB may delay its access to FMO, which has little opportunity for N-oxidation of JB and yield low JB N-oxidation.

In conclusion, we have purified carboxylesterase from guinea pig liver and assayed for hydrolytic activity toward the PAs JB and SN. This study presented evidence that the combination of high pyrrole activation and low rates of detoxification via N-oxidation and hydrolysis are the major factors in the unusual susceptibility of guinea pigs to intoxication by the PA JB.

Table V-1. Purification of carboxylesterases from guinea pig hepatic microsomes.

	Protein (mg)	Specific activity*	Total activity#	Recovery (%)
Microsomes	672	10.9 ± 1.0	7318	100.0
Solubilized†	601	9.9 ± 1.1	5964	81.5
Con A-Sep.	29.8	85.9 ± 6.4	2559	35.0
DE 52-1	2.86	239.1 ± 13	684	9.3
HAP 1 (GPL1)	0.37	485.9 ± 10	178	2.4
DE 52-2	5.61	88.3 ± 14	496	6.8
HAP 2 (GPL1)	0.23	270.0 ± 32	61	0.8
DE 52-3	2.05	65.0 ± 6.2	133	1.8
HAP 31 (GPL1)	0.04	159.1 ± 11	6.8	0.09
HAP 32 (GPL1)	0.14	24.3 ± 3.0	3.5	0.05
HAP 33 (GPH1)	0.03	42.7 ± 2.5	1.4	0.02
HAP 34 (GPH1)	0.07	85.8 ± 5.9	6.4	0.08

*Specific activities toward NPA are the mean ± SD for 4 incubations and expressed as μmole p-nitrophenol/min/mg protein.

#Total activities are expressed as μmole/min.

†Solubilized means the supernatant of solubilized microsomes.

Table V-2. Hydrolysis of pyrrolizidine alkaloids by purified carboxylesterases.

	HAP 1 (GPL1)	HAP 2 (GPL1)	HAP 31 (GPL1)	HAP 32 (GPL1)	HAP 33 (GPH1)	HAP 34 (GPH1)
[^{3}H]-JB	ND*	ND	ND	ND	ND	4.5
[^{3}H]-SN	ND	ND	ND	ND	4.2	11.5

#Activities are expressed as nmole retronecine/min/mg protein. Data are represented as the mean for duplicate incubations.

*ND means "not detectable".

Table V-3. Conversion of the PAs senecionine and jacobine to DHP and N-oxide by guinea pig liver microsomes.

	DHP	N-oxide	DHP/N-oxide
[^{3}H]-JB	0.329*	0.104	3.16
[^{3}H]-SN	0.460	0.865	0.53

*Activities are expressed as nmole/min/mg protein. Data are represented as the mean for duplicate incubations.

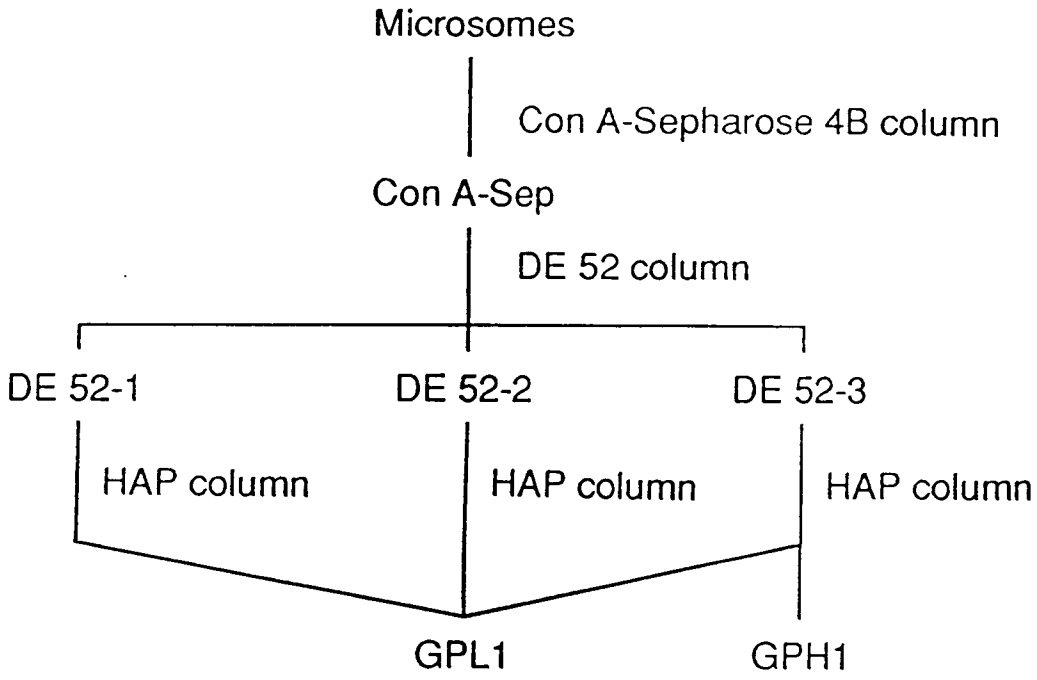

Fig. V-1. An outline of the purification of guinea pig liver carboxylesterases.

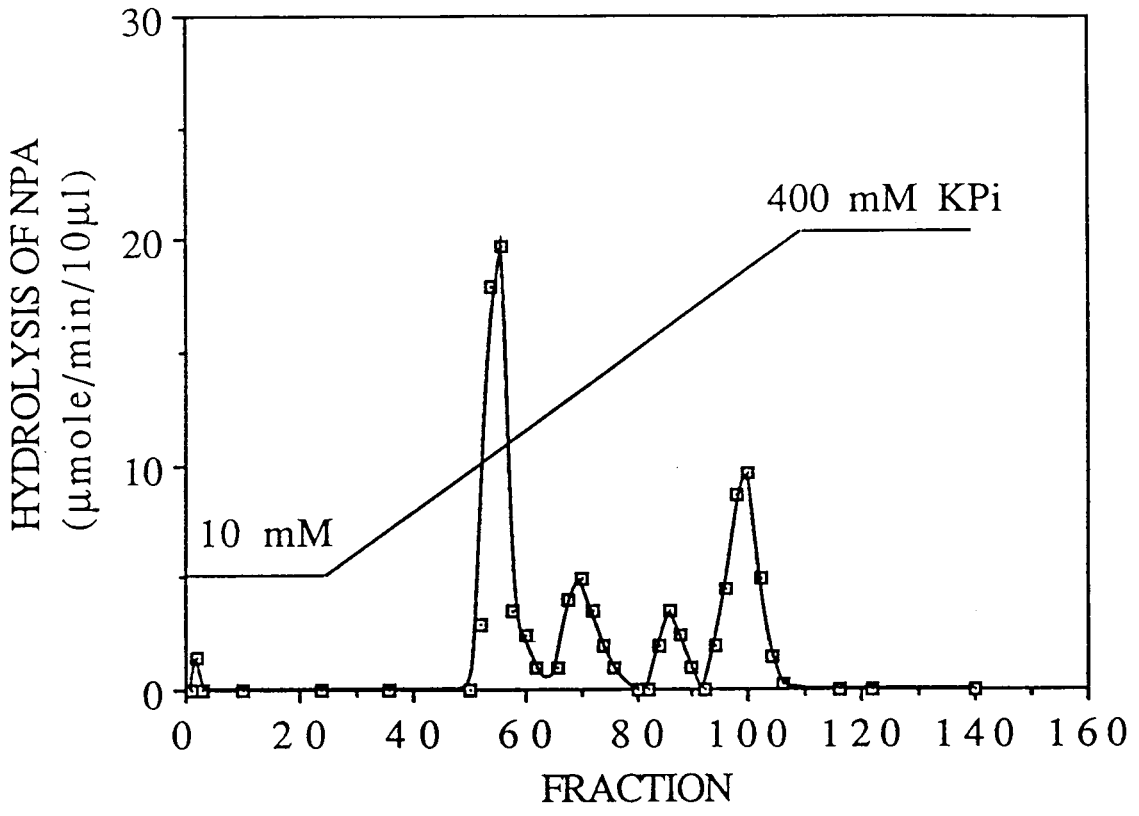

Fig. V-2. Carboxylesterase isolation. Hydrolysis of p-nitrophenyl acetate by hydroxylapatite column fractions. The first peak was defined as HAP 31 and then as HAP 32, HAP 33 and HAP 34. The elution of HAP 1 and HAP 2 in hydroxylapatite column appeared identical to that of HAP 31.

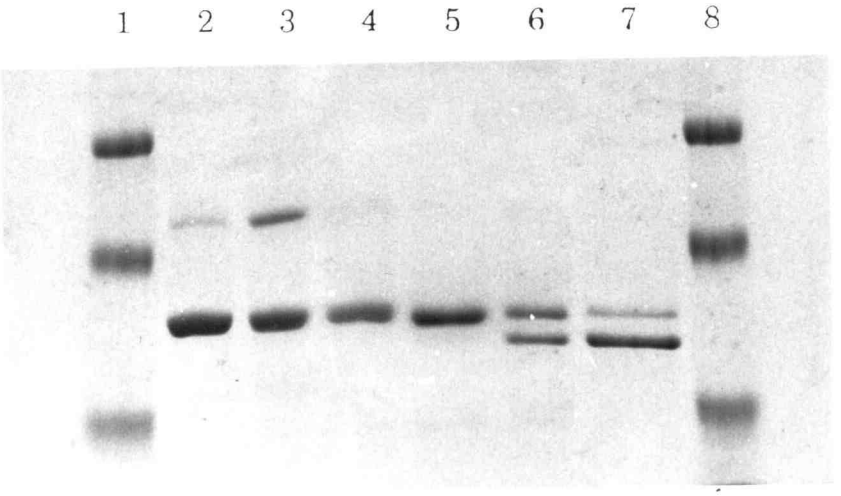

Fig. V-3. SDS-PAGE of purified guinea pig carboxylesterases. Lane 1 and 8; prestained molecular marker (Phosphorylase B; 106,000, Bovine serum albumin; 80,000, Ovalbumin 49,500), Lane 2; HAP 1, Lane 3; HAP 2, Lane 4; HAP 31, Lane 5; HAP 32, Lane 6; HAP 33, Lane 7; HAP 34. All carboxylesterases are loaded as 2 μg.

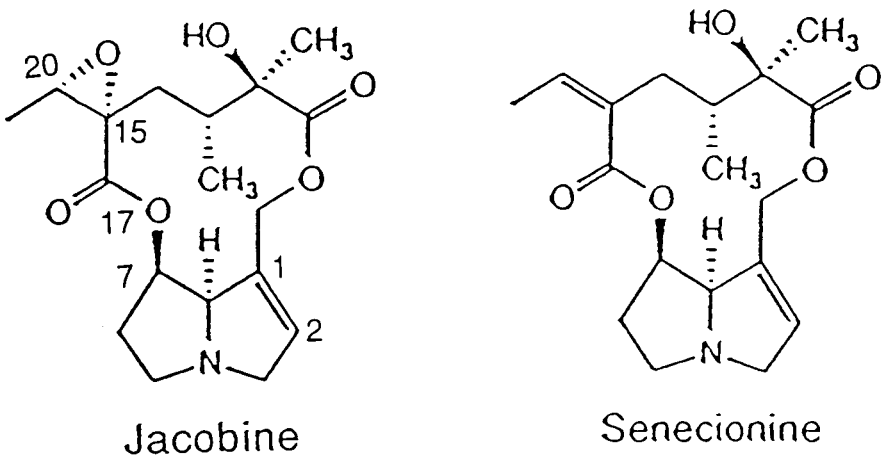

Fig. V-4. Structures of jacobine and senecionine.

VI. Conclusions

Once ingested by an animal, PAs may be excreted, be activated by conversion to toxic metabolites or be detoxified to harmless metabolites which can then be eliminated from the body. The biological effects of ingested PAs will depend on the balance of these three pathways. The major known routes of metabolism of PAs are hydrolysis, N-oxidation and dehydrogenation to pyrrole derivatives. The latter is the only one known to be associated with cytotoxicity.

In this study, we provided evidence about species/strain differences between the rat, a susceptible species, and the guinea pig, a resistant species, in PAs bioactivation and detoxication in terms of the role of P450s, flavin-containing monooxygenases (FMOs) and carboxylesterases. FMO was responsible for N-oxidation (detoxication) of the PA senecionine (SN) in both sexes of Fischer 344 (F344) rats, compared to P4502C11 in Sprague-Dawley (SD) rats. Also SN N-oxidation in F344 rats was higher than in SD rats. These results suggest that F344 rats may be refractory to the PAs intoxication because their enzymes preferentially N-oxidize PAs, therefore, leaving less opportunity for the products of cytotoxic dehydrogenated pyrrole derivatives. Cytochrome P4502B played the most important role in the bioactivation of the PA SN in guinea pigs while cytochrome P4503A2 is a major enzyme for pyrrole

formation from SN in SD rats. The combination of high capacity for pyrrole activation and low detoxification via N-oxidation and hydrolysis were the major factors of the susceptibility of guinea pigs to the unusual toxic PA jacobine.

A single factor can not explain the species/strain differences in susceptibility of an animal to the toxicities of PAs. Distinct enzyme systems and/or amounts available and unique substrate specificities altogether may explain the differences between animal species in toxicities of the PAs.

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