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MOLECULAR BASIS FOR THE SPECIES-DEPENDENT BIOACTIVATION OF VINYLCYCLOHEXENE: ROLE OF HEPATIC CYTOCHROME P450 INDUCTION

by

Susan M. Fontaine

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOctor of Philosophy

James R. Halpert	11-30-00 Date
Daniel C. Liebler	11/30/00 Date
Eugene A. Mash	11-30-00 Date
Patricia B. Hoyer	11-30-00 Date
I. Glenn Sipes	11-30-00 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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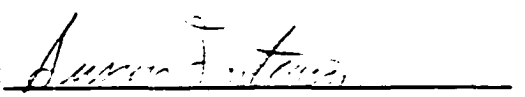
Signed: 

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DEDICATION

I dedicate this dissertation to my parents, since their love and support made this possible. You have given me the confidence to continuously strive to be my best.

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ABBREVIATIONS

VCH:	Vinylcyclohexene
(*R*)-VCH:	(*R*)-4-Vinyl-1-cyclohexene
(*S*)-VCH:	(*S*)-4-Vinyl-1-cyclohexene
VCH-1,2-Epoxide:	4-Vinyl-1-cyclohexene-1,2-Epoxide
VCH-7,8-Epoxide:	4-Vinyl-1-cyclohexene-7,8-Epoxide
VCD:	4-Vinyl-1-cyclohexene Diepoxide
CYP:	Cytochrome P450
GC:	Gas Chromatography
i.p.:	Intraparitoneal
p.o.:	Oral Gavage
PB:	Phenobarbital
ACE:	Acetone

ABSTRACT

4-Vinylcyclohexene (VCH) is an intermediate in the production of rubber and styrene. Following repeated exposure, VCH depletes the small pre-antral follicles of B6C3F$_1$ mice. Interestingly, the female rat is resistant to VCH-induced ovotoxicity, which can be partially attributed to a reduced ability in forming the toxic metabolites (VCH-1,2-epoxide, VCH-7-8-epoxide, and ultimately, 4-vinyl-1-cyclohexene diepoxide [VCD]), as compared to mice. Current studies have focused on species-dependent bioactivation of VCH, stereochemical considerations in VCH bioactivation, and the role of cytochrome P450 (CYP), particularly following repeated exposure to VCH. Hepatic microsomes isolated from mice pretreated with VCH converted VCH to epoxides to a greater extent than control mice or rats, or VCH-pretreated rats. In microsomes from both control animals and those pretreated with VCH, (*R*)-VCH formed more VCH-1,2-epoxide while (*S*)-VCH formed more VCH-7,8-epoxide, demonstrating enantioselectivity in metabolism. Rodents were also dosed with equitoxic doses of VCH, VCH-1,2-epoxide, or VCD for 10d and investigated for CYP expression. VCH or VCH-1,2-epoxide pretreatments were effective in increasing total CYP levels in the mouse only. Of the multiple specific CYP isoforms tested, expression of only CYP2A and CYP2B were induced following VCH and VCH-1,2-epoxide pretreatment in the mouse. Neither CYP2A nor CYP2B were

induced in the rat. While CYP2E1 plays a role in metabolism of the structurally related 1,3-butadiene, current studies demonstrate that these pretreatments caused no increases in CYP2E1 protein levels/activity. Those data, combined with the data showing no differences in epoxidation of VCH or its monoepoxides in CYP2E1-deficient mouse hepatic microsomes compared to those of mice that do have CYP2E1, indicated that CYP2E1 is not an important isoform in the species-specific bioactivation of VCH. These results have interesting correlations with human protein metabolism studies. Of several human hepatic CYP isoforms tested, CYP2E1 and CYP2B6 were the only isoforms that significantly catalyzed the epoxidation of VCH. In conclusion, CYP2A and CYP2B are critical isoforms in species-dependent VCH bioactivation, and the rat is resistant to VCH ovotoxicity, at least in part, because CYP induction does not occur following repeated exposure to VCH.

CHAPTER 1: INTRODUCTION

4-Vinylcyclohexene: Sources of Production, Uses, and Exposure

4-Vinylcyclohexene (VCH) is produced as a byproduct during rubber curing through the spontaneous dimerization of two molecules of 1,3-butadiene (Rappaport and Fraser, 1976). This compound is most commonly formed as a racemic mixture of (*R*)-VCH and (*S*)-VCH. Studies using laboratory models of rubber vulcanization have shown that VCH is a major volatile compound released into the air during this process (Rappaport and Fraser, 1977). These studies demonstrated air concentrations of VCH ranging from 54 to 118 ppb in passenger tire curing rooms. Further work by Cocheo et al. (1983) analyzed air samples from several rubber goods manufacturing processes. Up to 830 $\mu g/m^3$ cycloalkenes (including VCH) were measured in the air of shoe-sole factory vulcanization areas, and up to 2600 $\mu g/m^3$ of these particular cycloalkenes were detected in the air of tire retreading factory vulcanization areas. Lesser levels were found in extrusion areas of tire retreading factories and electrical cables insulation plants.

VCH has commercial applications as an intermediate in the chemical synthesis of other compounds such as vinylcyclohexene diepoxide (which is used as a diluent in epoxy resin manufacturing), and styrene (for food packaging) (International Agency for Research on Cancer (IARC), 1976; Tan et al., 1989). VCH is also used as a precursor in the manufacturing of certain insecticides,

flame retardants, and ethylcyclohexyl carbinol plasticizers (Rappaport and Fraser, 1977; IARC, 1982). It was estimated that 1.2 to 12.1 million pounds of VCH were produced in the United States in 1977 (USEPA, 1980).

Exposure of Humans to 4-Vinylcyclohexene

There is strong epidemiological evidence of sites of exposure of humans to VCH. In the Soviet Union in 1968, extremely high concentrations of VCH were measured at the site of production (maximum concentrations of 677 X 10^3 ppb) (Bykov, 1986). Workers exposed at these sites reported suffering occupationally related illnesses including keratitis, rhinitis, headache, hypotonia, leukopenia, neutrophilia, lymphocytosis, and impairment of pigment and carbohydrate metabolism. Mancusco et al. (1968) revealed early reports of consistently higher mortality rates in rubber curing workers compared to those in other departmental groups within the factory site. Fox et al. (1974) also showed through a 5-year study that bronchial cancer was more prevalent ($p < .05$) among vulcanization workers in England's rubber and cablemaking industries. However, the small sample sizes reported in these studies trivialized the findings, and it was not possible to define a clear relationship between VCH and elevated levels of cancer mortality characteristic of rubber workers.

4-Vinylcyclohexene as a Toxicant

Because of the human exposure to VCH and its structural similarity to butadiene, VCH was tested for its carcinogenic potential in the NTP subchronic assay. A 13 week oral gavage toxicity study in male and female $B6C3F_1$ mice with VCH (0-1200 mg/kg/day) and Fischer 344 rats (0-800 mg/kg/day) demonstrated a significant reduction in the number of primary and mature grafian follicles in the ovaries of mice receiving the highest dose (NTP, 1986; Collins and Manus, 1987). The only other observed effect was hyaline droplet degeneration of the proximal convoluted tubules of the kidney in male rats observed (800 mg/kg/day).

Similar results were seen in an inhalation study with male and female $B6C3F_1$ mice (0-1000 ppm VCH, 6 hours a day, 5 days a week) and male and female Sprague-Dawley rats (0-1500 ppm VCH, 6 hours a day, 5 days a week) (Bevan et al., 1994). In this study, the most adverse pathological effect noted was ovarian atrophy, and this occurred only in the mice receiving the highest dose. Again, there was also noted hyaline droplet degeneration of the proximal convoluted tubules.

Other studies confirmed that VCH is also ovotoxic in the mouse but not the rat following intraperitoneal and oral administration (Smith et al., 1990b; Hooser et al., 1993, 1994). Depletion of mouse primordial and growing follicles (85% and 75%, respectively) was observed following administration of VCH for 30 days

(7.5 mmol/kg/day, ip) (Smith et al., 1990b). Studies conducted by Hooser et al. (1994) characterized the long-term ovarian changes that occur following VCH-induced follicular loss. Correlations were made between follicular loss, elevated plasma follicle stimulating hormone (FSH) levels, and the development of preneoplastic ovarian lesions following administration of VCH for 30 days (7.5 mmol/kg/day, ip). Observations were made at 30, 60, 120, 240, and 360 days following the beginning of treatment. At the end of the 30 d of treatment, there was considerable follicular loss. Follicular populations decreased in a time-dependent manner. By 360 d, the ovaries of the VCH-treated mice were void of follicles at any stage of development, and the mice were acyclic (defined as cessation of estrous cyclicity). Elevated plasma concentrations of FSH, as well as preneoplastic lesions, were observed at 240 and 360 d. The lesions were characterized as irregularly shaped foci of hypertrophic cells. There were also 1 to 2 mm blood-filled cystic structures found on the surface of the ovary in 80% of the VCH-treated mice at 360 days.

Ovary as a Target Site

Since VCH targets this particular primordial/primary population of ovarian follicles, there are irreversible consequences following exposure to VCH.

Females are born with a finite number of immature ovarian primordial follicles that exist in an arrested state of mitotic division (Hirshfield, 1991). After sexual maturation, these cells can undergo transformation to form primary, secondary, and ultimately pre-antral follicles. Throughout a life span, the number of primordial follicles selectively recruited to develop to the preovulatory stage is miniscule compared to the total number of primordial follicles present in the ovary at birth (Hirshfield, 1991; Baker, 1963). Human ovaries contain approximately 400,000 primordial follicles at birth, but by the time menupause occurs, approximately 99.9% of all follicles will undergo a physiological form of apoptotic cellular death known as atresia (Byskov, 1978; Hughes and Gorospe, 1991).

Since there are a finite number of primordial follicles in the ovary, compounds that deplete this particular population of follicles, such as VCH, 1,3-butadiene, and 3-methyl-chloranthrene, cause irreversible damage (Hoyer and Sipes, 1996). A consequence of destruction of small follicles is the disruption in cyclicity, which may not be detected for months. This is distinct from effects by compounds that target larger preovulatory follicles: reversible disruptions in cyclicity that is observed within a few cycles. A series of events follow the premature ovarian depletion caused by compounds such as VCH that target ovarian germ cells. Since preovulatory follicles are a source of 17β-estradiol, circulating levels of 17β-estradiol will decrease, and therefore eliminate the negative feedback on the hypothalamus and pituitary normally imposed by steriods from the ovary. Circulating levels of follicle stimulating hormone (FSH) will then increase, and

eventually circulating levels of lueteinizing hormone (LH) will increase as well. Since this increase in release of these hormones from the pituitary will not occur until a sufficient loss of ovarian cyclicity has resulted (e.g. several months), the earliest biomarker that reflects ovotoxicity to date identified in laboratory animals appears to be disruptions in estrous cyclicity. Therefore, exposure to VCH in mice causes irreversible damage to the reproductive system. Extrapolation of these events to what would occur in women would be changes in menstrual cyclicity resulting in premature menupause.

Effects of 4-Vinylcyclohexene on Reproductive Competence

Since there is a long latency period between irreversible ovarian failure caused by VCH and clinical signs of reproductive toxicity, it was questionable whether VCH had effects on fertility within this time. Studies performed by Grizzle et al. (1994) demonstrated that VCH has no significant effect on fertility. Reproductive toxicity was assessed in Swiss (CD-1) mice dosed with VCH (0-500 mg/kg/day) by oral gavage in a continuous breeding study for 14 weeks of cohabitation (F_0 generation). While high-dose females F_0 females exhibited minor general toxicity (significantly reduced body weights), VCH had no effect on reproductive competence in these mice, as measured by number of litters, pups per litter, or percentage of pups born alive. In addition, VCH had no effect on preweaning growth or survival of these pups. The progeny (F_1 generation) of the

control and 500 mg/kg VCH groups were also dosed with either corn oil (vehicle control) or VCH (500 mg/kg, po), beginning at 22 days of age. These animals mated at day 74, and reproductive performance determined. Again, no effects on reproductive competence were noted. While F_1 males had a 20% reduction in the number of spermatids in the absence of histopahtologic lesions, there were no effects on the reproductive competence of the males. Therefore, at doses of VCH which reduced the gamete pool in both the ovary and testis, no significant adverse effect on fertility or reproductive performance was observed in either the F_0 or F_1 generation. Overall, these observations suggest that the first clinical signs of VCH-induced ovotoxicity do not occur until irreversible premature ovarian failure occurs.

4-Vinylcyclohexene as a Carcinogen

Follicular loss and the subsequent hormonal alterations are believed to be associated with the carcinogenic effects of VCH. A two year carcinogenicity study was conducted in male and female B6C3F_1 mice and Fischer 344 rats, in which animals received 0-400 mg/kg of VCH by oral gavage 5 d/ week for 103 weeks (NTP, 1986; Collins et al., 1987). An extensive and early mortality of undetermined cause was observed in male and female rats and male mice at the doses of VCH tested. This latter observation, as well as the lack of conclusive evidence of a carcinogenic effect, led to the conclusion that those studies with

VCH in male and female rats and in male mice were inadequate studies of carcinogenicity. There was clear evidence, however, of carcinogenicity of VCH in female mice. An increased incidence in the occurrence of uncommon ovarian neoplasms; including mixed benign tumors, granulosa cell tumors, and granulosa cell carcinomas, was observed. The mixed benign tumors were noninvasive, nonmetastasizing growths composed of mixtures of proliferating germinal epithelial cells and stromal granulosa cells. The granulosa cell tumors were characterized by proliferations of granulosa cells. Conversely, the granulosa cell carcinomas were cystic and hemorrhagic. Upon histological evaluation, these tumors had replaced the entire ovary and metastasized to the lungs. Therefore, even when survival of VCH-treated rodents was poor (high doses), the induction of ovarian tumors was evident in those mice that did survive. Therefore, there appears to be a dramatic species difference between the female rat and mouse in the ability of VCH to induce tumors in the ovary.

Role of Epoxidation in 4-Vinylcyclohexene-Induced Ovotoxicity

The growing evidence of species differences in the ovotoxicity of VCH led researchers to hypothesize that there may be differences in the metabolic fate of VCH in the mouse and the rat. The two double bonds on VCH it make it a likely candidate for extensive biotransformation in the liver. Early studies showed that

VCH formed epoxide metabolites in hepatic rat microsomes (Watabe et al., 1981). Microsomal epoxidation of VCH occurred preferentially at the C_1-double bond in the rat compared to the C_7-double bond. These epoxidation reactions have since proven to be catalyzed by CYP enzymes, as seen by decreased circulating epoxide levels in the blood of mice (60% of control mice) when the CYP inhibitor chloramphenicol was administered 1 hr prior to administration of VCH (Smith et al., 1990b).

Further investigation proved that the cytochrome P450-catalyzed bioactivation of VCH to epoxide metabolites (1,2-vinylcyclohexene [VCH-1,2-epoxide], 7,8-vinylcyclohexene [VCH-7,8-epoxide], and ultimately, VCD), is necessary for this ovarian toxicity to occur in the $B6C3F_1$ mouse (Smith et al., 1990b, Doerr et al., 1996). In one study, female $B6C3F_1$ mice and Fischer 344 rats received VCH (100-800 mg/kg), VCH-1,2-epoxide or VCH-7,8-epoxide (42-340 mg/kg), or VCD (10-80 mg/kg) ip daily for 30 d. Following 30 d of treatment, ovarian follicle counts were determined and these counts were utilized to define the ED50 values (dose which reduces follicular counts to 50% of control). A dose dependent depletion of small (primordial) follicles was observed in mice following VCH treatment, whereas ovotoxicity was not observed in rats at any dose tested. At the dose which depleted 85% of this follicular population in mice (800 mg/kg), no significant depletion of rat follicles was noted. The epoxides of VCH, however, were ovotoxic in both species and were clearly more potent ovarian toxicants than the parent compound. The ED_{50} value of VCH in mice was 2.7

mmol/kg, a dose 4 to 5-fold higher than the ED_{50} value in mice for VCH-7,8-epoxide (0.7 mmol/kg) and VCH-1,2-epoxide (0.5 mmol/kg), respectively. Since VCH did not cause ovotoxicity in the rat at the highest dose tested, an ED_{50} value could not be determined. The ED_{50} value for VCH-1,2-epoxide in rats was 1.4 mmol/kg. The most potent ovarian toxicant in either species was VCD, with ED_{50} values of 0.2 and 0.4 mmol/kg in mice and rats, respectively (Smith et al., 1990b).

To further asses the role of epoxidiation in the ovarian toxicity of VCH, studies were conducted to determine whether inhibition of VCH metabolism results in protection from VCH-induced ovarian injury (Smith et al., 1990b). Female $B6C3F_1$ mice were treated with 200 mg/kg i.p. of the CYP inhibitor chloramphenicol (Halpert et al., 1985) 1 hr prior to VCH pretreatment (800 mg/kg, ip) for either 1 d or 15 d. The 1 d experiments were conducted to determine blood levels of VCH-1,2-epoxide 2 hr after VCH treatment, whereas 15 d experiments were conducted to determine follicle counts. Pretreatment of mice with chloramphenicol prior to treatment with VCH reduced blood levels of VCH-1,2-epoxide to 60% of controls, as previously mentioned. Partial, but not complete protection from VCH-induced ovarian toxicity was observed in the 15 d dosing study, since the blood concentrations of VCH-1,2-epoxide in chloramphenicol-pretreated mice were not reduced to subtoxic levels (the blood level of the epoxide in chloramphenicol treated mice after 800 mg/kg of VCH was similar to that produced by 400 mg/kg of VCH in untreated mice, and this dose is

known to produce oocyte loss after repeated administration). Taken together, these results demonstrate that bioactivation of VCH to epoxides is required for ovotoxicity.

Additional studies supported the crucial role of epoxide formation in VCH-induced ovotoxicity. Structure activity studies demonstrated that compounds structurally related to VCH, but could only form monoepoxide metabolites, (eg. ethlycyclohexene, vinylcyclohexane, cyclohexene, phenylcyclohexene) were incapable of depleting small follicles in the $B6C3F_1$ mouse at the dose that VCH significantly depleted follicles (Doerr et al., 1996; Hooser et al., 1993). However, compounds that could form diepoxide metabolites (eg. butadiene monoepoxide, isoprene) did cause a significant depletion of follicles.

Effects of VCH compared to those of VCH-1,2-epoxide and VCD on testicular function and reproductive competence were also investigated in male $B6C3F_1$ mice (DeMerell et al., 1992; Hooser et al., 1992, 1995). Although VCH was administered at higher doses for a shorter time period compared to the Grizzle study (800 mg/kg, ip for 30 d) no adverse testicular effects were again reported. Interestingly, both VCH-1,2-epoxide (400 mg/kg, ip) and VCD (160 mg/kg ip) administration resulted in decreased testis weight. Treatment with VCD caused destruction of the germinal epithelium, decreased seminal vesicle weights, and increased concentrations of FSH (Hooser et al., 1992). In a similar dosing regimen, male mice were dosed daily with VCD for 30 d (320 mg/kg/d, ip), but then allowed to recover for 30 d. After this 30-day recovery period, the germinal

epithelium had returned to the normal state (Hooser et al., 1995). Conversely, in these studies, neither VCH (800 mg/kg, i.p) nor VCH-1,2-epoxide (200 mg/kg, i.p.) caused testicular damage. Therefore, while VCH, VCH-1,2-epoxide, and VCD all destroy germ cells in female mice, neither VCH nor VCH-1,2-epoxide casued testciular germ cell destruction. The reason for this sex difference in VCH-induced gonadal injury is unknown, although it may be due to differences in bioactivation/detoxification of VCH between the two species.

Further evidence that VCH requires bioactivation to elicit its toxicity is that VCH was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1537, or TA98 in the presence or absence of Arochlor 1254-induced male Sprague Dawley rat or male Syrian hamster liver S9 (Haworth et al., 1983). However, several metabolites of VCH, including VCD and VCH-1,2-epoxide, were mutagenic in Salmonella and/or produced chromosomal damage in vitro (Murray and Cummins, 1979; Simmon and Baden, 1980; Watabe et al., 1980; Turchi et al., 1981). The lack of mutagenicity of VCH in the presence of rat liver S-9 fractions may be due to insufficient epoxidation of VCH to epoxides or to rapid hydrolysis of these epoxides.

Bioactivation of 4-Vinylcyclohexene in Mice Compared to Rats

The studies by Smith et al. (1990b) demonstrated that VCD is a more potent ovarian toxicant than VCH and the VCH monoepoxides. In addition, the

epoxides of VCH are mutagens and carcinogens. The resistance of female rats to the ovarian toxicity caused by treatment with VCH prompted an investigation into comparing metabolism of VCH in the rat compared to the mouse. It was hypothesized that species differences in the capability to bioactivate VCH could account, in part, for the sensitivity of mice and the resistance of rats to VCH-induced ovarian tumors. Initial studies showed that after 5 min, VCH was metabolized by hepatic microsomes from both species to VCH-1,2-epoxide; however, a 6.5-fold greater rate of epoxidation was observed in mice compared to rats (Smith et al., 1990a). Kinetics of VCH epoxidation were determined in hepatic, pulmonary, and ovarian microsomes from female $B6C3F_1$ mice and Crl:CD rats (Keller et al., 1997). VCH was metabolized to VCH-1,2-epoxide and VCH-7,8-epoxide in hepatic microsomes of both species. Epoxidation at the 1,2-position was preferred. Mouse liver had a V_{max} for epoxidation at this position that was 56-fold higher than that for rat liver (11.1 and 0.20 nmol/min/mg protein, respectively). The V_{max} for epoxidation at the 1,2-position in mouse lung was 2-fold higher than that in rat lung, and this metabolite was not formed in detectable levels in ovarian microsomes from either species. Interestingly, the V_{max} values for metabolism of VCH-1,2-epoxide to the diepoxide were comparable in hepatic microsomes from mice and rats (5.35 and 3.69 nmol/min/mg protein, respectively).

These findings were supported by *in vivo* measurements of VCH metabolism in the two species (Smith et al., 1990a), in which a single dose (800 mg/kg, ip) of

VCH was administered and circulating levels of VCH-1,2-epoxide and VCH-7,8-epoxide were determined 0.5 to 6 hr after VCH administration. VCH-1,2-epoxide was present in the blood of mice with the highest concentration at 2 hr (41 nmol/ml), while the blood concentration of VCH-1,2-epoxide in rats was < 2.5 nmol/ml at all times tested. VCH-7,8-epoxide was not detected in the blood of rats at the limit of detection (2.5 nmolmL). It was noted that, while there was a 6.5-fold greater rate of formation of VCH-1,2-epoxide in the hepatic microsomes from mice compared to rats, the species differences observed in VCH-1,2-epoxide levels following a single dose of VCH was substantially greater (up to 16-fold differences). It was then hypothesized that the species differences in VCH ovotoxicity may not only lie exclusively in differences in bioactivating capabilities, but possibly also differences in detoxifying capabilities. Salyers (1995) provided evidence that mice were ineffective in forming and/or excreting diol conjugates of VCD. Thus, it was concluded that female mice are more susceptible to VCH-induced ovarian damage becasue of an enhanced rate of bioactivation of VCH to VCD, the ultimate ovarian toxicant, coupled with a reduced capacity to detoxify VCD via epoxide hydrolase. Thus, this prompted investigation into the molecular basis of the species-differences in the bioactivation of VCH.

Specific CYP Isoforms Involved in 4-Vinylcyclohexene Bioactivation in the Mouse

The species-dependent ovotoxicity of VCH led to an investigation into the roles of specific CYP isoforms in female B6C3F$_1$ mice through chemical inhibition and immunoinhibition techniques (Smith et al., 1990c; Doerr-Stevens et al., 1999). Testosterone 6β-hydroxylase and 15α-hydroxylase activities and VCH epoxidation were decreased in microsomes from chloramphenicol-treated mice, which suggested the involvement of CYP2A and CYP3A in VCH metabolism. However, anti-rat CYP3A IgG did not inhibit VCH epoxidase activity in mouse microsomes at a concentration that inhibited testosterone 6β-hydroxylase activity (68%). Conversely, anti-mouse CYP2A inhibited VCH epoxidase activity by 48% in mouse microsomes at a concentration that inhibited testosterone 15α-hydroxylase activity by 86%. Furthermore, microsomal VCH epoxidase and testosterone 16α-hydroxylase activities were 34% lower in female 129/J mice (deficient in constitutive expression of CYP2B forms) than in B6C3F$_1$ mice. These results suggested partial involvement of CYP2A and CYP2B forms in the microsomal epoxidation of VCH in the mouse, although little could be said about relative activities in the rat (Smith et al., 1990c).

Because significant depletion of small oocyte counts in mice is not detectable until after 15 days of repeated dosing with VCH (7.5 mmol/kg/day) (Smith et al., 1990b), studies were conducted to determine whether repeated dosing with VCH resulted in enhanced metabolism of VCH to the epoxides. This repeated dosing

with VCH (7.5 mmol/kg/day for 5, 10, or 15 d) increased the total hepatic microsomal CYP protein in mice (Doerr-Stevens et al., 1999). Accompanying the increased concentration of hepatic CYP was an increase in the hepatic microsomal conversion of VCH to VCH-1,2-epoxide and VCD. Parallel *in vivo* studies demonstrated that VCH-1,2-epoxide blood levels were elevated after repeated administration of VCH for 5 or 10 d as compared to controls. Furthermore, blood levels of VCD were significantly elevated at 10 d.

While there was evidence of the roles of CYP2A and CYP2B in the bioactivation of VCH in the mouse, parallel studies had not been performed in the female rat. It was hypothesized that if the rat did not induce CYP following repeated dosing with VCH, it could be the underlying reason behind the rat's resistance to the ovotoxicity of VCH. Studies by Smith and Doerr indicate that CYP2A and CYP2B may play a role in the species-dependent bioactivation of VCH. Metabolism studies with other small molecular weight compounds such as 1,3-butadiene, styrene, and benzene indicate that CYP2E1 may also be a critical hepatic CYP isoform in VCH bioactivation (Valentine et al., 1996; Lieber, 1997; Guengerich et al., 1991). CYP2E1 plays a major role in the epoxidation of 1,3-butadiene (BD) and its metabolite, 1,3-butadiene monoepoxide (BMO) (Duescher and Elfarra, 1994). However, since CYP represents a large family of enyzymes, there may be other isoforms besides CYP2A, CYP2B, and CYP2E1 that are important in VCH bioactivation, and therefore, ovotoxicity.

Extrapolation of Rodent Metabolism of 4-Vinylcyclohexene to that in Humans

It is important to determine the reason behind the species-differences in VCH-induced ovotoxicity to better estimate risk to humans. To date, there are limited data with regards to VCH metabolism in humans. In a study by Smith and Sipes (1991), female human microsomes epoxidized VCH to VCH-1,2-epoxide at rates 13-fold and 2-fold less than that in mice and rats, respectively. VCH-1,2-epoxide was the major metabolite, while VCH-7,8-epoxide formation was about 6-fold lower and in mose cases was below the limit of detection. Therefore, if the rate of hepatic VCH epoxiation is the major factor that determines the ovotoxicity of VCH, then the results of these studies provide initial evidence that humans would be less susceptible than the mouse and rat. Due to the recent availability of "Supersomes" containing several human CYP isoforms (GENTEST, Woburn, MA), epoxidation of VCH, VCH-1,2-epoxide, and VCH-7,8-epoxide can be determined to gain further insight into how humans metabolize VCH.

Statement of the Problem

The separate observations that (1) VCH is ovotoxic in B6C3F$_1$ mice but not the F-344 rat, (2) VCH requires multiple days of dosing to initiate ovotoxicity in the mouse, and (3) VCH and VCH-1,2-epoxide epoxidation is enhanced in murine microsomes after consecutive treatment with VCH *in vivo*, together raised the possibility that induction of VCH epoxidation may play a critical role in its species-dependent ovotoxicity. This prompted investigation into which CYP isoforms are induced in the mouse and/or rat following repeated dosing with VCH, which isoforms are capable of epoxidizing VCH as well as determine if there are stereochemical considerations in VCH bioactivation and ovotoxicity. Understanding the molecular reasons for the enhanced ability of the mouse to bioactivate VCH is necessary to better extrapolate which of these animal models, mouse or rat, would better predict the ability of humans to bioactivate VCH.

Research Objectives

The hypothesis tested by the experiments presented in this dissertation was that the molecular basis for the VCH-induced ovarian toxicity in the mouse relates to induction of hepatic CYP isoforms responsible for conversion of VCH to ovotoxic epoxides. Three major research objectives were developed to test this hypothesis, and are outlined below.

Chapter 2. The first objective was to determine the role of CYP2E1 in the bioactivation of VCH. Since CYP2E1 is responsible for the bioactivation of an extensive list of several small molecular weight compounds, including styrene and 1,3-butadiene, there is a strong possibility of CYP2E1 playing a similar role in the bioactiation of VCH.

Chapter 3. The second objective was to focus on the role of CYP2A, CYP2B, and several other subfamilies of hepatic CYP in the bioactivation of VCH. Through chemical inhibition and immunoinhibition techniques, it was demonstrated that CYP2A3 and CYP2B9/10 are involved in VCH bioactivation in the $B6C3F_1$ mouse, although parallel studies have not yet been performed in the rat. While it was previously suggested that CYP3A is not an important isoform through immunoinhibition studies, little is known about other hepatic CYP isoforms in VCH bioactivation.

Chapter 4. The third objective was to focus on the role of stereochemistry in VCH bioactivation. VCH is most commonly formed as a racemic mixture of (*R*)-VCH and (*S*)-VCH. There are numerous examples in which one enantiomer of a compound is more toxic than the racemic mixture or the other enantiomer. For example, the (*S*)- enantiomer of thalidomide causes severe limb abnormalities in offspring of pregnant rabbits, but the (*R*)- enantiomer is not toxic (Heger et al., 1994). Furthermore, CYP enzymes have been shown to have varying degrees of

stereoselectivity in catalyzing epoxidation reactions in compounds such as polycyclic aromatic hydrocarbons (Yang, 1988). VCH may therefore also undergo stereoselective bioactivation in cytochrome P450 systems.

Chapter 5. This chapter contains a comprehensive summary of the results and a discussion of the significance of the findings.

CHAPTER 2: EVALUATION OF HEPATIC CYTOCHROME P4502E1 IN THE SPECIES-DEPENDENT BIOACTIVATION OF 4-VINYLCYCLOHEXENE

ABSTRACT

4-Vinyl-1-cyclohexene (VCH), is converted by multiple forms of cytochrome P450 (CYP) to two monoepoxides (4-vinyl-1-cyclohexene 1,2-epoxide [VCH-1,2-epoxide], 4-vinyl-1-cyclohexene 7,8-epoxide [VCH-7,8-epoxide]), and 4-vinyl-1-cyclohexene diepoxide (VCD). A greater degree of formation of these epoxides by female B6C3F$_1$ mice as compared to Fischer 344 rats correlates with the ovarian toxicity observed only in the mice. Understanding which isoforms of CYP are involved in VCH bioactivation will better explain the species-dependent ovotoxicity of VCH. Present studies focus on the role of CYP2E1, as this isoform is responsible for the bioactivation of several structurally related small molecular weight compounds, including 1,3-butadiene. Hepatic microsomes prepared from either mice or rats pretreated with the CYP inducer acetone demonstrated 2-fold increases in the formation of VCH-1,2-epoxide. However, incubations with microsomes from CYP2E1-deficient mice compared to those from wild type mice revealed no differences in the rates of bioactivation of VCH to the monoepoxides. Since repeated exposure to VCH is required for VCH-induced ovotoxicity, rodents were dosed with VCH for 5 or 10 d to observe effects on the hepatic concentration of CYP2E1 and/or associated activities. VCH pretreatment failed to increase the concentration of CYP2E1 or CYP2E1 activity in either species, as measured by immunoblotting analysis and p-nitrophenol hydroxylation. Based on these data, it is concluded that CYP2E1 does not play a role in the species

differences between mice and rats in the bioactivation of VCH following repeated exposure to VCH.

INTRODUCTION

Many chemicals require bioactivation *in vivo* to elicit toxic effects. Cytochrome P450- (CYP) catalyzed bioactivation of the rubber curing by-product, 4-vinyl-1-cyclohexene, (VCH), to epoxide metabolites (4-vinyl-1-cyclohexene 1,2-epoxide [VCH-1,2-epoxide], 4-vinyl-1-cyclohexene 7,8-epoxide [VCH-7,8-epoxide], and ultimately, 4-vinyl-1-cyclohexene diepoxide [VCD]), is responsible for VCH-induced ovarian toxicity observed in B6C3F$_1$ mice (Smith et al., 1990a; Doerr and Sipes, 1996). Repeated daily dosing with these epoxide metabolites irreversibly depletes ovarian primordial and primary follicles (Collins and Manus, 1987; Smith et al., 1990b; Kao et al., 1999). In mice, the loss of these follicles results in premature ovarian failure and the subsequent development of ovarian neoplasms (NTP, 1986; Collins et al., 1987; Hooser et al., 1994). Interestingly, female rats are resistant to this ovarian toxicity caused by treatment with VCH. This resistance has been attributed to a reduced capacity of CYP to bioactivate VCH to the epoxide metabolites in rats as compared to mice (Smith et al., 1990c). Previous work also demonstrated that repeated exposure of mice to VCH results in induction of hepatic CYP levels and increased epoxidation of VCH (Doerr-Stevens et al., 1999). Comparing these induction effects in the mouse to those in F-344 rats will determine if the VCH-induced CYP activity following repeated exposure is the link to the species-dependent toxicity of VCH.

The studies reported here focus on the role of CYP2E1 in hepatic VCH bioactivation in female mice and rats. The CYP2E1 isoform is constitutively found in many mammals, including the mouse, rat, rabbit, and human (Lewis et al., 1997; Shimada et al., 1994), and represents approximately 7% and 10% of total human and rodent hepatic CYP (Shimada et al., 1994; Imaoka et al., 1991). CYP2E1 is responsible for the bioactivation of an extensive list of several small molecular weight compounds, including carbon tetrachloride, styrene, and benzene (Valentine et al., 1996; Lieber, 1997; Guengerich et al., 1991). CYP2E1 also plays a major role in the epoxidation of 1,3-butadiene (BD) and its metabolite, 1,3-butadiene monoepoxide (BMO) (Duescher and Elfarra, 1994). A recent study demonstrated the abilities of rabbit, rat, and human recombinant CYP2E1 isozymes to bioactivate BD to its toxic epoxide metabolites (Nieusma et al., 1998). CYP2E1 is readily induced by compounds such as ethanol (Coop and Koop, 1987) and acetone (Koop et al., 1985), as well as by fasting (Koop and Tierney, 1990) and by obesity (Salazar et al., 1988).

The studies presented here focus on the role of CYP2E1 in VCH bioactivation using approaches that include CYP2E1 immunoblotting and catalytic activity studies following repeated exposure to VCH or acetone. These studies also utilize hepatic microsomes from a transgenic mouse line, which has been successful in determining the role of CYP2E1 in the bioactivation and toxicity of several other compounds, such as benzene (Valentine et al., 1996),

acetominophen (Lee et al., 1996), methyl tert-butyl ether (Hong et al., 1999), and acetone (Bondoc et al., 1999).

EXPERIMENTAL PROCEDURES

Animals and Treatments. Female B6C3F$_1$ mice and Fischer 344 rats (approx. 28 to 38 d of age) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were housed in cages with sawdust bedding and had free access to food (Teklad, Harlan Sprague-Dawley, Inc., Madison, WI) and water. Animals were maintained on 12-h light/dark cycle and acclimated to this environment for at least 7 d before dosing and preparation of hepatic microsomes. Animals were dosed with either acetone (1% in the drinking water (v/v) for 5 d) or racemic VCH (7.5 mmol/kg i.p in sesame oil for 5 or 10 d) (Doerr-Stevens et al., 1999).

Chemicals. VCH, VCH-1,2-epoxide, VCD, methylcyclohexene, and acetone were purchased from Aldrich Chemical Co. (St. Louis, MO). NADP+, G6PDH, G6P, p-nitrophenol, and p-nitrocatechol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide was purchased from Fisher Scientific (Pittsburgh, PA). Magnesium chloride and perchloric acid were purchased from Mallinckrodt Laboratory Chemicals (Hazelwood, MO). Cyclohexene oxide was donated by NTP/RTI (Research Triangle Park, NC). VCH-7,8-epoxide was synthesized by the method of Watabe et al. (1981).

Microsomal Preparations. Animals were euthanized by inhalation of carbon dioxide 24 h after final dosing. Livers were excised and homogenized in a 50

mM Tris-HCl buffer (pH 7.4) using a drill motor and teflon-glass homogenizer. Microsomes were prepared by subjecting this homogenate to differential centrifugation as described by Guengerich et al. (1989). Hepatic microsomes from female wild-type ($cyp2e1^{+/+}$) and mice deficient of cytochrome P4502E1 ($cyp2e1^{-/-}$) of mixed background (C57BL/6N X Sv129) were prepared by Dr. Jun—Yan Hong of Rutgers University. Protein concentrations of all microsomal preparations were determined by using a Bicinchoninic Acid kit (Pierce, Rockford, IL). Total P450 concentration (nmol/mg microsomal protein) was determined using the carbon monoxide-binding assay as described by Omura and Sato (1964).

Capillary Gas-Liquid Chromatographic Conditions for Epoxide Analysis. Analyses were performed on a Hewlett-Packard HP 5890A gas chromatograph (GC) equipped with a DB-624 capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector (FID). The nitrogen carrier gas flow rate was 1 ml/min. The FID gas flow rates for H_2, N_2, and air were 42, 35, and 400 ml/min, respectively. Splitless injection was used with the purge off from time 0 to 1.0 min, with a 2 µl injection volume. The injection and detector temperatures were held isothermally at 200 °C and 250 °C, respectively. The oven temperature was held at 60 °C for 10 min, then increased to 230 °C at a rate of 12 °C/min. Final temperature was held for 3 min to ensure elution of the diepoxide. Retention times were 11.9 min for methylcyclohexene, 15.2 min for cyclohexene oxide, 16.6 min for VCH, 20.4 min for VCH-1,2-epoxide, 21.4 min for VCH-7,8-epoxide,

and 25.0 min for VCD. Quantities of these epoxides were determined by comparing the peak areas to those in standard curves prepared with known amounts of the epoxides. The level of sensitivity in detecting the epoxide metabolites was approximately 1 nmol.

Mouse and Rat Microsomal Incubations. To initiate incubations, 1 mg/ml microsomal protein was added to a 50 mM HEPES/ 0.1mM EDTA buffer containing a recycling NADPH system (0.5 mM $NADP^+$, 1 unit/ml G6PDH, and 10 mM G6P), 2 mM epoxide hydrolase inhibitor cyclohexene oxide (Guest and Dent, 1980), and 1 mM either VCH, VCH-1,2-epoxide, or VCH-7,8-epoxide. Cyclohexene oxide was tested previously to ensure that it is an effective inhibitor of epoxide hydrolase for VCH metabolism studies (data not shown). After the appropriate amount of time (ranging from 0 to 30 min), reactions were terminated by submersion in liquid nitrogen. VCH and its epoxide metabolites were extracted with ethyl acetate containing 1 µl/ml methylcyclohexene as an internal standard. The epoxides were identified and quantified using gas chromatography as described above. Data are presented as nmol/mg protein. The values were adjusted for percent recovery of epoxides, which were 96% for VCH-1,2-epoxide, 92% for VCH-7,8-epoxide, and 76% for VCD. The percent recoveries were calculated by adding known amounts of the chemical into denatured microsomes (heated for 30 min at 60 °C) prior to extraction with ethyl acetate and analyzed by GC.

Effects on CYP2E1 Activity in Microsomes from Mice or Rats Pretreated with VCH (p-Nitrophenol Hydroxylation). Hepatic microsomes from mice or rats pretreated with VCH (7.5 mmol/kg i.p. for 5 or 10 d), or acetone (1% in the drinking water (v/v) for 5 d), were compared for their ability to hydroxylate p-nitrophenol, a model substrate of CYP2E1 (Tassaneeyakul et al., 1993). Methods have been previously described by Forkert et al. (1996).

Effects on CYP2E1 Levels in Microsomes from Mice or Rats Pretreated with VCH. To determine the effect of VCH pretreatment on CYP2E1 levels, hepatic microsomal protein from B6C3F_1 mice or F-344 rats pretreated with acetone or VCH were measured for CYP2E1 protein levels. Protein separations were performed on 10% SDS-PAGE gels and proteins were electrophoretically transferred to nitrocellulose. Blots were incubated with an anti-goat polyclonal human CYP2E1 primary antibody (GENTEST Corporation, Woburn, MA) for 1 h at room temperature. Blots were then incubated with anti-goat secondary antibody and developed using an alkaline phosphatase color development buffer. Semi-quantitative comparisons of the relative densities of the different bands were made using densitometric analysis using a Scion Image Computer Program.

Studies were also performed to determine whether acetone increases CYP subfamilies in the mouse or rat other than CYP2E1, such as CYP2A, CYP2B, CYP2C, or CYP4A through immunoblotting techniques. Microsomal proteins (5 μg) were separated by SDS-PAGE, transferred to nitrocellulose filters, and

probed with polyclonal antibodies raised to either purified rat CYP1A1, CYP2A6, CYP2B1, CYP2C6, or CYP4A11 enzymes. Immunoblots were quantified by densitometric analysis and data are represented as % CYP expression in microsomes from acetone pretreated mice or rats as compared to controls

To confirm lack of CYP2E1 protein in the CYP2E1-null mice, CYP2E1 levels were also measured in the microsomes from cyp2e1$^{-/-}$ and cyp2e1$^{+/+}$ C57BL/6N X Sv129 mice. Semi-quantitative comparisons of the relative densities of the different bands were made as mentioned above.

Statistical Analysis. Student s t-test was used to compare between means of two different samples. Data were considered significantly different at $p < 0.05$.

RESULTS

Effects of Acetone Pretreatment on Microsomal Metabolism of VCH and Epoxides. Hepatic microsomes prepared from control mice formed greater amounts of VCH-1,2-epoxide and VCH-7,8-epoxide than those from control rats (Table 1). VCD was not detected in hepatic microsomes obtained from either the control mouse or rat. Acetone pretreatment resulted in approximately 2-fold increases in the epoxidation of VCH to VCH-1,2-epoxide in both mouse and rat hepatic microsomes, but did not cause an increase in the formation of VCH-7,8-epoxide in microsomes from either species. VCD could be quantified in hepatic microsomes from acetone pretreated mice but not acetone pretreated rats.

Studies were performed to determine whether acetone pretreatment increases CYP isoforms other than CYP2E1 in the mouse and rat. Immunoblots **(Figure 1)** were quantified by densitometric analysis and data are represented as % CYP expression in microsomes from acetone pretreated mice or rats as compared to controls **(Table 2)**. Acetone pretreatment caused 2.0- and 1.4-fold increases in mouse and rat hepatic CYP2A expression (CYP2A5 in the mouse and CYP2A1 in the rat), and1.3- and 2.9-fold increases in mouse and rat hepatic CYP2B expression (CYP2B9/10 in the mouse and CYP2B1/2 in the rat). Acetone pretreatment slightly decreased expression of other hepatic subfamilies in the mouse and rat microsomes (CYP1A, CYP2C, and CYP4A).

Bioactivation Differences in Microsomes from cyp2e1$^{-/-}$ Compared to cyp2e1$^{+/+}$ C57BL/6N X Sv129 Mice. Bioactivation of VCH in mice that lacked CYP2E1, (cyp2e1$^{-/-}$), ccmpared to wild-type mice of the same strain, (cyp2e1$^{+/+}$), showed no significant differences in bioactivation of VCH to form VCH-1,2-epoxide or VCH-7,8-epoxide **(Figures 2a and b)**. Furthermore, there were no differences between cyp2e1$^{-/-}$ and cyp2e1$^{+/+}$ C57BL/6N X Sv129 mice in the conversion of VCH-1,2-epoxide or VCH-7,8-epoxide to VCD **(Figures 2c and d)**. Hepatic microsomes from cyp2e1$^{+/+}$ mice bioactivated VCH, VCH-1,2-epoxide, and VCH-7,8-epoxide to a greater extent than those obtained from B6C3F$_1$ mice. Lack of CYP2E1 enyzme expression in the cyp2e1$^{-/-}$ mice was confirmed by western blot analysis **(Figure 3)**, and a greatly reduced activity towards p-nitrophenol compared to wild type (0.12 and 1.19 nmol p-nitrocatechol formed/mg/min after 30 min in cyp2e1$^{-/-}$ and cyp2e1$^{+/+}$ mice, respectively. Data not shown).

Effects of Repeated VCH Exposure on Cytochrome P4502E1 Levels and Catalytic Activity in B6C3F$_1$ Mice and F-344 Rats. CYP2E1 protein expression in different pretreatment groups was examined using a polyclonal anti-human CYP2E1, which recognizes both mouse and rat CYP2E1 (Figures 4a and b). VCH pretreatment (both 5 d and 10 d) failed to increase CYP2E1 levels in either species. The CYP2E1 inducer acetone increased CYP2E1 levels in microsomes from both species. Results obtained in the p-nitrophenol

hydroxylation assay demonstrated that there were no increases in CYP2E1 catalytic activity in microsomes from mice or rats pretreated with VCH for 5 or 10 d (Figure 5). However, acetone significantly induced CYP2E1 activity in microsomes from both species.

TABLE 1:

Effects of pretreatment with the CYP inducer acetone on the epoxidation of VCH.

Metabolite	Treatment Group	Species	
		Mouse	Rat
VCH-1,2-Epoxide	Control	44.2–4.0	19.5–2.3
	Acetone	76.5–3.4 **(1.7)**	45.1–3.6 **(2.3)**
VCH-7,8-Epoxide	Control	21.1–5.2	15.3–4.3
	Acetone	22.3–4.0 **(0)**	18.0–5.1 **(0)**
VCD	Control	n.d.	n.d.
	Acetone	3.1–1.9	n.d.

Table 1: Formation of VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD (nmol/mg protein) from VCH after 30 min incubations with hepatic microsomes from B6C3F$_1$ mice or Fischer 344 rats pretreated with acetone (1.0% in drinking water X 5 d). n.d: not detectable. Data are represented as nmol epoxide formed/mg protein after 30 min incubation with 1 mM VCH. Numbers in parenthesis represent significant ($p < 0.05$) fold-increase in epoxide metabolite formation over control microsomal incubations.

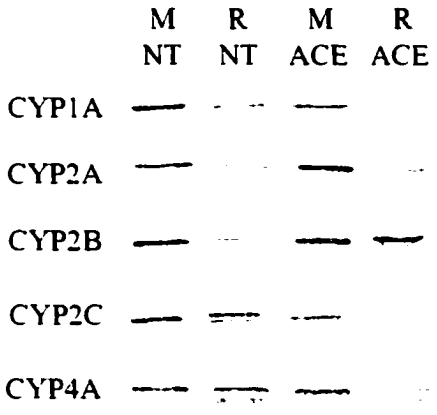
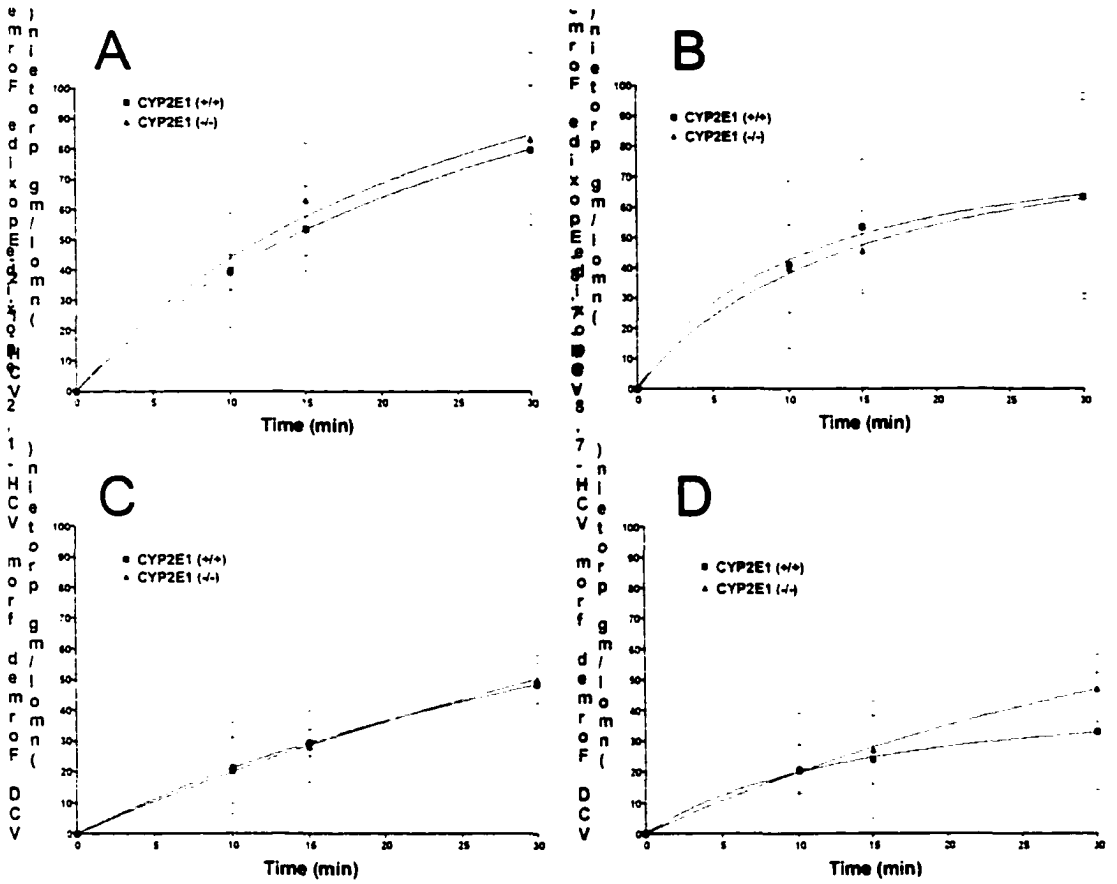


TABLE 2:

% CYP Expression in Hepatic Microsomes from Acetone Pretreated Animals Compared to Control

CYP Subfamily	Species	
	Mouse	Rat
CYP1A	80	60
CYP2A	200	140
CYP2B	130	290
CYP2C	70	50
CYP4A	90	60

Figure 1 and Table 2: Western blots of different CYP levels in hepatic microsomes isolated from B6C3F$_1$ mice and Fischer 344 rats after treatment with acetone. Rodents were treated with acetone 1% in drinking water for 5 d. Microsomal proteins (5 μg) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with polyclonal antibodies raised to either purified rat CYP1A1, CYP2B1, CYP2C6, or CYP4A11 enzymes, or to purified rabbit CYP2A6 (Figure 1). Immunoblots were quantified by densitometric analysis and data are represented as % CYP expression in microsomes from acetone pretreated mice or rats as compared to controls (Table 2). Treatment groups: nontreated (NT), acetone (ACE).



Figures 2a - d: Comparison of epoxide formation from VCH (Figures 2a and b), and diepoxide formation from VCH-1,2-epoxide and VCH-7,8-epoxide (Figures 2c and d) in female C57BL/6N X Sv129 CYP2E1-null (cyp2e1$^{-/-}$) and wild-type (cyp2e1$^{+/+}$) mouse hepatic microsomes.

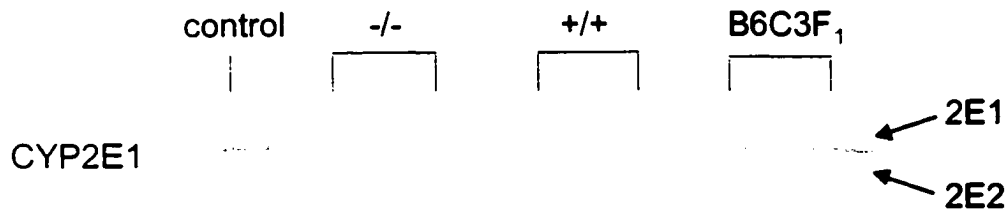
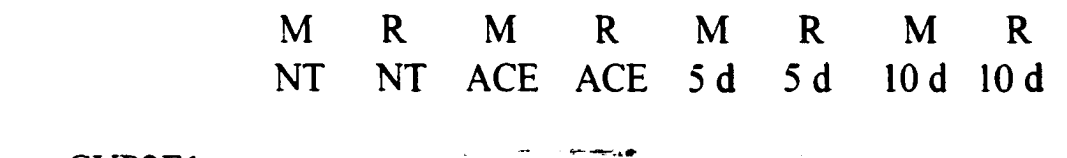
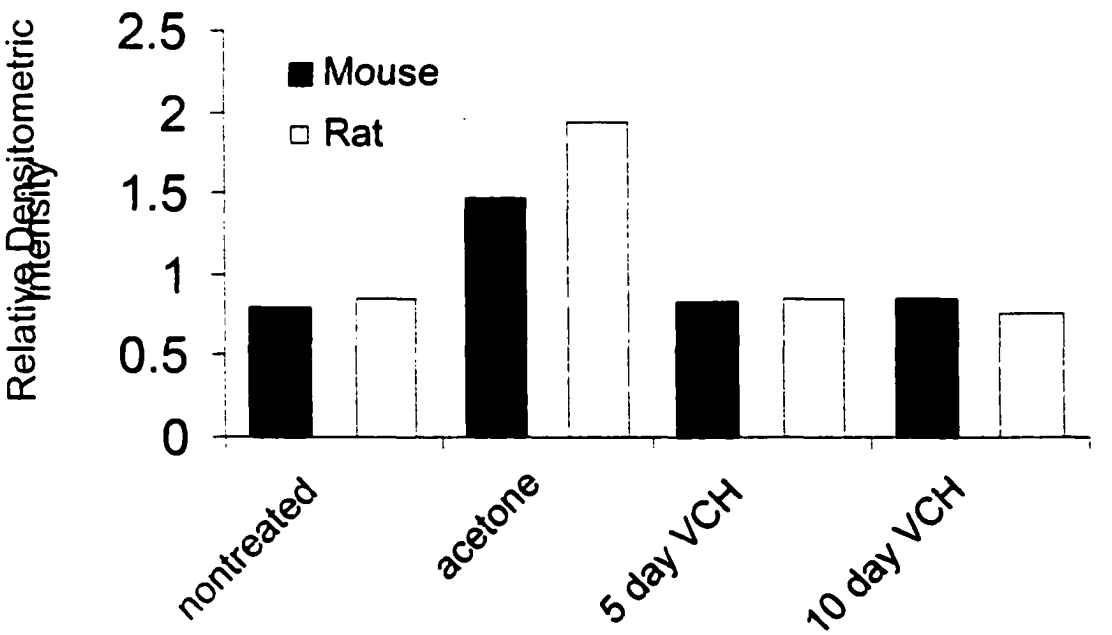


Figure 3. Immunoblots of CYP2E1 in hepatic microsomes isolated from CYP2E1 wild-type (cyp2e1$^{+/+}$) and P4502E1-null (cyp2e1$^{-/-}$) C57BL/129 mice, and B6C3F$_1$ mice. To confirm that P4502E1-null C57BL/129 mouse microsomes lacked CYP2E1, microsomal protein (5 μg) from cyp2e1$^{-/-}$ and cyp2e1$^{+/+}$ C57BL/129 mice, and B6C3F$_1$ mice were measured for protein levels of hepatic CYP2E1. Immunoblots were quantifed by densitometric analysis (data not shown).

A



B



Figures 4a and b. Western blots and relative densitometry of CYP2E1 levels in hepatic microsomes isolated from B6C3F$_1$ mice and Fischer 344 rats after treatment with various inducing agents. Rodents were treated with VCH or acetone, as described in *Materials and Methods*. Microsomal proteins (5 μg) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with polyclonal antibodies raised to purified human CYP2E1 enzymes (Figure 5a). Immunoblots were quantified by densitometric analysis (Figure 5b). Treatment groups: nontreated (NT), acetone (ACE), 4-Vinylcyclohexene (VCH). Western blots were performed three times in different sets of microsomes.

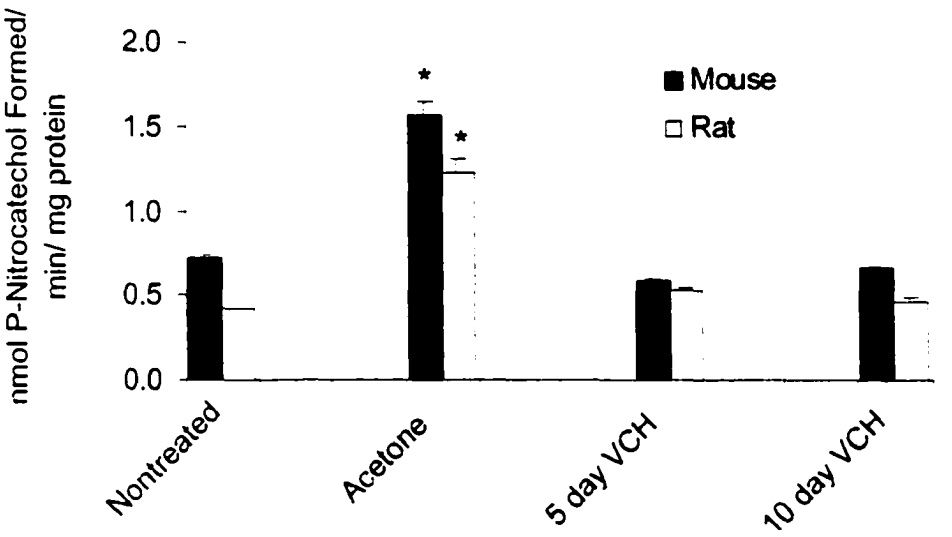


Figure 5. P-Nitrophenol hydroxylase activities in hepatic microsomes from VCH-treated or acetone-treated B6C3F$_1$ mice or Fischer 344 rats. Hepatic microsomes were isolated from mice or rats administered acetone (1% in drinking water for 5 d) or VCH (7.5 mmol/kg for 5 or 10 d). Microsomes (1.0 mg/ml) were incubated for 30 min with 100 mM p-nitrophenol. Data represent the mean ± S.D. of either 4 individual rats or 4 groups of pooled mice (4 mice/group) at $p < 0.05$. Statistically significant compared to nontreated animal of the same species (*).

DISCUSSION

The separate observations that (1) VCH is ovotoxic in B6C3F$_1$ mice but not in the F-344 rat, (2) VCH requires multiple days of dosing to initiate ovotoxicity in the mouse, and (3) the epoxidation of VCH and VCH-1,2-epoxide is enhanced in murine microsomes after consecutive treatment with VCH *in vivo*, together raised the possibility that induction of the enzyme(s) responsible for VCH epoxidation may play a critical role in its species-dependent ovotoxicity (Doerr-Stevens et al., 1999). This prompted an investigation into which CYP isoforms are responsible for the epoxidation of VCH. The experiments represented in this paper focused on CYP2E1 due to its role in the bioactivation of several other small molecular weight compounds, including 1,3-butadiene (Lieber, 1997; Valentine et al., 1996; Guengerich et al., 1991).

Initial studies focused on the effects of pretreatment with acetone, a known inducer of CYP2E1, on VCH bioactivation. Acetone pretreatment in mice and rats did indeed cause increases in both hydroxylation of p-nitrophenol and epoxidation of VCH. However, further investigation revealed that acetone is not a selective inducer of CYP2E1, and the acetone-dependent increases in VCH bioactivation may be due to increases in activities of other CYP isoforms besides CYP2E1. Previous studies have shown that acetone increases CYP2B1 expression in the rat (Ronis et al., 1991; Longo and Ingelman-Sindberg, 1993), and this has been confirmed in our laboratory with both the rat (CYP2B1/2 and CYP2A1) and mouse (CYP2B9/10 and CYP2A4/5) through immunoblotting

techniques. Through previous chemical induction and immunoinhibition studies, CYP 2A and CYP2B9/10 have been shown to catalyze the epoxidation of VCH in the mouse (Smith et al., 1990c). Also, these isoforms are induced in the mouse following repeated exposure to VCH (Doerr-Stevens et al., 1999). Thus, it was concluded that other isoforms of CYP present in hepatic microsomes from acetone pretreated animals may explain the increased rates of formation of the VCH epoxides.

The failure to find differences in the epoxidation of VCH between hepatic microsomes prepared from cyp2e1$^{-/-}$ and cyp2e1$^{+/+}$ mice provides further evidence that CYP2E1 is not a key enzyme in the bioactivation of VCH. It was demonstrated that cyp2e1$^{-/-}$ mice lacked the CYP2E1 enzyme through immunoblotting and greatly reduced catalytic activity towards p-nitrophenol. However, the knockout microsomes converted VCH to the various epoxides at the same rate as those obtained from the wild-type counterparts. Therefore, other isoforms of CYP must catalyze the epoxidation of VCH in the C57BL/6N X Sv129 mouse.

Further strong evidence indicating that CYP2E1 is not a critical isoform in VCH bioactivation is the fact that this isoform is not induced in the mouse liver following repeated dosing with VCH. Since previous studies show that repeated exposure with VCH is required to elicit ovotoxicity in the mouse, and this repeated dosing coincides with increases in total hepatic CYP levels in the mouse (but not the rat) [Fontaine, Unpublished data], one would expect that the

isoforms responsible for bioactivating VCH be increased in the mouse. Neither CYP2E1 protein expression nor CYP2E1 activity were increased in the mouse or the rat following repeated dosing with VCH. Again, these results support the role of other isoforms of CYP.

In summary, the sensitivity of mice and the resistance of rats to VCH-induced ovarian toxicity relate in part to differences in the bioactivation of VCH, which are due to the expression and activities of certain isoforms of cytochrome P450. Although hepatic microsomes from mice and rats pretreated with acetone showed increases in VCH-1,2-epoxide formation from VCH, hepatic microsomes from mice or rats pretreated with VCH for 5 or 10 d demonstrated no increases in CYP2E1 protein levels or activity. These data, combined with the data showing no differences in epoxidation of VCH or its monoepoxides in CYP2E1-deficient mouse hepatic microsomes compared to those of mice that do have CYP2E1, indicate that CYP2E1 is not an important isoform in the species-specific bioactivation of VCH. Results thus far indicate that CYP isoforms from the CYP2A and CYP2B subfamilies play important roles in the species-dependent bioactivation, and therefore ovotoxicity, of VCH.

CHAPTER 3: EVALUATION OF SPECIFIC HEPATIC CYTOCHROME P450 ISOFORMS IN THE SPECIES-DEPENDENT BIOACTIVATION OF VINYLCYCLOHEXENE

ABSTRACT

4-Vinyl-1-cyclohexene (VCH), is ovotoxic in B6C3F$_1$ mice, but not in Fischer 344 rats, which can be partially attributed to greater formation of toxic epoxides from VCH in mice compared with rats. Since repeated exposure to VCH is necessary to cause ovotoxicity in mice, it is important to determine if repeated exposure results in induction of cytochrome P450 (CYP) enzymes involved in its bioactivation. Hepatic microsomes prepared from either mice or rats treated repeatedly with VCH demonstrated significantly increased VCH bioactivation *in vitro* as assessed by epoxide formation. Mice and rats were then dosed with VCH, VCH-1,2-epoxide, or vinylcyclohexene diepoxide (VCD) for 10d, and measured for increases in hepatic microsomal CYP levels or activities. Total hepatic CYP levels were elevated only in microsomes from mice pretreated with VCH or VCH-1,2-epoxide. Immunoblotting analysis of microsomes from VCH-treated rodents revealed elevated levels of CYP2A and CYP2B in mice but not in rats. VCH-1,2-epoxide pretreatment also increased CYP2B levels in the mouse. Activities toward specific substrates for CYP2A and CYP2B (coumarin and pentoxyresorufin, respectively) confirmed that VCH and VCH-1,2-epoxide pretreatments resulted in increased catalytic activities of CYP2A and CYP2B in the mouse but not in the rat. Pretreatment with phenobarbital, a known inducer of CYP2A and CYP2B, increased VCH bioactivation *in vitro* in both species.

Interestingly, metabolism studies with human CYP Supersomes reveal that, of eight isoforms tested, only human CYP2E1 and CYP2B6 are capable of significantly bioactivating VCH, while CYP2B6, CYP2A6, CYP2E1, and CYP3A4 were capable of catalyzing the epoxidation of the monoepoxides.

INTRODUCTION

4-Vinylcyclohexene (VCH) is formed by the spontaneous dimerization of two molecules of 1,3-butadiene during the rubber curing process (Rappaport et al., 1976, International Agency for Research on Cancer, 1994). VCH is also an intermediate in the synthesis of styrene and vinylcyclohexene diepoxide (VCD) for epoxy resin formation (International Agency for Research on Cancer, 1976). Repeated exposure of mice to VCH causes premature ovarian failure by depletion of ovarian primordial and primary follicles (Collins and Manus, 1987; Smith et al., 1990a; Hooser et al., 1994). This loss of follicles results in premature ovarian failure, which may be associated with the ovarian neoplasms that develop in mice chronically exposed to VCH (National Toxicology Program, 1986; Collins, 1987a and 1987b). Cytochrome P450 (CYP)-catalyzed bioactivation of VCH to epoxide metabolites (VCH-1,2-epoxide, VCH-7,8-epoxide, and ultimately, VCD) **(Figure 6)**, is necessary for this ovarian toxicity to occur (Smith et al., 1990b, Doerr et al., 1996). Interestingly, female rats are resistant to the ovarian toxicity caused by treatment with VCH, which is at least partially related to a reduced capacity to bioactivate VCH to the epoxide metabolites (Smith et al., 1990c). Understanding the molecular reasons for this enhanced ability of the mouse to bioactivate VCH is necessary to better extrapolate which of these animal models, mouse or rat, would better predict the risk to humans exposed to VCH.

Studies in the mouse have established that repeated daily dosing with VCH is necessary for depletion of ovarian follicles (Smith et al., 1990b), and causes increases in total CYP protein as well as increases in VCH bioactivation *in vitro* (Doerr-Stevens et al., 1999). Furthermore, repeated exposure to VCH led to significantly elevated circulating levels of VCH-1,2-epoxide and VCD in the mouse *in vivo*. These results have prompted an investigation into the roles of specific CYP isoforms in hepatic VCH bioactivation in female $B6C3F_1$ mice and F-344 rats. Through chemical inhibition and immunoinhibition techniques, it was demonstrated that CYP2A3 and CYP2B9/10 are involved in VCH bioactivation in the $B6C3F_1$ mouse (Smith et al., 1990c). Current studies focus on the roles of these and several other CYP subfamilies in the species-dependent VCH bioactivation using techniques such as chemical induction, specific CYP activity studies and immunoblotting, and metabolism in different human heterologously expressed CYP proteins. In addition, mice and rats were dosed with VCH-1,2-epoxide or VCD (equitoxic doses to that of VCH in the mouse, Smith et al., 1990b), to determine whether VCH or the epoxide metabolites of VCH were causing the CYP induction.

EXPERIMENTAL PROCEDURE

Animals and Treatments. Female B6C3F$_1$ mice and Fischer 344 rats (approximately 28 to 38 days of age) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were housed in cages with sawdust bedding and had free access to food (Teklad, Harlan Sprague-Dawley, Inc., Madison, WI) and water. Animals were maintained on 12-hr light/dark cycle and acclimated to this environment for at least 7 days before dosing and/or preparation of hepatic microsomes. Animals were dosed with either VCH (7.5 mmol/kg for 10 d), VCH-1,2-epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d) (Doerr-Stevens *et al.*, 1999), or phenobarbital (PB) (80 mg/kg i.p. for 5 d). VCH-7,8-epoxide was not tested based on its limited availability. PB was prepared as a 3.2% solution (w/v) in 0.9% NaCl. VCH, VCH-1,2-epoxide, and VCD were prepared in sesame oil. In each treatment group, microsomes were prepared from 4 individual rats, or were pooled from 4 mice/ group (16 mice total).

Chemicals. VCH, VCH-1,2-epoxide, VCD, and methylcyclohexene were purchased from Aldrich Chemical Co. (St. Louis, MO). $NADP^+$, G6PDH, G6P, NADPH, trichloroacetic acid, coumarin, 7-hydroxycoumarin, pentoxyresorufin, ethoxyresorufin, resorufin, p-nitrophenol, p-nitrocatechol, and sodium borate were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide was purchased from Fisher Scientific (Pittsburgh, PA). Magnesium chloride,

phenobarbital sodium, and perchloric acid were purchased from Mallinckrodt Laboratory Chemicals (Hazelwood, MO). Chloroform was purchased from Burdick and Jackson Products (Muskegon, MI). Cyclohexene oxide was donated as a gift from NTP/RTI (Research Triangle Park, NC). VCH-7,8-epoxide was synthesized by the method of Watabe *et al.* (1981). For immunoblotting studies, anti-human polyclonal CYP2E1 primary antibody, and anti-rat polyclonal CYP1A1, CYP2B1, CYP2C6, CYP3A2, and CYP4A1 primary antibodies were purchased from GENTEST (Woburn, MA), while the corresponding anti-goat secondary antibodies were purchased from SIGMA Chemical Co. (St. Louis, MO). Anti-rat polyclonal CYP2A6 was purchased from Affinity Bioreagents (Golden, CO), and anti-rabbit secondary antibody was purchased from SIGMA Chemical Co. (St. Louis, MO).

Subcellular Preparations and Characterization. Animals were killed by inhalation of carbon dioxide 24 hr after final dosing. Livers were excised and homogenized in a 50 mM Tris-HCl buffer (pH 7.4) using a drill motor and teflon-glass homogenizer. Microsomes were prepared by subjecting this homogenate to differential centrifugation as described by Guengerich et al. (1989). Protein concentrations were determined by using a Bicinchoninic Acid kit (Pierce, Rockford, IL). Total P450 concentration (nmol/mg microsomal protein) was determined using the carbon monoxide-binding spectrophotometric assay as described by Omura and Sato (1964).

Capillary Gas-Liquid Chromatographic Conditions for Epoxide Analysis. Analyses were performed on a Hewlett-Packard HP 5890A gas chromatograph (GC) equipped with a .25 mm diameter DB-624 capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector. The nitrogen carrier gas flow rate was 1 ml/min. The FID gas flow rates for H_2, N_2, and air were 42, 35, and 400 ml/min, respectively. Splitless injection was used with the purge off from time 0 to 1.0 min, with a 2 µl injection volume. The injection and detector temperatures were held isothermally at 200 °C and 250 °C, respectively. The oven temperature was held at 60 °C for 10 min, then increased to 230 °C at a rate of 12 °C/min. Final temperature was held for 3 min to ensure elution of the diepoxide. Retention times were 11.9 min for methylcyclohexene, 15.2 min for cyclohexene oxide, 16.6 min for VCH, 20.4 min for VCH-1,2-epoxide, 21.4 min for VCH-7,8-epoxide, and 25.0 min for VCD. VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD formation (nmol/mg microsomal protein) was quantified by comparing the peak areas to those in standard curves of known amounts of the epoxides.

***In Vitro* Metabolism of VCH, VCH-1,2-epoxide, and VCH-7,8-epoxide in the Mouse and Rat.** Microsomal protein (1 mg/ml) was added to a 50 mM HEPES/ 0.1mM EDTA buffer containing a recycling NADPH system (0.5 mM $NADP^+$, 1 unit/ml G6PDH, and 10 mM G6P), 2 mM cyclohexene oxide (an epoxide hydrolase inhibitor, Guest and Dent, 1980), and 1 mM VCH. Previous studies demonstrated that these reactions are linear up to 1 mg/ml protein (data

not shown). After 60 min, the cytochrome P450 reactions were terminated by submersion in liquid nitrogen. VCH and its epoxide metabolites (VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD) were extracted with ethyl acetate containing 1 µl/ml methylcyclohexene as an internal standard. The epoxide metabolites were identified and quantified using gas chromatography as described above. Data are presented as nmol/mg microsomal protein formed. The values were adjusted for percent recovery of epoxides, which were approximately 96% for VCH-1,2-epoxide, 92% for VCH-7,8-epoxide, and 76% for VCD. The percent recoveries were calculated by injecting a known amount of compound into denatured microsomes (first heated for 30 min at 60 °C) and then measured by gas chromatography.

Analysis of Specific CYP Levels in Microsomal Samples from Mice or Rats Pretreated with VCH. Depending on the CYP isoform to be assayed, either 1 or 10 µg of hepatic microsomal protein from each treatment group was used per gel lane. Proteins were separated on 10% SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Membranes were incubated with polyclonal antibody to rat CYP2A6, polyclonal antibody to human CYP2E1, or polyclonal antibody to rat CYP1A1, CYP2B1, CYP2C6, CYP3A2, or CYP4A11, for 1 hr at room temperature. Blots were then incubated with anti-goat secondary antibody (for CYP1A1, CYP2B1, CYP2C6, CYP2E1, CYP3A2 or CYP4A11) or anti-rabbit secondary antibody (CYP2A6), all conjugated with alkaline phosphatase (AP), and developed using an AP color development buffer.

Different positive control microsomes were supplemented with the different CYP IgG, (eg. microsomes from acetone-treated rats for CYP2E1, microsomes from phenobarbital-treated rats for CYP2A6, CYP2B1, and CYP2C6; microsomes from 3-methylcholanthrene-treated rats for CYP1A; microsomes from clofibrate-treated rats for CYP4A11) to ensure there was proper binding of the antibody (data not shown). Semi-quantitative comparisons of the relative densities of the different bands were made using densitometric analysis using a Scion Image Computer Program. All western blots were performed 4 times/antibody probed using different sets of microsomes.

Analysis of Specific CYP Enzyme Activities in Microsomal Samples from Mice or Rats Pretreated with VCH, VCH-1,2-Epoxide, or VCD. Microsomal protein samples from mice or rats pretreated as described as in Materials and Methods were compared in their ability to biotransform model substrates of different CYP isoforms. Ethoxyresorufin has been utilized for measuring mouse CYP1A1 activity (Dickerson et al., 1999), rat CYP1A1 activity (Hashemi et al., 2000) and human hepatic CYP1A2 activity (Hengstler et al., 1997). Likewise, pentoxyresorufin has served as a model substrate for measuring CYP2B activity in different species, including mouse CYP2B9/10 (Posti et al., 1999), rat CYP2B1/2 (McKim et al., 1999), and human CYP2B6 (Gervot et. al., 1999). The procedure of Lubet et al. (1985) was used for assessing the metabolism of ethoxy- or pentoxyresorufin.

Conversion of coumarin to 7-hydroxycoumarin was used to assess CYP2A activity. Methods have been previously described by Waxman (1982). Honkakoski and Negishi (1997) have classified the CYP2A subfamily that describes the catalytic specificities and relates the activities of rat CYP2A3, mouse CYP2A5, and human CYP2A6. All of these enzymes catalyze coumarin 7-hydroxylation.

Microsomal hydroxylation of p-nitrophenol was used to assess CYP2E1 activity (Tassaneeyakul et al., 1993). Methods have been previously described by Forkert et al. (1996).

Metabolism of VCH, VCH-1,2-epoxide, of VCH-7,8-epoxide in Supersomes Containing Human CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, CYP3A4, CYP4A11, or Aromatase. To investigate whether expressed individual human CYP proteins were capable of mediating the epoxidation of VCH and its monoepoxide metabolites, the Supersome system (GENTEST Corporation, Woburn, MA) was utilized (Human CYP + P450 Reductase + Cytochrome b_5). The 0.5 mL total incubation volume consisted of 50 pmol CYP protein, 1 mM racemic VCH, and a recycling NADPH system (0.5 mM $NADP^+$, 1 unit/ml G6PDH, and 10 mM G6P). Samples were incubated at 37 °C for up to 30 min. Epoxide metabolites were identified and quantified using gas chromatography. Blank incubations lacked G6P. Substrates were also incubated with control insect microsomes to ensure there was no metabolism without CYP enzyme.

Statistical Analysis. Student s t-test was used to compare between means of two different samples. Data were considered significantly different at $p < 0.05$.

RESULTS

Effects of VCH Pretreatment on *In Vitro* Metabolism of VCH, VCH-1,2-epoxide, and VCH-7,8-epoxide in Mouse and Rat Microsomes. Hepatic microsomes from untreated mice formed greater amounts of VCH-1,2-epoxide and VCH-7,8-epoxide compared to rats (Table 3). Incubations were conducted for up to 60 min to allow possible VCD formation, but none was detected in microsomes obtained from either the control mice or rats. Pretreatment of mice or rats with VCH caused 3.8- and 2.0-fold increases in the formation of VCH-1,2-epoxide in hepatic microsomes, respectively. VCH pretreatment also resulted in a 2.0-fold increase in VCH-7,8-epoxide formation by mouse hepatic microsomes and a 1.8-fold increase by rat hepatic microsomes. Only in the mouse did VCH pretreatment result in microsomal formation of VCD. Microsomes from phenobarbital pretreated mice and rats exhibited 5.9- and 2.8-fold increases in VCH-1,2-epoxide formation, respectively, and 2.1- and 1.6-fold increases in VCH-7,8-epoxide formation, respectively.

Effects of VCH, VCH-1,2-epoxide, or VCD Exposure on Total Cytochrome P450 Levels in Hepatic Microsomes from Mice or Rats. There were significant increases in total hepatic microsomal CYP levels in the mouse following repeated exposure to VCH or VCH-1,2-epoxide compared to

nontreated mice (Table 4). Neither VCH, VCH-1,2-epoxide, or VCD caused significant increases in total CYP levels in the rat compared to control. As expected, PB caused significant increases in CYP levels in both species.

Effects of VCH Exposure on Specific Cytochrome P450 Levels in Microsomes from Mice or Rats. The levels of specific hepatic CYP enzymes in mice and rats exposed to VCH, VCH-1,2-epoxide, or VCD were examined with polyclonal antibodies to human CYP2E1, rat CYP1A1, rat CYP2A6, CYP2B1, CYP2C6, CYP3A2, and CYP4A1 (Figure 7, Tables 5 and 6). All western blots were performed 4 times/antibody probed using different sets of microsomes. In the mouse, there were 2.5-fold and 1.9-fold increases in CYP2B protein levels following 10 d treatments with VCH or VCH-1,2-epoxide, respectively. There was also a 2.1-fold increase in CYP2A protein levels with 10 d VCH pretreatment in the mouse. There were not significant increases in CYP2A or CYP2B levels in the rat with either VCH, VCH-1,2-epoxide, or VCD pretreatment. Instead, CYP2A expression was decreased with VCH pretreatment, and CYP2B expression was decreased with VCH-1,2-epoxide or VCD pretreatment. There were no increases in CYP1A or CYP4A levels in microsomes from either species pretreated with VCH. Pretreatment with VCD caused elevated levels of CYP1A and CYP2C in the rat.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP1A Activity in Microsomes from Mice or Rats. To assess the effect of repeated treatment with VCH, VCH-1,2-epoxide, or VCD on the activity of

CYP1A, the hepatic dealkylation of ethoxyresorufin was measured (Figure 8). After repeated treatment with VCH or VCH-1,2-epoxide, small but significant increases of ethoxyresorufin dealkylation were observed in the mouse but not the rat. There were also significant increases in ethoxyresorufin dealkylation in the microsomes from mice pretreated with PB.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP2A Activity in Hepatic Microsomes from Mice or Rats. Repeated exposure to VCH or VCH-1,2-epoxide significantly increased CYP2A activity in mouse hepatic microsomes, as measured by coumarin 7-hydroxylation (Figure 9). Pretreatment of rats with VCH, VCH-1,2-epoxide, or VCD did not result in increased hepatic microsomal activity with respect to 7-hydroxylation of coumarin. Phenobarbital pretreatment increased coumarin hydroxylation in microsomes obtained from both species.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP2B Activity in Hepatic Microsomes from Mice or Rats. The hepatic microsomal dealkylation of pentoxyresorufin, a model substrate of CYP2B, was enhanced by pretreatment of mice with either VCH or VCH-1,2-epoxide (Figure 10). VCH pretreatment resulted in a 4.1-fold increase of pentoxyresorufin dealkylation over control, while VCH-1,2-epoxide caused a 2.2-fold increase over control. No increases in pentoxyresorufin dealkylation were observed in microsomes from rats pretreated with VCH, VCH-1,2-epoxide. VCD pretreatment caused a small, but significant decrease in pentoxyresorufin

dealkylation in rat hepatic microsomes. Phenobarbital pretreatment caused large increases in pentoxyresorufin dealkylation in both species.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP2E1 Activity in Hepatic Microsomes from Mice or Rats. The microsomal hydroxylation of p-nitrophenol, a model substrate of CYP2E1 (Tassaneeyakul et al., 1993), was not effected by pretreatment with either VCH, 1-2epoxide, or VCD in either the mouse or the rat (Figure 11). Previous studies with acetone pretreated microsomes from mice or rats pretreated with the CYP2E1 inducer acetone (1% in drinking water for 5 d) demonstrated significant increases in p-nitrophenol hydroxylation in the mouse and rat compared to control (Fontaine et al., submitted).

Incubations with Supersomes Containing Specific Human CYP Enzymes. Purified protein systems were utilized to determine which human CYP isoforms are capable of catalyzing the epoxidation of VCH and its epoxide metabolites (Figures 12a and b). Of the isoforms tested, only human CYP2B6 and CYP2E1 showed substantial catalytic towards VCH in forming VCH-1,2-epoxide and VCH-7,8-epoxide. The major product formed by CYP2B6 was VCH-7,8-epoxide, while the major product formed by CYP2E1 was VCH-1,2-epoxide. CYP2A6, CYP2B6, CYP2E1, and CYP3A4 were capable of catalyzing the epoxidation of both monoepoxides to form the diepoxide (Figures 13a and b).

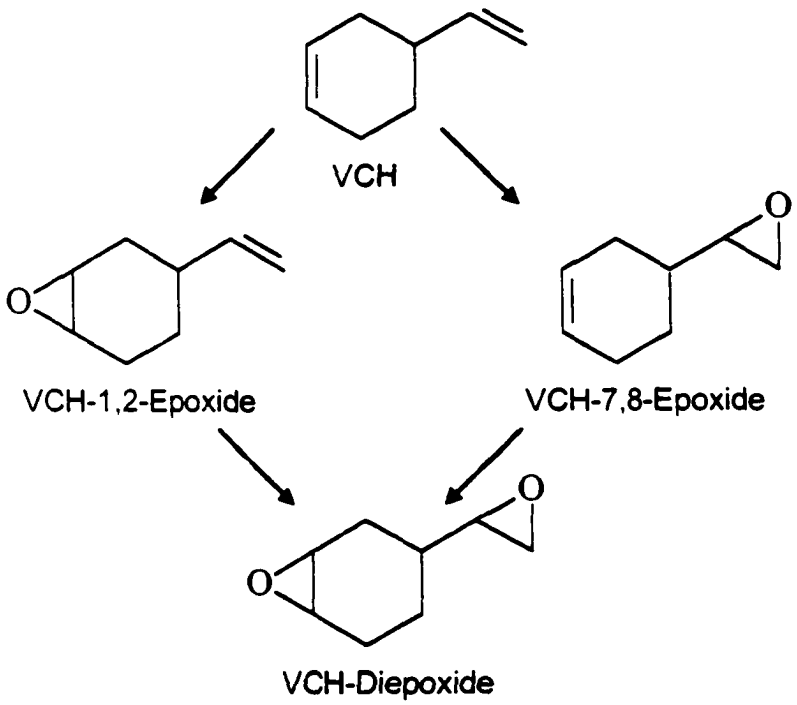


Figure 6. Proposed scheme for the hepatic bioactivation of VCH.

TABLE 3:

Comparison of epoxide formation from VCH in microsomes from female B6C3F$_1$ mice [a] or Fischer 344 rats treated with either 7.5 mmol/kg VCH for 10 d, or 80 mg/kg i.p. PB for 5 d.

Metabolite	Treatment Group	nmol/mg microsomal protein/60 min	
		Mouse	Rat
VCH-1,2-Epoxide	Control	54.0–8.0[b]	28.3–2.8
	Phenobarbital	318.6–8.7 ***(5.9)***[c]	79.3–7.1 ***(2.8)***
	VCH	205.2–36.5 ***(3.8)***	56.1–5.3 ***(2.0)***
VCH-7,8-Epoxide	Control	36.6–5.2	22.0–5.3
	Phenobarbital	75.7–1.3 ***(2.1)***	35.0–3.3 ***(1.6)***
	VCH	75.0–5.0 ***(2.0)***	39.6–5.3 ***(1.8)***
VCD	Control	n.d.[d]	n.d.
	Phenobarbital	7.6–0.9	n.d.
	VCH	6.3–0.9	n.d.

[a] n = 4. Microsomes were pooled from mice (4 mice/group: 16 mice total/treatment). Microsomes were prepared from individual rats (4 rats/treatment).

[b] Values represent mean (±SD) nmol epoxide formed/mg protein after 60 min.

[c] Values in parenthesis represent significant ($p<0.05$) fold-increase in epoxide metabolite formation over control microsomal incubations.

[d] Not detectable.

TABLE 4

Total cytochrome P450 levels in hepatic microsomes from VCH, VCH-1,2-epoxide-, VCD-, or phenobarbital- treated B6C3F$_1$ mice [a] or Fischer 344 rats.

	Total Cytochrome P450 (nmol/ mg Microsomal Protein)				
	Nontreated	**VCH**	**VCH-1,2-epoxide**	**VCD**	**PB**
Mouse	0.89–0.08	1.31–0.04 [b]	1.48–0.06 [b]	1.05–0.18	1.73–0.10 [b]
Rat	0.91–0.09	0.93–0.12	1.00–0.06	0.92–0.08	1.64–0.12 [b]

[a] n = 8 groups of microsomes were pooled from mice (4 mice/group: 16 mice total/treatment). Microsomes were prepared from individual rats (4 rats/treatment).
[b] n = 8 separate rats.
[c] Significantly different ($p<0.05$) from control.

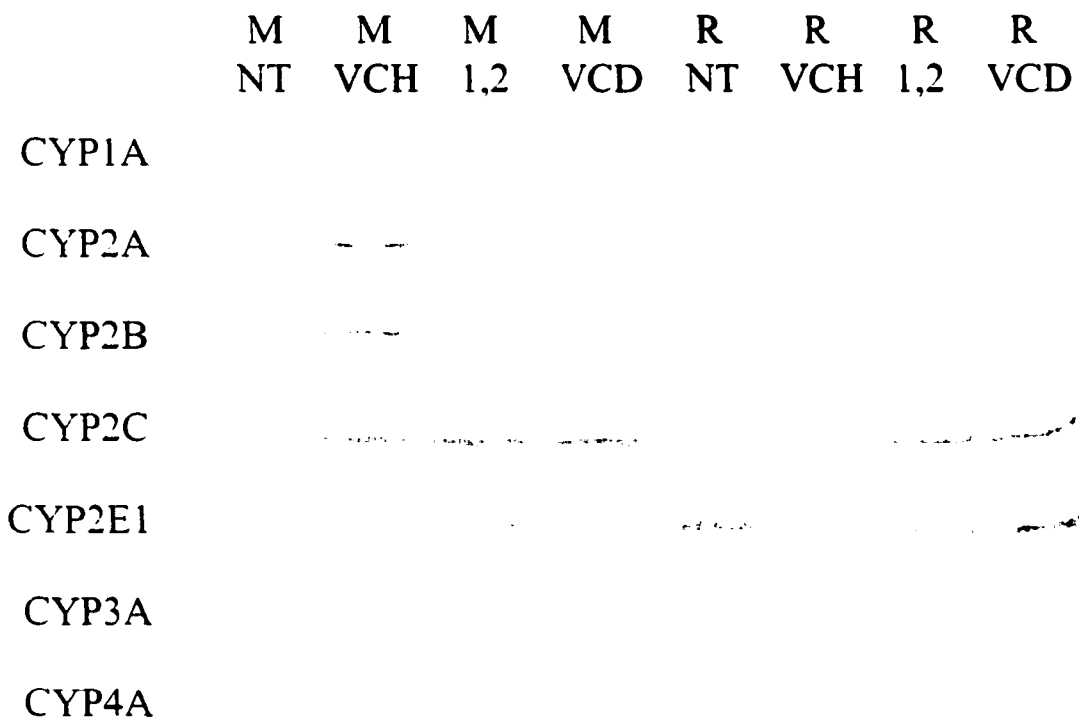


Figure 7. Western blots of hepatic microsomes isolated from VCH-1,2-Epoxide-, or VCD-treated B6C3F$_1$ mice and Fischer 344 rats.

Mice and rats were treated as described in Materials and Methods. Microsomal proteins (1 or 10 μg) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with polyclonal antibodies raised to purified rat or human CYP enzymes. Immunoblots were quantitated by densitometric analysis **(Tables 5 and 6)**. Microsomes were prepared from pooled livers from mice (4 mice/group: 16 mice/treatment), or individual rats (4 rats/treatment). All Western blots were performed 4 times/antibody probed, using different sets of microsomes. Treatment group abbreviations: mouse (M), rat (R), nontreated (NT), VCH-1,2-epoxide (1,2).

TABLE 5:

Densitometry of Western blotting experiments.

Densitometric analyses of western blots (**Figure 7**) using microsomes from $B6C3F_1$ mice treated with VCH (7.5 mmol/kg for 10 d), VCH-1,2-Epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d) as previously described in Materials and Methods. Densitometric analyses have been normalized to control (i.e. nontreated mice). Blots represent pooled microsomes from mice (4 mice/ group). All Western blots were performed 4 times/antibody probed using different sets of microsomes.

Treatment	Fold of Control						
	1A	2A	2B	2C	2E	3A	4A
None	1.0	1.0	1.0	1.0	1.0	1.0	1.0
VCH	0.8	2.1	2.5	1.2	1.0	1.3	1.3
VCH-1,2-epoxide	0.7	1.0	1.9	1.4	1.1	1.3	1.1
VCD	1.0	1.2	1.3	1.0	1.0	1.2	1.3

TABLE 6:

Densitometry of Western blotting experiments.

Densitometric analyses of western blots (**Figure 7**) using microsomes from Fischer 344 rats treated with VCH (7.5 mmol/kg for 10 d), VCH-1,2-epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d) as previously described in Materials and Methods. Densitometric analyses have been normalized to control (i.e. nontreated rats). Blots represent microsomes prepared from individual rats (4 rats/treatment). All Western blots were performed 4 times/antibody probed using different sets of microsomes.

Treatment	Fold of Control						
	1A	2A	2B	2C	2E	3A	4A
None	1.0	1.0	1.0	1.0	1.0	1.0	1.0
VCH	1.1	0.5	0.7	0.9	0.8	1.0	0.9
VCH-1,2-epoxide	1.9	1.5	0.6	1.3	0.9	1.1	1.2
VCD	2.3	1.1	0.7	1.7	1.0	1.0	1.2

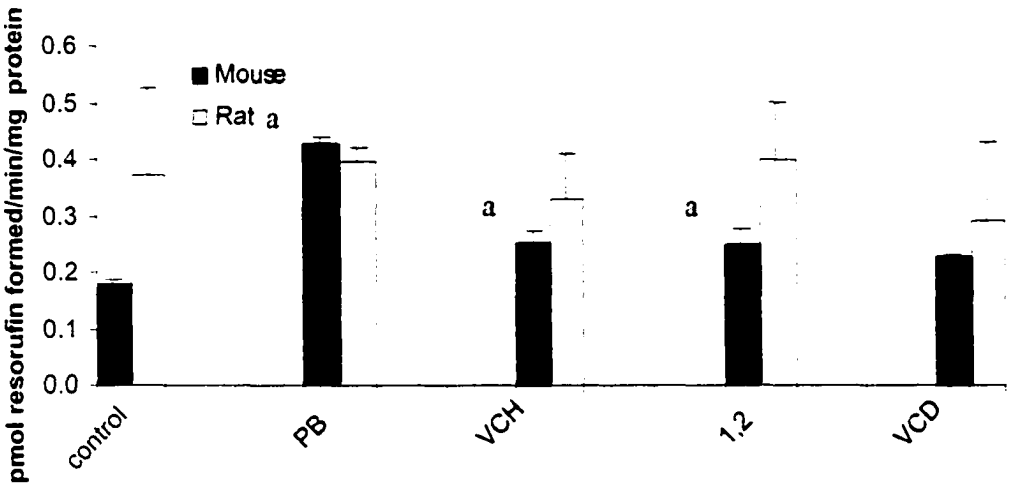


Figure 8. Ethoxyresorufin dealkylation activity in hepatic microsomes from VCH, VCH-1,2-epoxide, VCD-, or PB- treated B6C3F$_1$ mice and Fischer 344 rats.

Hepatic microsomes were isolated from mice or rats administered PB (80 mg/kg i.p. for 5 d), VCH (7.5 mmol/kg for 5 or 10 d), VCH-1,2-Epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d). Microsomes (0.25 mg/ml) were incubated for 10 min with 10 μM ethoxyresorufin. Data represent the mean ± S.D. of either 4 individual rats or 4 groups of pooled microsomes from mice (4 mice/ group: 16 mice/ treatment). Statistically significant ($p < 0.05$) compared to control of same species (**a**).

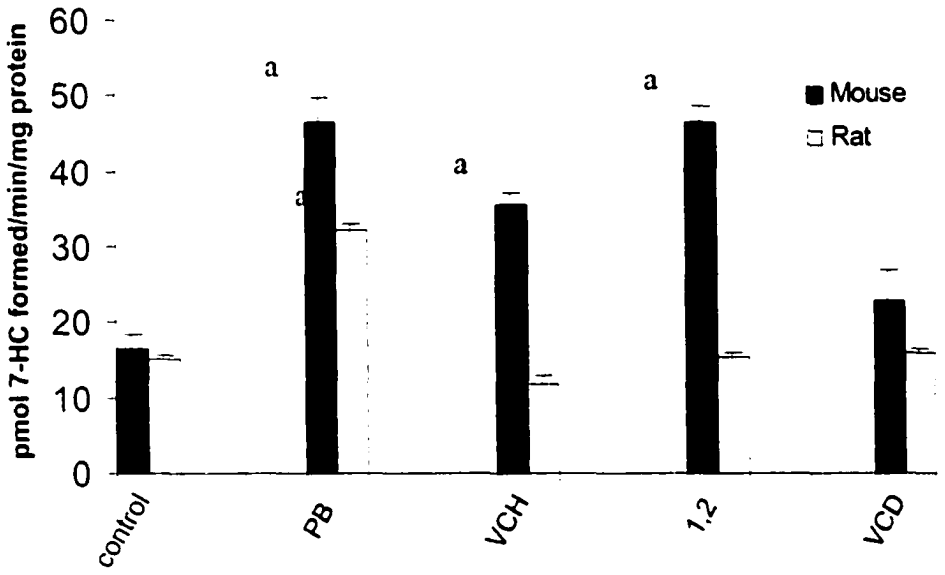


Figure 9. Coumarin hydroxylation activity in hepatic microsomes from VCH, VCH-1,2-epoxide, VCD-, or PB- treated B6C3F$_1$ mice and Fischer 344 rats.

Hepatic microsomes were isolated from mice or rats administered PB (80 mg/kg i.p. for 5 d), VCH (7.5 mmol/kg for 5 or 10 d), VCH-1,2-Epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d). Microsomes (0.1 mg/ml) were incubated for 15 min with 50 μM coumarin. Data represent the mean ± S.D. of either 4 individual rats or 4 groups of pooled microsomes from mice (4 mice/ group: 16 mice/ treatment). Statistically significant ($p < 0.05$) compared to control of same species (**a**).

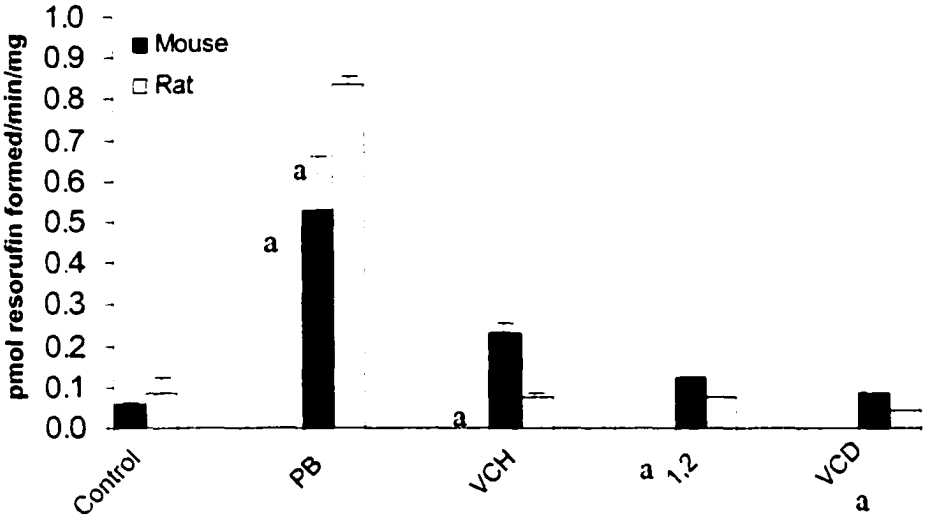


Figure 10. Pentoxyresorufin dealkylation activity in hepatic microsomes from VCH, VCH-1,2-epoxide, VCD-, or PB- treated B6C3F$_1$ mice and Fischer 344 rats.

Hepatic microsomes were isolated from mice or rats administered PB (80 mg/kg i.p. for 5 d), VCH (7.5 mmol/kg for 5 or 10 d), VCH-1,2-epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d). Microsomes (0.25 mg/ml) were incubated for 10 min with 10 μM pentoxyresorufin. Data represent the mean ± S.D. of either 4 individual rats or 4 groups of pooled microsomes from mice (4 mice/ group: 16 mice/ treatment). Statistically significant ($p < 0.05$) compared to control of same species (**a**).

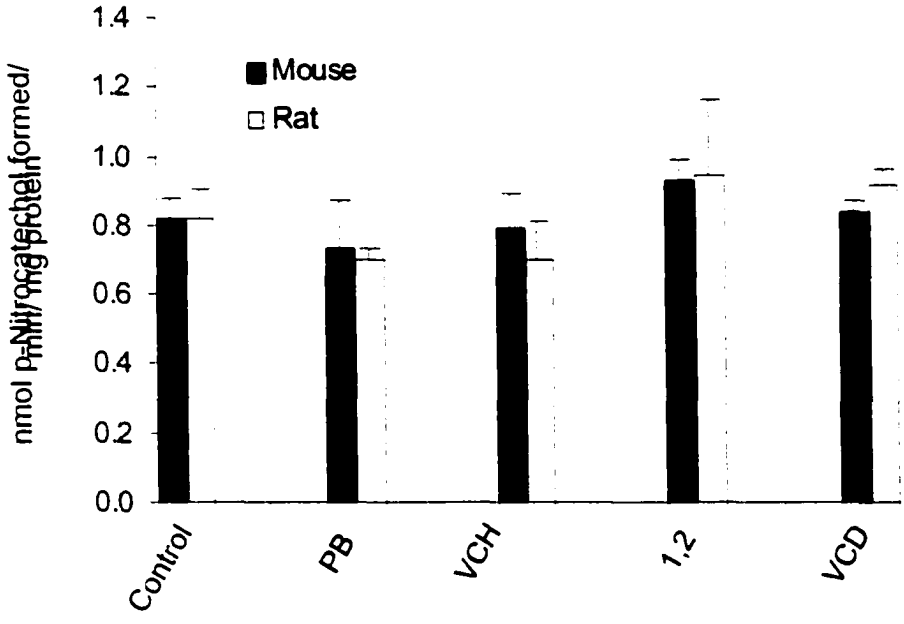
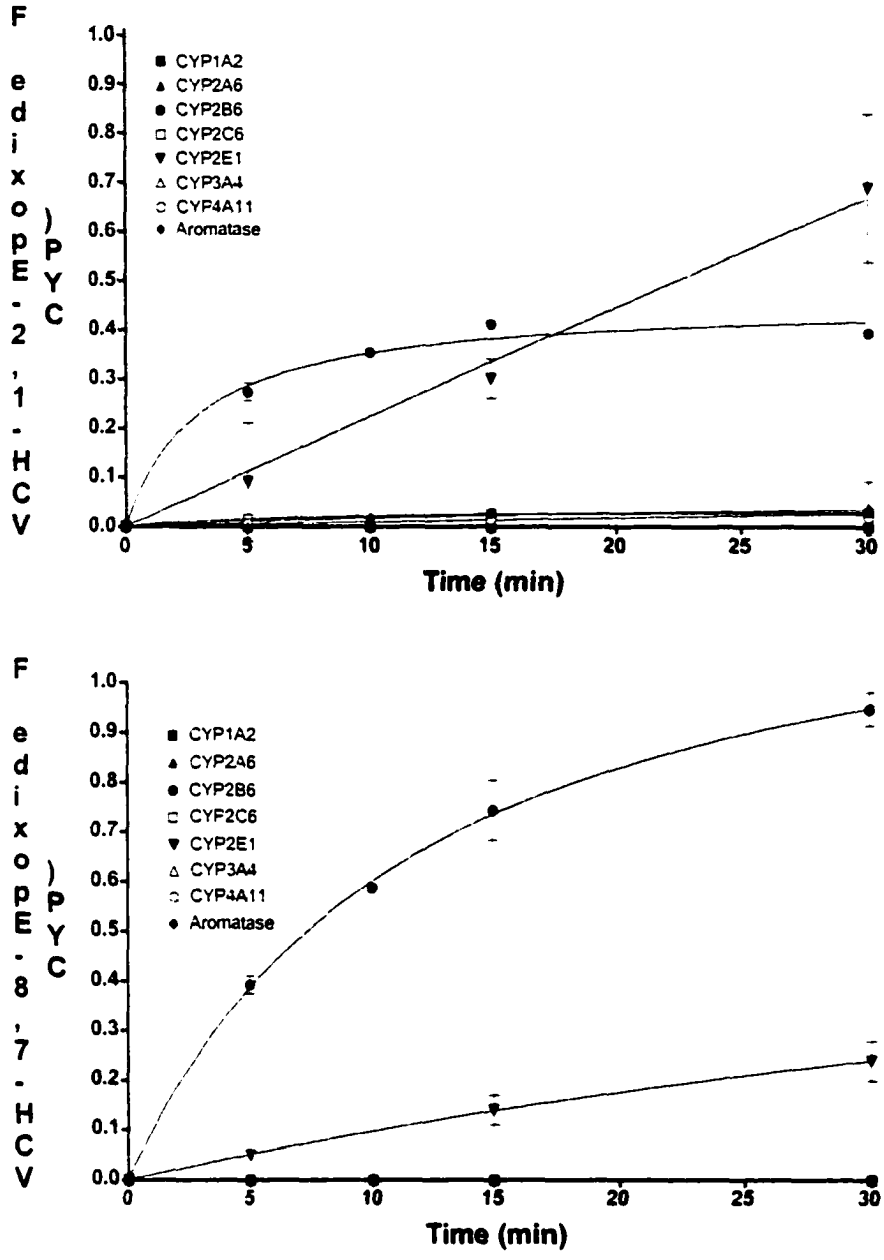
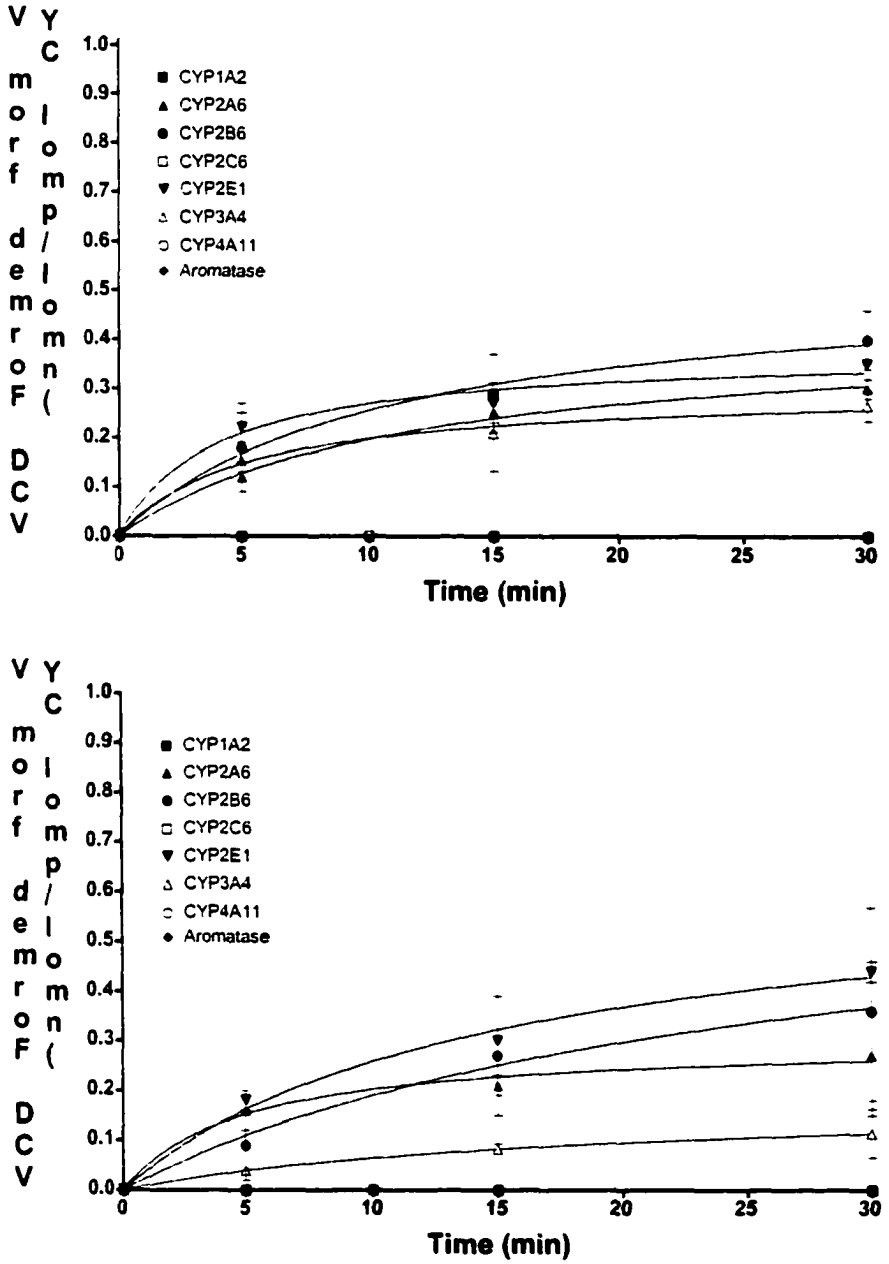


Figure 11. p-Nitrophenol hydroxylase activity in hepatic microsomes from VCH,VCH-1,2-epoxide, VCD-, or PB- treated B6C3F$_1$ mice and Fischer 344 rats.

Hepatic microsomes were isolated from mice or rats administered PB (80 mg/kg i.p. for 5 d), VCH (7.5 mmol/kg for 5 or 10 d), VCH-1,2-epoxide (1.75 mmol/kg for 10 d), or VCD (0.4 mmol/kg for 10 d). Microsomes were incubated for 10 min with 200 μM p-nitrophenol. Data represent the mean ± S.D. of either 4 individual rats or 4 groups of pooled microsomes from mice (4 mice/ group: 16 mice/ treatment). Statistically significant ($p < 0.05$) compared to control of same species (**a**).



Figures 12a and b: Comparison of VCH-1,2-epoxide and VCH-7,8-epoxide formation from VCH in Supersomes containing 100 nM (50 pmol) either human CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2E1, CYP3A4, CYP4A11, or aromatase. There was no detectable metabolism of VCH in forming VCH-7,8-epoxide by aromatase, CYP1A2, CYP2A6, CYP2C6, CYP3A4, or CYP4A11 by 30 min.



Figures 13a and b: Comparison of VCD formation from VCH-1,2-epoxide or VCH-7,8-epoxide in Supersomes containing 100 nM (50 pmol) either human CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2E1, CYP3A4, CYP4A11, or aromatase cDNA. There was no detectable metabolism of VCH-1,2-epoxide or VCH-7,8-epoxide by aromatase, CYP1A2, CYP2C6, or CYP4A11 by 30 min.

DISCUSSION:

While VCH is ovotoxic in female mice, female rats are resistant due to limitations in forming the toxic metabolites (Smith et al., 1990a). Furthermore, repeated exposure to VCH is required to elicit this ovotoxicity in mice, which also causes an increase in VCH bioactivation *in vitro* (Doerr-Stevens et al., 1999). Therefore, elevated protein levels and activities of the cytochrome P450 isoforms responsible for the bioactivation of VCH would be expected following repeated exposure of mice to VCH. Through chemical induction studies and examination of total and specific CYP levels and activities following repeated exposure to VCH, the roles of at least two hepatic CYP isoforms in the metabolism of VCH have now been revealed.

There is substantial evidence for the role of bioactivation in the species-dependent ovotoxicity of VCH. Repeated exposure to VCH caused greater increases in VCH epoxidation to VCH-1,2-epoxide and VCD in the mouse compared to the rat. There were also significant increases in total hepatic CYP levels in these mice and in mice pretreated with VCH-1,2-epoxide, while neither of these pretreatments caused significant increases in total CYP levels in the rat. Although VCH-1,2-epoxide pretreatment resulted in slightly greater increases in total hepatic CYP levels than VCH, VCH-1,2-epoxide did not increase specific CYP levels tested to the extent as VCH. Therefore, other CYP isoforms may play a role in the biotransformation of VCH and/or VCH-1,2-epoxide. Since

repeated treatment with VCH is required for ovotoxicity, and the mouse forms more VCH-1,2-epoxide than the rat, this metabolite may be causing the enhanced VCH bioactivation in the mouse (although the role of VCH-7,8-epoxide has not been investigated due to limited availability).

The current study defined the roles of CYP2B and CYP2A in the species-dependent bioactivation of VCH. Significant increases in CYP2B levels were seen in microsomes from mice pretreated with VCH or VCH-1,2-epoxide. These data correlate with the increases in pentoxyresorufin dealkylation in microsomes from VCH and VCH-1,2-epoxide pretreated mice. In contrast, there were no increases in CYP2B levels or activity seen with pretreatments other than PB in the rat. Instead, these pretreatments appeared to decrease levels in rat hepatic microsomes. The associated CYP2B activity was also reduced in rats pretreated with VCD.

There were also significant increases in CYP2A protein levels seen in microsomes from mice pretreated with VCH, and significant increases in coumarin 7-hydroxylation in microsomes from mice pretreated with either VCH and VCH-1,2-epoxide. In contrast, there were no increases in CYP2A levels or activity in microsomes by VCH-, VCH-1,2-epoxide-, or VCD-pretreament in rats. Again, these results correlate with other data that suggest that VCH pretreatment results in greater *in vitro* bioactivation of VCH in the mouse than in the rat.

Previous reports show that PB is a potent inducer of CYP2A, CYP2B, and CYP3A in humans and rodents (Gervot et al., 1999; Cheng et al., 1982). Current

studies show that pretreatment with PB resulted in significant increases in total hepatic CYP, CYP2A and CYP2B activities, and levels (data not shown), as well as up to 6-fold increases in VCH epoxidation in microsomes from mice and rats. Although PB is known to induce CYP3A, previous studies eliminated this subfamily as a critical hepatic CYP isoform in VCH bioactivation in the mouse. For example, anti-rat P4503A IgG inhibited testosterone 6β-hydroxylation by 68% but did not effect VCH epoxidase activity in murine microsomes (Smith et al., 1990c). Furthermore, CYP3A levels and activities were not significantly increased in the mouse following either 5 or 10 d pretreatment (Doerr-Stevens et al., 1999). Moreover, immunoblotting data and CYP3A4 Supersomes metabolism described in current studies suggest that CYP3A does not contribute to VCH bioactivation.

Female human microsomes demonstrated epoxidation of VCH at rates 13-fold and 2-fold less than those in mice and rats, respectively (Smith and Sipes, 1991), and total hepatic CYP/ mg protein is significantly lower in humans than in rodents (Shimada et al., 1994; Imaoka et al., 1996). It is interesting that out of the eight human hepatic CYP isoforms tested in current studies, CYP2E1 and CYP2B6 were the only isoforms that significantly catalyzed the epoxidation of VCH. However, CYP2E1 and CYP2B6 only represent approximately 6% and < 1% of total human hepatic CYP, respectively (Shimada et al., 1994). Previous experiments focused on the role of CYP2E1 in the epoxidation of VCH because it has been reported to metabolize the structurally related compounds styrene and

1,3-butadiene (Lieber et al., 1997; Nieusma et al., 1998; Fontaine et al., submitted). Those studies showed that, although hepatic microsomes from mice and rats pretreated with acetone showed increases in VCH-1,2-epoxide formation from VCH, hepatic microsomes from mice or rats pretreated with VCH for 5 or 10 d demonstrated no increases in CYP2E1 protein levels or activity (Fontaine et al., submitted). Those data, combined with the data showing no differences in epoxidation of VCH or its monoepoxides in CYP2E1-deficient mouse hepatic microsomes compared to those of mice that do have CYP2E1, indicated that CYP2E1 is not an important isoform in mouse- and rat-specific bioactivation of VCH. Current studies reconfirm this conclusion, as neither VCH, VCH-1,2-epoxide, or VCD pretreatment for 10 d effected CYP2E1 levels or activity in mice or rats.

It is also interesting that, although CYP2B6 and CYP2E1 were the only CYP isoforms that catalyzed VCH epoxidation in humans, CYP2B6, CYP2A6, CYP2E1, and CYP3A4 catalyzed the epoxidation of both monoepoxides to form the diepoxide. While CYP3A4 is the major hepatic CYP isoform in humans, human liver CYP2A6 expression is relatively low (approximately 4%) (Cheng and Schenkman, 1982; Shimada et al., 1994). However, since the rate of formation of VCH-1,2-epoxide was shown to be significantly limited in humans compared to the mouse or rat, the possibility of VCH-1,2-epoxide or VCH-7,8-epoxide bioactivation to VCD by these particular enzymes is unlikely in humans exposed to VCH.

In conclusion, comparisons of VCH metabolism and hepatic CYP induction demonstrate that CYP2A and CYP2B are important CYP isoforms in the species-dependent bioactivation, and therefore ovotoxicity, of VCH in mice and rats. The increased expression of CYP2A and CYP2B seen exclusively in the mouse appears to be due to repeated treatment with VCH or VCH-1,2-epoxide. This indicates that, with repeated exposure to VCH, the mouse is exposed to a greater concentration of the ovotoxic metabolites via enhanced bioactivation. The rat is resistant to the ovotoxicity of VCH, at least in part, because the increases in CYP levels/activities are not occurring following repeated exposure to VCH. It would be interesting to determine how repeated exposure of VCH to human hepatocytes would influence the ability to bioactivate VCH.

CHAPTER 4: STEREOCHEMICAL ASPECTS OF VINYLCYCLOHEXENE BIOACTIVATION IN RODENT HEPATIC MICROSOMES AND PURIFIED HUMAN CYTOCHROME P450 ENZYME SYSTEMS

ABSTRACT

The racemic mixture of 4-Vinylcyclohexene (VCH) forms ovotoxic epoxides [vinylcyclohexene-1,2-epoxide (VCH-1,2-epoxide), vinylcyclohexene-7,8-epoxide (VCH-7,8-epoxide), and vinylcyclohexene diepoxide (VCD)] by cytochrome P450 (CYP) in $B6C3F_1$ female mice. These epoxides deplete primordial and primary follicles. Current studies compared *in vitro* epoxidation of (*R*)-VCH to that of (*S*)-VCH in hepatic microsomes prepared from adult female $B6C3F_1$ mice and Fischer 344 rats. Bioactivation of VCH in the rat was significantly less compared to that in the mouse. (*R*)-VCH formed significantly more VCH-1,2-epoxide as compared to (*S*)-VCH in both species, and less VCH-7,8-epoxide in the mouse. Neither of the enantiomers formed detectable amounts of VCD in the mouse or rat. Hepatic microsomes prepared from mice and rats pretreated with CYP-inducing agents (phenobarbital and acetone), were also incubated with (*R*)-VCH or (*S*)-VCH. While monoepoxide formation was not increased enantioselectively in the mouse, VCD was formed preferentially from (*R*)-VCH as compared to (*S*)-VCH. Pretreatment with VCH resulted in nonstereoselective increases in both monoepoxide and diepoxide formation. In the rat, these pretreatments resulted in nonstereoselective increases in monoepoxide formation, but VCD formation was not detectable. Incubations with human CYP2E1 enzyme revealed that (*R*)-VCH formed significantly more VCH-1,2-epoxide and less VCH-7,8-epoxide than (*S*)-VCH. Human CYP2A6 was limited in its ability to form epoxides from either

enantiomer of VCH. Human CYP2B6 preferentially formed VCH-7,8-epoxide as compared with VCH-1,2-epoxide, and to a greater extent from (*R*)-VCH than from (*S*)-VCH. These results demonstrate regioselectivity and enantioselectivity in the bioactivation of VCH in rodent hepatic microsomes as well as in expressed human CYP enzymes.

INTRODUCTION

4-Vinylcyclohexene (VCH) is formed by the spontaneous dimerization of two molecules of 1,3-butadiene during the rubber curing process (Rappaport et al., 1976, International Agency for Research on Cancer, 1994). VCH is also an intermediate in the synthesis of styrene and vinylcyclohexene diepoxide (VCD) for epoxy resin formation. Repeated exposure of mice to VCH causes premature ovarian failure by depletion of ovarian primordial and primary follicles (Collins and Manus, 1987; Smith et al., 1990a). This premature ovarian failure may be associated with the ovarian neoplasms that develop in mice chronically exposed to VCH (National Toxicology Program, 1986; Collins et al., 1987). Cytochrome P450 (CYP)-catalyzed bioactivation of VCH to metabolites 1,2-vinylcyclohexene monoepoxide (VCH-1,2-epoxide), 7,8-vinylcyclohexene monoepoxide (VCH-7,8-epoxide), and ultimately, VCD (Figure 14), is necessary for VCH-induced ovotoxicity to occur (Smith et al., 1990b, Doerr et al., 1996). Interestingly, female Fischer 344 (F-344) rats are resistant to the ovarian toxicity caused by treatment with VCH. This resistance in female rats is at least partially related to a low capacity to bioactivate VCH to epoxide metabolites (Smith et al., 1990c).

VCH is most commonly formed as a racemic mixture of (*R*)-VCH and (*S*)-VCH. There are numerous examples in which one enantiomer of a compound is more toxic than the racemic mixture or the other enantiomer (D Arcy and Griffin, 1994; Braun et al., 1986). Furthermore, CYP enzymes have been shown to have varying degrees of stereoselectivity in catalyzing epoxidation reactions in

compounds such as polycyclic aromatic hydrocarbons and warfarin (Yang, 1988 Heimark et al., 1985). Therefore, it is important to determine if the enantiomers of VCH also demonstrate selective bioactivation and ultimately, differential toxicity. The effects of stereochemistry on the bioactivation of the related compound 1,3-butadiene have recently been investigated. This prochiral compound can form multiple mono- and diepoxide stereoisomers or diastereoisomers through CYP-catalyzed epoxidation, as can VCH. Neiusma et al. (1997,1998) demonstrated stereoselectivity of 1,3-butadiene bioactivation in both mouse and rat hepatic microsomes, as well as with purified human CYP2E1 protein. VCH may also undergo stereoselective bioactivation in cytochrome P450 systems. Sixteen possible monoepoxide and diepoxide diastereomers can be formed from a racemic mixture of VCH (Figure 1). Perhaps only one or two of these possible metabolites are formed *in vivo* and responsible for VCH-induced toxicity.

The studies reported here compared the epoxidation of (*R*)-VCH and (*S*)-VCH in microsomes obtained from female B6C3F$_1$ mice and F-344 rats. The effects of pretreatment with various CYP inducing agents on the epoxidation of the enantiomers of VCH were also examined in both species. Furthermore, since initiating a toxic response in mice with VCH requires several days of repeated exposure (Doerr-Stevens et al., 1999), microsomes prepared from mice or rats pretreated with racemic VCH for 10 days were also incubated with (*R*)-VCH or (*S*)-VCH to determine how this would affect stereoselective bioactivation. Lastly,

several human CYP isoforms were incubated with (*R*)-VCH and (*S*)-VCH to gain insight into how humans metabolize VCH.

EXPERIMENTAL PROCEDURE

Animals and Treatments. Female B6C3F$_1$ mice and F-344 rats (approximately 28 to 38 days of age) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were housed in cages with sawdust bedding and had free access to food (Teklad, Harlan Sprague-Dawley, Inc., Madison, WI) and water. Animals were maintained on 12-hr light/dark cycle and acclimated to this environment for at least 7 d before dosing and/or preparation of hepatic microsomes. Treated animals were dosed with either racemic VCH (7.5 mmol/kg i.p per day for 10 d, as previously described by Doerr-Stevens et al., 1999), phenobarbital (PB) (80 mg/kg i.p. per day for 5 d), or acetone (1% in the drinking water for 5 d).

Chemicals. (*R,S*)-4-vinylcyclohexene, vinylcyclohexene 1,2-monoepoxide, vinylcyclohexene diepoxide, methylcyclohexene, and acetone were purchased from Aldrich Chemical Co. (Milwaukee, WI). NADP+, G6PDH, and G6P were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclohexene oxide was produced by NTP/RTI (Research Triangle Park, NC). Vinylcyclohexene 7,8-monoepoxide was synthesized by the method of Watabe et al. (1981). (*R*)-VCH and (*S*)-VCH were synthesized as previously reported (Mash et al., 1995). *(Caution: VCH and its epoxides are either potential carcinogens or are carcinogens in animals and should be handled with appropriate precautions).*

Subcellular Preparation and Characterization. Animals were euthanized by inhalation of carbon dioxide 24 h after final dosing. Livers were excised and

homogenized in a 50 mM Tris-HCl buffer (pH 7.6) using a drill motor and teflon-glass homogenizer. Microsomes were prepared by differential centrifugation of the homogenate, as described by Guengerich et al. (1989). Mouse microsomes were prepared and pooled as 4 mice/group. Protein concentrations were determined by using a Bicinchoninic Acid kit (Pierce, Rockford, IL). Total P450 concentrations (nmol/mg microsomal protein) were determined by the carbon monoxide-binding spectra as described by Omura and Sato (1964).

Capillary Gas-Liquid Chromatographic Conditions for Epoxide Analysis. Analyses were performed on a Hewlett-Packard HP 5890A gas chromatograph equipped with a DB-624 capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector (FID). The nitrogen carrier gas flow rate was 1 mL/min. The FID gas flow rates for H_2, N_2, and air were 42, 35, and 400 mL/min, respectively. Splitless injection was used with the purge off from time 0 to 1.0 min, with 2 uL injection volume. The injection and detector temperatures were 200 °C and 250 °C, respectively. The oven temperature was held at 60 °C for 10 min, then increased to 230 °C at a rate of 12 °C/min. The final temperature was held for 3 min to ensure elution of the diepoxide. Retention times were 11.9 min for methylcyclohexene, 15.2 min for cyclohexene oxide, 16.6 min for VCH, 20.4 min for VCH-1,2-epoxide, 21.4 min for VCH-7,8-epoxide, and 25.0 min for VCD. Formation of VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD (nmol/mg microsomal protein) was quantified by comparing the peak areas to those in

standard curves prepared with known amounts of the epoxides. Detection limits for the epoxides were each approximately 1 nmol.

Microsomal Incubations. Microsomal protein (final concentration of 1 mg/ml in 1 ml total volume) was added to a 50 mM HEPES/EDTA buffer solution containing a recycling $NADP^+$ system (0.5 mM $NADP^+$, 1 unit G6PDH/mL, and 10 mM G6P), 2 mM cyclohexene oxide (an epoxide hydrolase inhibitor, Guest and Dent, 1980), and 1 mM (*R,S*)-, (*R*)-, or (*S*)-VCH. Previous studies were conducted to demonstrate that reactions were linear up to 1 mg/ml protein using the individual enantiomers of VCH, as well as racemic VCH. Samples were incubated in a 37 °C water bath. After the appropriate amount of time (ranging from 0 to 60 min), the CYP reactions were terminated by submersion in liquid nitrogen. VCH and its epoxide metabolites (VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD) were extracted with ethyl acetate containing 1 ug/mL methylcyclohexene as an internal standard. The epoxide metabolites were identified and quantified using gas chromatography (DB-624 capillary column). Blanks included incubations containing denatured microsomes (preheated at 60 °C for 30 min before incubating), and incubations lacking glucose-6-phosphate. Data are presented as nmol epoxide formed/mg microsomal protein. The extraction efficiencies of VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD were 96%, 92%, and 76%, respectively. Reported values were corrected for recovery.

Incubations of Supersomes containing human CYP2A6, CYP2B6, CYP2E1 or CYP3A4. To investigate whether individually expressed human CYP

protein can catalyze the epoxidation of (*R*)- or (*S*)-VCH to the epoxide metabolites, the Supersome system was utilized (Human CYP + P450 Reductase + Cytochrome b_5, GENTEST Corporation, Woburn, MA). Supersomes containing either human CYP2A6, CYP2B6, CYP2E1, or CYP3A4 (100 pmol total P450/mL) were combined with 1 mM (*R*)-VCH or (*S*)-VCH and a recycling system for NADPH (0.5 mM $NADP^+$, 1 unit G6PDH/mL, and 10 mM G6P), for a total volume of 500 µL. Samples were incubated at 37 °C for up to 30 min. Epoxide metabolites were identified and quantified using gas chromatography.

Statistical Analysis. A two-sample inference was made using a Student s t-test. Means were considered significantly different at $p<0.05$.

RESULTS

Biotransformation of (*R*)- and (*S*)-VCH in Mouse Hepatic Microsomes. There were clear differences in epoxidation of (*R*)-VCH and (*S*)-VCH in mouse hepatic microsomes. After 60 min incubations with control mouse microsomes, (*R*)-VCH formed more than twice the amount of VCH-1,2-epoxide as did (*S*)-VCH (72.5±9.0 and 29.8±3.7 nmol, respectively), but significantly less VCH-7,8-epoxide (30.4±4.0 and 56.3±7.3 nmol, respectively). (Figures 15a and c). Neither enantiomer formed the diepoxide at detectable levels. Comparative incubations with (*R,S*)-VCH demonstrated epoxidation to the monoepoxides at rates approximately average of that seen with (*R*)-VCH and (*S*)-VCH (data not shown).

Hepatic microsomes from mice pretreated with phenobarbital, acetone, or VCH all resulted in increased *in vitro* epoxidation of (*R*)-VCH and (*S*)-VCH to VCH-1,2-epoxide (Table 7). Of all the pretreatments, phenobarbital pretreatment resulted in the greatest induction of VCH bioactivation, with VCH-1,2-epoxide as the major metabolite. However, this induction was not enantioselective, as both (*R*)-VCH and (*S*)-VCH bioactivation were increased approximately 6-fold over controls. VCD was formed in the incubations with microsomes from PB pretreated mice, with (*R*)-VCH forming more VCD than (*S*)-VCH (8.3±1.7 and 3.1±1.5 nmol, respectively). With microsomes from acetone pretreated mice, there were 2-fold increases in VCH-1,2-epoxide formation from both (*R*)- and (*S*)-

VCH, but no increase in VCH-7,8-epoxide formation from either enantiomer. Under these conditions, VCD was again formed preferentially from (*R*)-VCH as compared with (*S*)-VCH (6.7±1.2 and 1.6±0.8 nmol, respectively). Hepatic microsomes obtained from mice pretreated with VCH were also more active than control microsomes with respect to epoxidation of (*R*)- and (*S*)-VCH. Formation of VCH-1,2-epoxide and VCH-7,8-epoxide increased approximately 3- and 2-fold, respectively, from both VCH enantiomers. Although VCD formation was detected in the microsomes obtained from mice pretreated with VCH, there was no significant difference in the amounts formed from (*R*)-VCH or (*S*)-VCH.

Biotransformation of (*R*)- and (*S*)-VCH in Rat Hepatic Microsomes. Parallel studies performed in hepatic microsomes from adult female F-344 rats demonstrated lower overall bioactivation of (*R*)- and (*S*)-VCH compared with the mouse. The metabolic profiles in the control rat microsomes were stereochemically similar to those in the mouse, as (*R*)-VCH was converted to approximately twice as much VCH-1,2-epoxide as was (*S*)-VCH after 60 min, but equal amounts of VCH-7,8-epoxide (Figures 15b and d). Neither enantiomer was converted to the diepoxide at detectable levels in control microsomes. All pretreatments caused relatively nonstereoselective induction of VCH bioactivation *in vitro*, but induction overall was still much less in the rat compared to the mouse (Table 1). Furthermore, none of the pretreatments in the rat resulted in VCD formation from either enantiomer *in vitro*.

Biotransformation of (*R*)- and (*S*)-VCH in Human CYP2E1 Supersomes. Human CYP2E1 formed both epoxides when incubated with (*R*)- or (*S*)-VCH (Figures 16a and b). By 30 minutes, (*R*)- and (*S*)-VCH were converted to 0.7±0.0 and 0.4±0.0 nmol VCH-1,2-epoxide/pmol CYP2E1, respectively, and 0.3±0.0 and 0.5±0.0 nmol VCH-7,8-epoxide/pmol CYP2E1, respectively. Neither enantiomer was converted to VCD at detectable levels when incubated with human CYP2E1. There was no epoxide formation in parallel incubations with control insect microsomes lacking CYP2E1.

Biotransformation of (*R*)- and (*S*)-VCH in Human CYP2A6 Supersomes. Human CYP2A6 Supersomes were limited in their ability to form epoxides from either enantiomer of VCH (Figure 17). Furthermore, VCH-7,8-epoxide was not formed in detectable levels from (*R*)- or (*S*)-VCH by human CYP2A6. (*R*)-VCH was converted to more VCH-1,2-epoxide than (*S*)-VCH at later time points, and neither enantiomer formed VCD at detectable levels. Activity of the Human CYP2A6 Supersomes was confirmed by coumarin hydroxylation.

Biotransformation of (*R*)- and (*S*)-VCH in Human CYP2B6 Supersomes. Human CYP2B6 Supersomes demonstrated a greater ability to form epoxides from (*R*)- and (*S*)-VCH than human CYP2A6. After 30 min, 0.4±0.0 and 0.2±0.0 nmol VCH-1,2-epoxide/pmol CYP2B6, and 0.9±0.1 and 0.7±0.0 nmol VCH-7,8-epoxide/pmol CYP2B6 were formed from (*R*)- and (*S*)-VCH, respectively (Figures 18a and b). Thus, human CYP2B6 is a more

selective catalyst for epoxidation of (*R*)- and (*S*)-VCH to the epoxide at the 7,8-position over the 1,2- position. Neither enantiomer formed VCD at detectable levels when incubated with human CYP2B6. There was no epoxide formation in parallel incubations with control insect microsomes lacking CYP2B6.

Biotransformation of (*R*)- and (*S*)-VCH in Human CYP3A4 Supersomes . Neither (*R*)- or (*S*)-VCH were converted to epoxide metabolites at detectable levels by human CYP3A4.

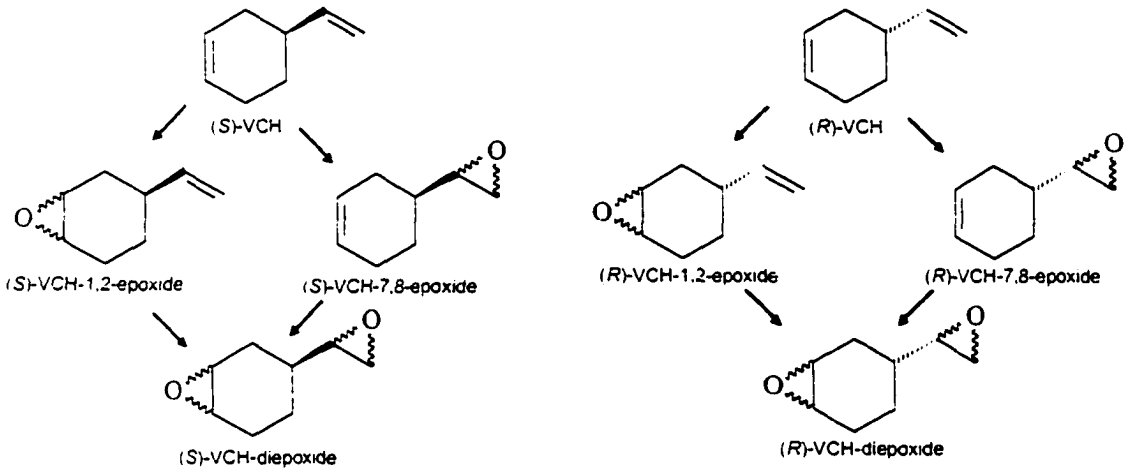
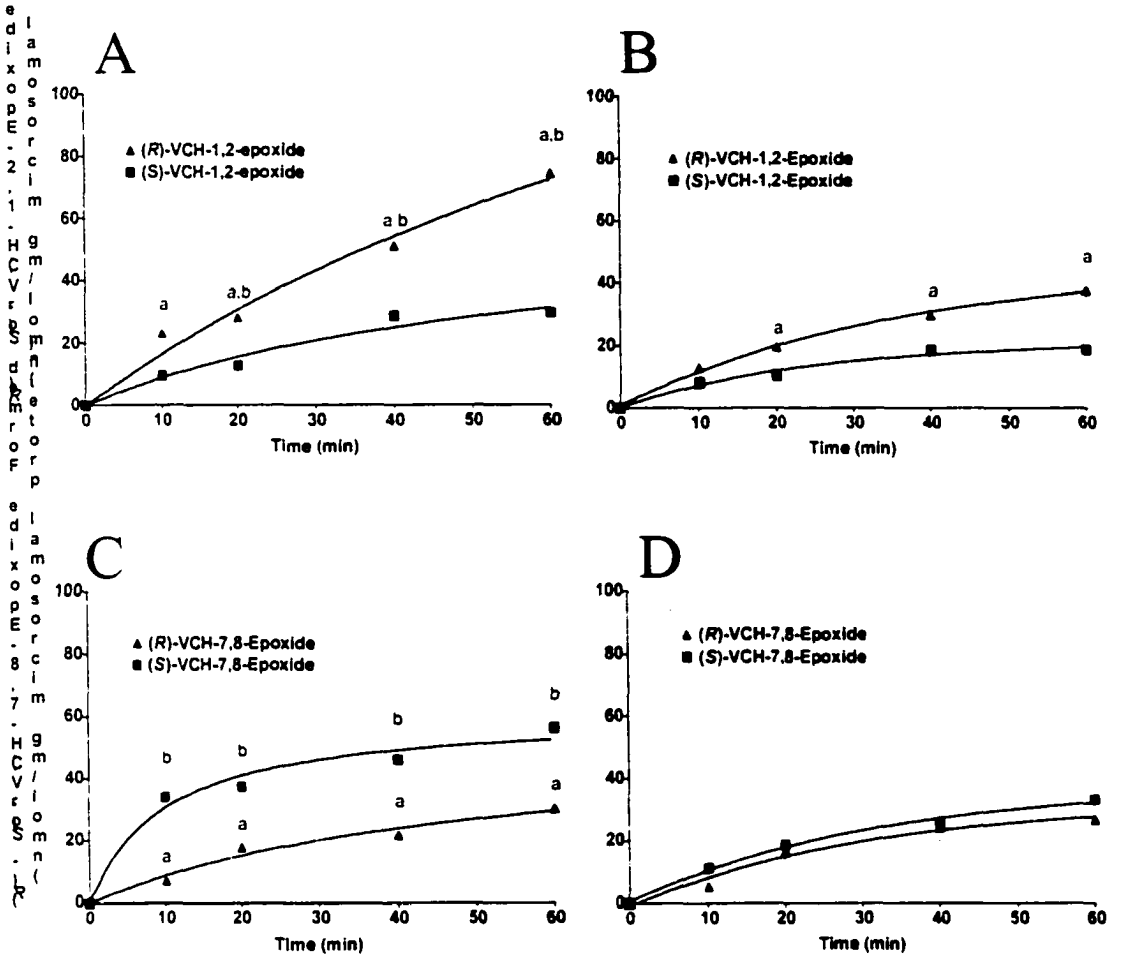


Figure 14: Schematic of all possible stereoisomers of VCH and its toxic metabolites.

Bioactivation of VCH to epoxides by CYP. Fundamentals of stereochemistry prove that it is impossible for (*R*)- and (*S*)-VCH to form the same epoxide metabolite stereoisomers.



Figures 15A-D: (R)-VCH and (S)-VCH Bioactivation in mouse and rat microsomes.

Comparison of VCH-1,2-epoxide formation (Figure A and Figure B) and VCH-7,8-epoxide formation (Figure C and Figure D) from (*R*)-VCH (_) and (*S*)-VCH (_) by hepatic microsomes obtained from female B6C3F$_1$ mice (Figure A and Figure C) and Fischer 344 rats (Figure B and Figure D). Data are expressed as mean ± standard deviation (n = 3-4 in duplicate; mice microsomes were prepared from 4 mice/group). (a) Significantly different amount of epoxide formed than from (*S*)-VCH in the same species, at $p<0.05$ (Student s t-test.). (b) Significantly different from epoxide formed from same enantiomer in the rat, at $p < 0.05$ (Student s t-test.).

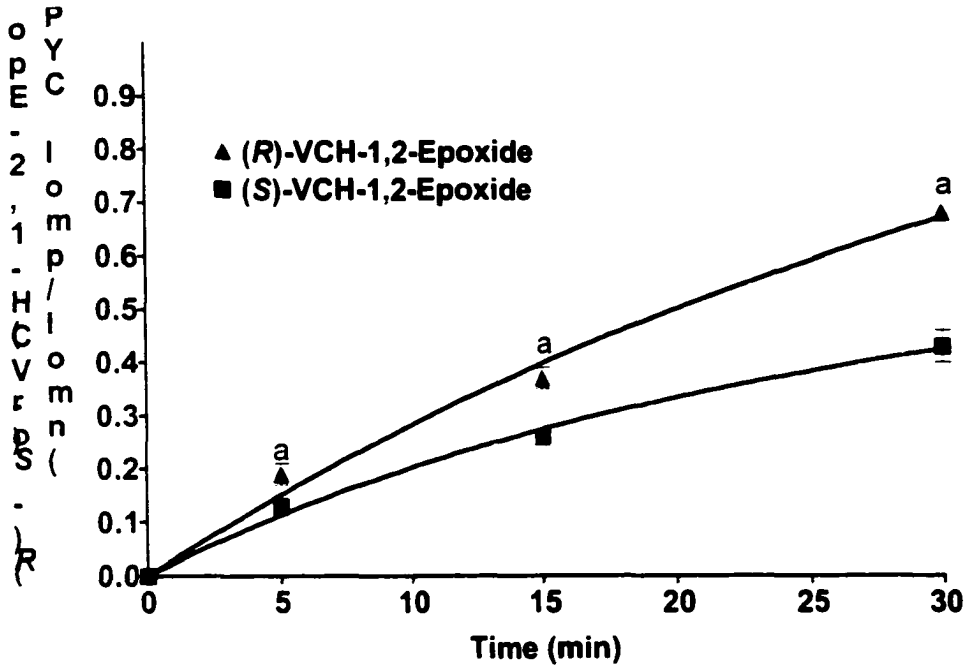
TABLE 7:

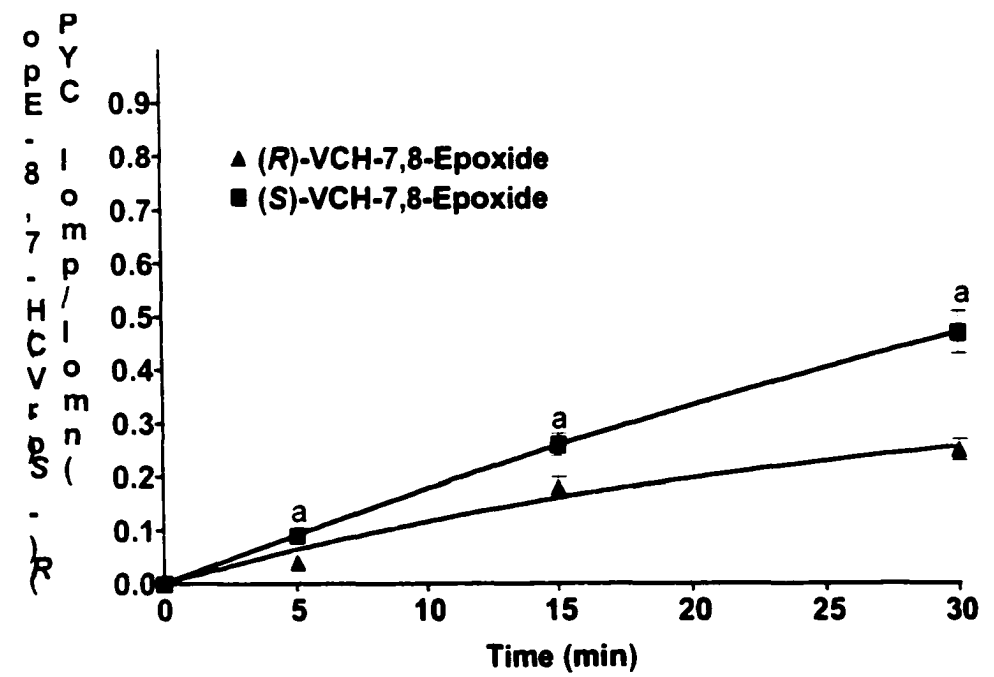
Effects of pretreatment with CYP enzyme inducers on the epoxidation of (R)-VCH and (S)-VCH (mean (±SD) nmol epoxide formed/mg protein after 60 min).

		MOUSE		RAT	
Metabolite	Treatment Group	(*R*)-VCH	(*S*)-VCH	(*R*)-VCH	(*S*)-VCH
VCH-1,2-Epoxide	Control	75 – 9[a]	30 – 4	38 – 7	19 – 7
	Acetone	134 – 11 (**1.8**[b])	66 – 8 (**2.2**)	79 – 7 (**2.1**)	37 – 0 (**2.0**)
	Phenobarbital	462 – 30 (**6.2**)	182 – 13 (**6.1**)	83 – 8 (**2.2**)	36 – 11 (**1.9**)
	VCH	231 – 16 (**3.1**)	101 – 16 (**3.4**)	68 – 8 (**1.8**)	68 – 8 (**1.8**)
VCH-7,8-Epoxide	Control	30 – 4	56 – 7	30 – 4	33 – 7
	Acetone	40 – 6	56 – 7	30 – 4	37 – 4
	Phenobarbital	58 – 2 (**1.9**)	90 – 12 (**1.6**)	30 – 3	37 – 6
	VCH	58 – 8 (**1.9**)	107 – 11 (**1.9**)	35 – 12	38 – 5
VCD	Control	n.d.[c]	n.d.	n.d.	n.d.
	Acetone	7 – 1*	2 – 1	n.d.	n.d.
	Phenobarbital	8 – 2*	3 – 3	n.d.	n.d.
	VCH	8 – 2	7 – 2	n.d.	n.d.

[a] Values represent mean (±SD) nmol epoxide formed/mg protein.
[b] Values in parenthesis represent significant ($p < 0.05$) fold-increase in epoxide metabolite formation over control microsomal incubations with that particular enantiomer.
[c] Not detectable.

Table 7: Formation of VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD (nmol/mg protein) from (*R*)-VCH or (*S*)-VCH after 60 min incubations with hepatic microsomes from B6C3F$_1$ mice or Fischer 344 rats pretreated with acetone (1.0% in drinking water X 5 d), phenobarbital (80 mg/kg X 5 d, i.p.) or VCH (7.5 mmol/kg X 10 d, i.p.). Data are represented as nmol epoxide formed/mg protein after 60 min incubations with 1mM (*R*)-VCH or (*S*)-VCH (n = 3-4 in duplicate; mice microsomes were prepared from 4 mice/group). Numbers in parenthesis represent significant ($p<0.05$) fold-increase in epoxide metabolite formation over control microsomal incubations with that particular enantiomer. (*) Significantly different amount of epoxide formation from (*S*)-VCH in the same species and treatment group, at $p < 0.05$.





Figures 16A and B: (R)-VCH and (S)-VCH Bioactivation in "Supersomes" containing Human CYP2E1.

Comparison of VCH-1,2-epoxide (**Figure A**) and VCH-7,8-epoxide (**Figure B**) formation from 1 mM (*R*)-VCH (_) or (*S*)-VCH (_) by human CYP2E1 "Supersomes" (100 pmol/mL). (a) Significantly different amount of epoxide formed than from (*S*)-VCH, at $p < 0.05$.

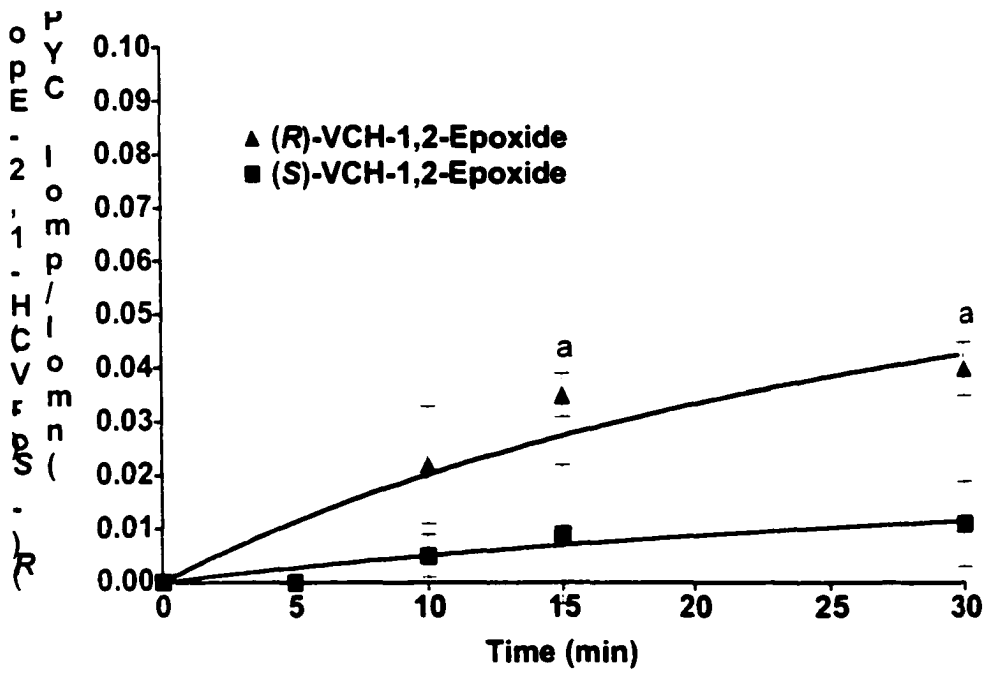
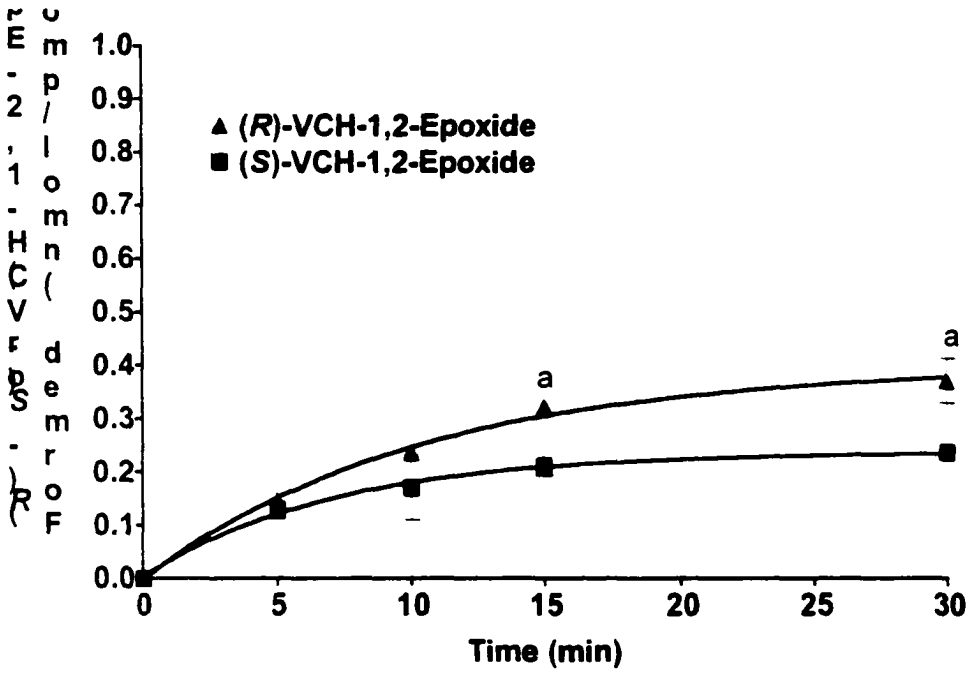
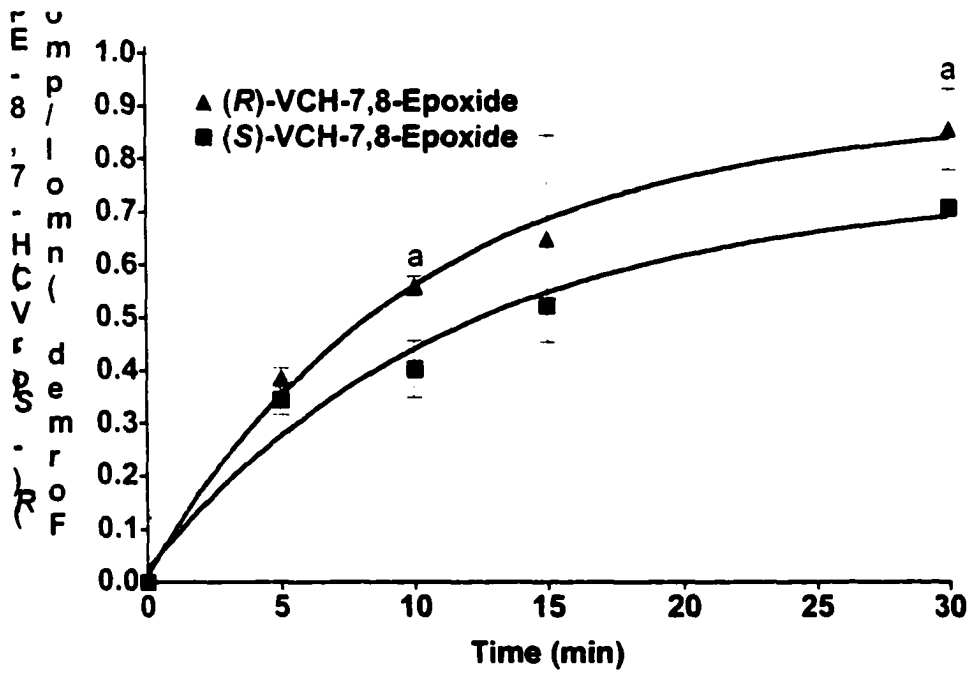


Figure 17: (R)-VCH and (S)-VCH Bioactivation in "Supersomes" containing Human CYP2A6.

Comparison of VCH-1,2-epoxide formation from 1 mM (*R*)-VCH (_) or (*S*)-VCH (_) by human CYP2A6 Supersomes (100 pmol/mL). (a) Significantly different amount of epoxide formed than from (*S*)-VCH, at $p < 0.05$.





Figures 18A and B: (R)-VCH and (S)-VCH Bioactivation in Supersomes" containing Human CYP2B6.

Comparison of VCH-1,2-epoxide (**Figure A**) and VCH-7,8-epoxide (**Figure B**) formation from 1 mM (*R*)-VCH (_) or (*S*)-VCH (_) by human CYP2B6 Supersomes (100 pmol/mL). (a) Significantly different amount of epoxide formed than from (*S*)-VCH, at $p < 0.05$.

DISCUSSION

These studies clearly demonstrated enantioselectivity and regioselectivity in the bioactivation of VCH in rodent hepatic microsomes as well as in expressed human CYP enzymes. In all microsomal incubations (from both treated and nontreated rodents), (*R*)-VCH formed significantly more of the 1,2 monoepoxide while (*S*)-VCH generally formed more of the 7,8- monoepoxide. In general, since the 1,2-monoepoxide was the major metabolite, more of the (*R*)- enantiomer was converted to monoepoxide compared to the (*S*)- enantiomer.

In both rodent species, the various CYP inducing agents caused greater induction of VCH-1,2-epoxide formation compared with VCH-7,8-epoxide formation from both enantiomers. In the mouse, phenobarbital pretreatment increased VCH-1,2-epoxide formation 6-fold and VCH-7,8-epoxide only 2-fold; acetone pretreatment increased VCH-1,2-epoxide formation 2-fold and did not affect VCH-7,8-epoxide formation; VCH pretreatment increased VCH-1,2-epoxide formation approximately 3-fold, and VCH-7,8-epoxide 2-fold. Since all pretreatments resulted in induction of bioactivation that was similar with both (*R*)- and (*S*)-VCH, it appears that these inductions did not result in increased enantioselective bioactivation. Although there was no formation of VCD, the ovotoxic metabolite of VCH, in any microsomal incubation from nontreated mice and rats, it was detected in incubations with microsomes obtained from mice that had been treated with the inducing agents. In both phenobarbital and acetone pretreatments groups, (*R*)-VCH formed more VCD than (*S*)-VCH. This indicates

that when there are elevated levels of certain CYP isoforms such as CYP2E1, CYP2A, or CYP2B in the liver, (*R*)-VCH may be more responsible for bioactivation to VCD than (*S*)-VCH. It is interesting, however, that VCH pretreatment in the mouse resulted in approximately the same microsomal capacity to form VCD from each VCH enantiomer. Therefore, VCH may induce isoforms other than those induced by acetone or PB, which catalyze the reactions forming VCD from (*S*)-VCH.

Induction of CYPs typically increases the concentration of CYP protein. Compounds such as phenobarbital achieve this by increasing transcription of mRNA encoding certain CYP genes (Waxman and Azaroff, 1992), whereas others (e.g. acetone, ethanol) stabilize the CYP protein (Forkert et al., 1991), or increase translational efficiency of mRNA into protein (Tsutsumi et al., 1993). Research in our laboratory has shown that repeated dosing with racemic VCH increases CYP2A and CYP2B protein levels in the mouse, but not in the rat (Doerr-Stevens et al., 1999; Fontaine et al., Unpublished observations). Since VCH pretreatment results in relatively the same amounts of VCD formation from each VCH enantiomer, it may be inducing both (*R*)-and (*S*)-VCH-metabolizing CYPs equally through one or more than one of the CYP-inducing pathways mentioned above. This has important stereochemical implications, since repeated exposure to VCH is required to cause ovotoxicity (Doerr-Stevens et al., 1999).

Human microsomes have been shown to metabolize VCH, but at rates lower than those of mouse or rat hepatic microsomes (Smith et al., 1991). To assess which CYP isoforms epoxidize VCH, the two separate VCH enantiomers were incubated with individual expressed human CYP proteins. Two other papers currently in preparation show that of 8 different human CYP isoform "Supersomes" tested, (CYP1A1, -2A6, -2B6, -2C9, -2E1, -3A4, -4A11, and Aromatase), only CYP2E1, -2A6, and -2B6 are capable of bioactivating racemic VCH (Fontaine et al., Unpublished observations). In studies represented here, CYP2A6, CYP2B6, and CYP2E1 metabolized the enantiomers of VCH to monoepoxides. Although human and rodent CYP isoforms cannot be directly compared, the human CYP2A and 2E1 protein systems used in these studies sterochemically mimicked metabolic profiles as seen with the mouse and rat microsomal systems. For example, in the CYP2E1 Supersome system, (*R*)-VCH preferentially formed more VCH-1,2-epoxide but less VCH-7,8-epoxide as compared to (*S*)-VCH. It is interesting that CYP2E1-inducing acetone pretreatment in both rodent species resulted in 2-fold increases in VCH-1,2-epoxide formation from both enantiomers, but no increases in VCH-7,8-epoxide formation from either enantiomer, and (*R*)-VCH still formed more VCH-1,2-epoxide but less VCH-7,8-epoxide as compared to (*S*)-VCH.

Since (*R*)-VCH preferentially forms VCH-1,2-epoxide and (*S*)-VCH forms more VCH-7,8-epoxide in microsomal samples, it is important to know whether one of these monoepoxides forms the toxic diepoxide at a more rapid rate.

Mouse hepatic microsomal incubations with either diastereometric VCH-1,2-epoxide or diastereometric VCH-7,8-epoxide demonstrated that the monoepoxides are bioactivated at comparable rates to form VCD (Fontaine et al., Unpublished observations). However, it is important to note that those studies measured metabolism of each monoepoxide from diastereometric mixtures [2(*R*)- and 2(*S*)-monoepoxides in total] rather than one diastereomer of the monoepoxide, so VCD formation represents only an average from both (*R*)- and (*S*)-VCH-monoepoxides.

VCH causes ovotoxicity in mice by depleting primordial and primary follicles through an apoptotic-like mechanism (Kao et al., 1999). Structure-activity studies have shown that the formation of the diepoxide is essential to cause ovotoxicity (Doerr and Sipes, 1996). Diepoxides such as butadiene diepoxide are highly electrophilic and may cause toxicity by cross-linking biomolecules (Hartley et al., 1999; Rydberg et al., 1996). Such cross-linking may require certain configurations of the diepoxides, as bio-molecules themselves have specific topographies. Therefore, it is possible that a limited number of the possible eight VCD diastereoisomers formed are responsible for the ovarian toxicity.

These studies represent the first important step in examining the stereochemistry of VCH bioactivation and toxicity. There were differences between the bioactivation of (*R*)- and (*S*)-VCH in rodent hepatic microsomes as well as in individual expressed human CYP isoforms. These differences in

metabolic profiles of (*R*)- and (*S*)-VCH may be due to, but are not limited to, different CYP isoforms involved in (*R*)- and (*S*)-VCH bioactivation for which there may be differences in binding affinities, different modes of binding based on how the molecule enters the active site, and different K_{cat} and/or K_{off} for each binding mode for each isoform. It is important to recognize, however, that *in vitro* bioactivation studies may not be entirely indicative of the physiological induction of ovarian toxicity by the VCH enantiomers. For example, one of the two VCH enantiomers may have a different distribution or partitioning *in vivo* than the other. Additionally, the epoxide metabolites of one VCH enantiomer may be more readily detoxified in the liver or ovary compared with the epoxide metabolites of the other enantiomer. Future studies that compare the ovarian toxicity of (*R,S*)-VCH to that of (*R*)- or (*S*)-VCH will further address whether stereochemistry is critical in VCH-induced ovarian toxicity.

CHAPTER 5

SUMMARY: MOLECULAR BASIS FOR THE SPECIES-DEPENDENT BIOACTIVATION OF VCH: ROLE OF HEPATIC CYTOCHROME P450 INDUCTION

Repeated exposure of female mice to VCH depletes primordial and primary ovarian follicles, resulting in premature ovarian failure (NTP, 1986; Collins and Manus, 1987; Bevan, 1994; Smith et al., 1990b). In chronic studies, this ovarian failure is followed by rare neoplasm formation in the ovary (NTP, 1986; Collins et al., 1987). It is known that bioactivation of VCH via CYP-catalyzed epoxidation is required to ellicit this ovotoxicity (Smith et al., 1990a,b; Doerr et al., 1996). Interestingly, female rats are resistant to VCH-induced ovotoxicity, which is at least in part due to limitations in forming the toxic metabolites (Smith et al., 1990a). Studies reported here also support differences in the bioactivation of VCH between the two species. Over a 60 min incubation period, hepatic microsomes from nontreated mice formed greater amounts of VCH-1,2-epoxide and VCH-7,8-epoxide compared to rats. The enhanced epoxidation by mouse microsomes was evident whether racemic, (*R*)- or (*S*)-VCH served as the substrate. (*R*)-VCH formed more VCH-1,2-epoxide and less VCH-7,8-epoxide than (*S*)-VCH in microsomes from both the mouse and the rat.

Since VCH requires multiple days of dosing to initiate ovotoxicity in the mouse, this prompted an investigation into which CYP isoforms are induced in the mouse and/or rat following repeated dosing with VCH, and if there are stereochemical considerations under these conditions. It was hypothesized that the underlining reason behind the resistance of the rat to the ovotoxicity of VCH is its inability to induce CYP following repeated dosing with VCH.

There is great evidence for the role of bioactivation in the species-dependent ovotoxicity of VCH, and a model has now been devoloped to describe these events (Figure 19). First, repeated exposure to VCH caused greater increases in VCH epoxidation to VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD in the mouse compared to the rat. In microsomes from VCH-pretreated rodents, (*R*)-VCH again formed significantly more of the 1,2 monoepoxide while (*S*)-VCH generally formed more of the 7,8- monoepoxide. In general, since the 1,2-monoepoxide was the major metabolite, more of the (*R*)- enantiomer was converted to monoepoxide compared to the (*S*)- enantiomer.

There were also significant increases in total hepatic CYP levels in mice pretreated with VCH or VCH-1,2-epoxide, while neither of these pretreatments caused significant increases in total CYP levels in the rat. Significant increases in CYP2B expression and activities were seen in microsomes from mice pretreated with VCH or VCH-1,2-epoxide. In contrast, there were no increases in CYP2B levels or activity seen with pretreatments other than PB in the rat. Rather, there were significant decreases in CYP2B levels and activity in microsomes from rats pretreated with VCD. There were also significant increases in CYP2A activity seen in microsomes from mice, but not rats, pretreated with VCH or VCH-1,2-epoxide. These results have important correlations with the fact that VCH pretreatment results in a greater fold increase in VCH bioactivation in vitro compared to controls in the mouse than in the rat, and they also correlate with the increases in VCH bioactivation in microsomes

pretreated with the known CYP2A and CYP2B inducer, PB. PB pretreatement not only caused increases in VCH epoxidation, but also caused increases in total hepatic CYP levels, and CYP2A and CYP2B levels/activities in both species, further supporting the role of these two CYP isoforms in VCH bioactivation.

As previously mentioned, both VCH and VCH-1,2-epoxide pretreatment resulted in increases in total and specific hepatic CYP expression in the mouse but not the rat (VCH-7,8-epoxide pretreatment was not tested due to limitations in abundance). This raised the interesting possibility that perhaps the monoepoxide was responsible for the CYP induction. Since repeated treatment with VCH is required for ovotoxicity, and the mouse forms more VCH-1,2-epoxide than the rat, this metabolite may be causing the enhanced VCH bioactivation in the mouse. In these studies, both VCH and VCH-1,2-epoxide pretreatment resulted in higher increases in total CYP levels, and CYP2A and CYP levels/activities. Therefore, these studies indicate that both VCH and VCH-1,2-epoxide may lead to CYP induction in the mouse. Furthermore, there is indication that the decreases in CYP2B levels/activity in microsomes from rats pretreated with VCH-1,2-epoxide or VCD may serve as protective measures in this species.

Although CYP2E1 is involved in the bioactivation and toxicity of structurally related compounds such as 1,3-butadiene, this isoform is not crucial in rodent VCH bioactivation. Hepatic microsomes from mice and rats pretreated with acetone showed increases in VCH-1,2-epoxide formation from VCH, however, hepatic microsomes from mice or rats pretreated with either VCH, VCH-1,2-

epoxide, or VCD demonstrated no increases in CYP2E1 protein levels or activity. Those data, combined with the data showing no differences in epoxidation of VCH or its monoepoxides in CYP2E1-deficient mouse hepatic microsomes compared to those of mice that do have CYP2E1, indicated that CYP2E1 is not an important isoform in the species-specific bioactivation of VCH.

Understanding the molecular reasons for the enhanced ability of the mouse to bioactivate VCH compared to the rat is necessary to better extrapolate which of these animal models would better predict the risk to humans exposed to VCH. While it is known that total hepatic CYP/ mg protein is significantly lower in humans than in rodents (Shimada et al., 1994; Imaoka et al., 1996), there is still little known about how humans would bioactivate VCH, particularly after repeated exposure. In a previous study, female human microsomes epoxidized VCH at rates 13-fold and 2-fold less than that in mice and rats, respectively (Smith and Sipes, 1991). It is interesting that out of the eight human hepatic CYP isoforms tested in current studies, CYP2B6 and CYP2E1 were the only isoforms that significantly catalyzed VCH. However, CYP2E1 and CYP2B6 only represent approximately 6% and < 1% of total human hepatic CYP (Shimada et al., 1994). It is also interesting that, although CYP2B6 and CYP2E1 were the only CYP isoforms that catalyzed VCH epoxidation, CYP2B6, CYP2A6, CYP2E1, and CYP3A4 catalyzed the epoxidation of both monoepoxides to form the diepoxide. While CYP3A4 is the major hepatic CYP isoform in humans, the rates of monoepoxide formation are significantly limited in humans compared to the

mouse or rat. Therefore, there may not be the opportunity for epoxidation of these monoepoxides to occur after being exposed to VCH. Hence, if VCH bioactivation alone is an indication of VCH-indcued ovotoxicity, the Smith study combined with present results indicate the humans will respond more like the rat than the mouse. While it is impossible to know how humans may respond to repeated exposure, in terms of inducing CYP capabilities and VCH bioactivation, it would be interesting to determine how human hepatocytes would handle VCH compared to those from mice and rats, particularly following repeated exposure.

While there are now clearly defined differences in VCH bioactivation between the mouse and rat, it is also important to consider differences in hepatic detoxification. As mentioned previously, there was a 6.5-fold greater rate of formation of VCH-1,2-epoxide in the hepatic microsomes from mice compared to rats, but the species differences observed in plasma levels of VCH-1,2-epoxide following a single dose of VCH was substantially greater (up to 16-fold differences) (Smith et al., 1990a). The detoxification pathway has been previously described (Doerr-Stevens et al., 1999) (Figure 20). The bioactivation of VCH is followed by subsequent hydrolysis of epoxides by a family of enzymes known as epoxide hydrolases, or conjugation with glutathione. Differences in the abilities of epoxide hydration of VCH-epoxides have been established in the mouse and rat (Keller et al., 1996). While glutathione conjugates have not been identified to date, increases in cytosolic glutathione S-transferase activity were observed in mouse liver after repeated 10 d VCH treatment (Doerr-Stevens et al.,

1999). Therefore, species-differences in epoxide hydrolase expression/activity could add to the understanding of the species-dependent ovotoxicity of VCH.

Species differences in the ability to bioactivate and detoxofiy VCH in the ovary may also exist. Studies have shown that rat ovaries were capable of detoxifying VCD by catalyzing the formation of tetrol from VCD (Flaws et al., 1994b). This study also demonstrated that the conversion of VCD to tetrol in large preantral follicles and hepatocytes of the adult rat was 3- and 10-fold greater than in small preantral follicles and interstitial cells. This may explain the increased susceptibility of this particular population of follicles to VCD-induced ovotoxicity. Furthermore, while the liver is suspect as the primary site of bioactivation of VCH, extrahepatic tissues also have CYP metabolizing capabilities (Farrell, 1987; Krishna and Klotz, 1994). The ovary has been characterized as expressing CYP enzymes capable of bioactivating several polycyclic aromatic hydrocarbons, such as 7,12-dimethylbenz[a]anthracene (Bengtsson et al., 1988, 1990). While the liver has shown to readily convert VCH to VCH-1,2-epoxide, and to a lesser extent, VCH-7,8-epoxide, formation of the ovotoxic metabolite VCD is not detectable in microsomes from control rodents, and is barely above the limit of detection in mice pretreated with VCH or CYP inducers. Therefore, the ovarian bioactivation of VCH, or more particularly, the subsequent bioactivation of monoepoxides to VCD should be considered.

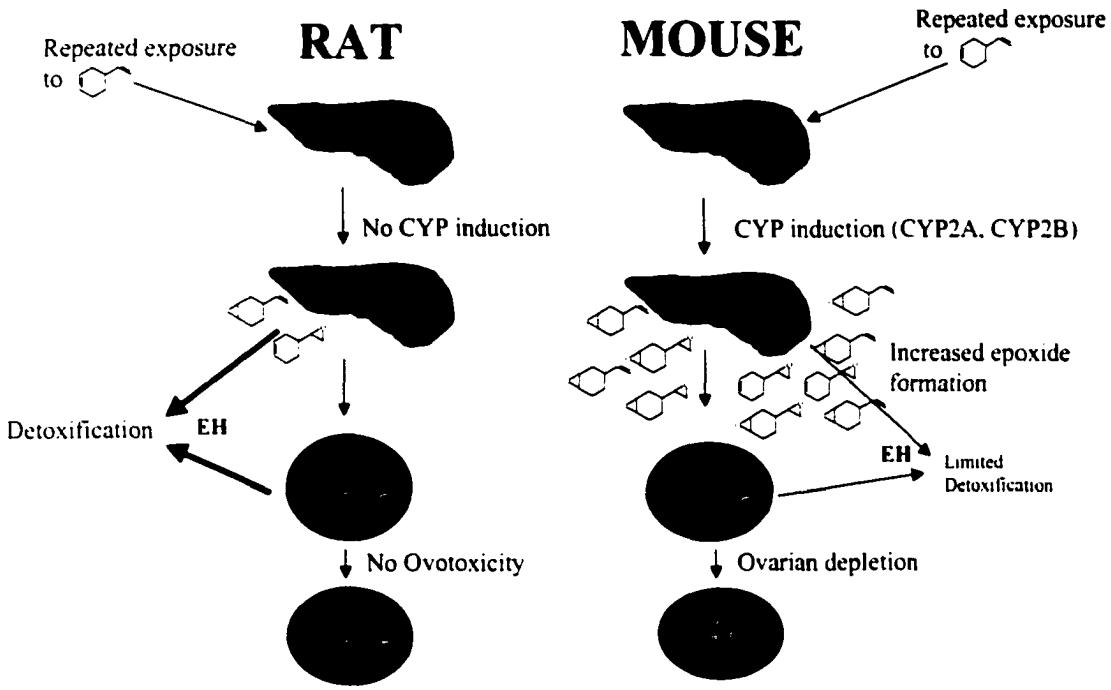


Figure 19. Proposed model for the mechanism of species-dependent ovotoxicity of VCH following repeated exposure to VCH. CYP =cytochrome P450 and EH = epoxide hydrolase.

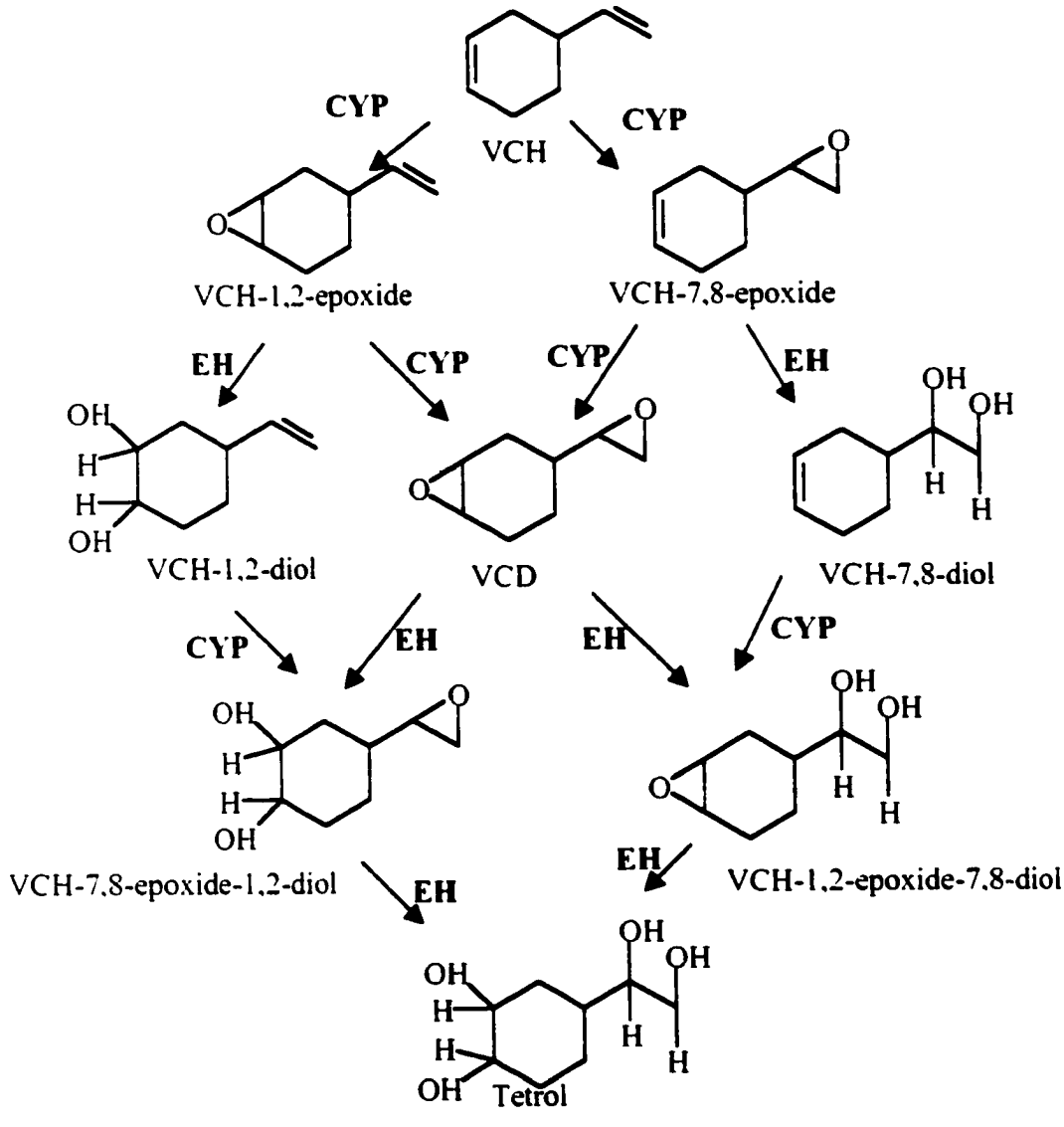


Figure 20: Proposed scheme for the bioactivation and subsequent detoxification of VCH.

APPENDIX A

REPRINT APPROVAL

December 8, 2000

Susan Fontaine
College of Pharmacy
Department of Pharmacology and Toxicology
The University of Arizona
PO Box 210207
Tucson, AZ 85721-0207

Fax: 520-626-2466

Dear Ms. Fontaine,

This is to grant you permission to include the following manuscript in its entirety in your doctoral dissertation for the University of Arizona:

S. M. Fontaine, E. A. Marsh, P. B. Hoyer, and J. G. Supco, "Stereochemical aspects of 4-vinylcyclohexene bioactivation in rodent hepatic microsomes and purified human cytochrom P450 systems". *Drug Metabolism and Disposition*, vol. 29, no. 2, February 2000 (scheduled date of publication)

On each copy of the manuscript that is included with your dissertation, please include the following:

Sincerely yours,

Richard Dodenhoff
Journals Director

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