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The role of epoxidation in 4-vinylcyclohexene-induced ovarian toxicity

Smith, Bill J., Ph.D.

The University of Arizona, 1990

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THE ROLE OF EPOXIDATION IN 4-VINYLCYCLOHEXENE-INDUCED
OVARIAN TOXICITY

by

Bill J. Smith

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
in the Graduate College
THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read
the dissertation prepared by Bill J. Smith

entitled The Role of Epoxidation in 4-Vinylcyclohexene-Induced
Ovarian Toxicity

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I dedicate this dissertation to my parents since it was through their continued support that this was possible.

ACKNOWLEDGEMENTS

I have been very fortunate to be under the direction of Dr. I. Glenn Sipes. Dr. Sipes, with my best interests in mind, was always creating new opportunities for me to learn. I can honestly say that my advisor was also a friend, something seldom said by graduate students. I thank you Glenn. Dr. Dean Carter took an interest in my personal and professional growth, knowing just how to inspire me. Inspiration usually came in the form of ridicule and embarrassment, however, it was never serious and always good natured. I appreciated your open door policy which led to many informative conversations concerning both personal and professional issues. A unique aspect of this dissertation was collaborative efforts with two of my committee members, Dr. Donald Mattison and Dr. James Halpert. The collaboration with Dr. Mattison facilitated the ovarian toxicity studies which were pivotal to the success of the work. Dr. Mattison treated me more like a colleague than a student, which provided an excellent model for my future interactions with other scientists. Dr. Halpert's enthusiasm for science was contagious. I will always be impressed by your knowledge and determination to find the answer. Dr. Klaus Brendel probably saw more of me than he would have preferred. He frequently caught me fraternizing with his student, Mary Stefaniak. Once caught, I would give an account of work both completed and in progress and through these interactions he was kept up to date and could offer suggestions for future studies. Finally, I thank Dr. Hugh Laird for serving as an alternate for my preliminary oral exam. I have learned that graduate school is a place where close personal friendships are formed. The love of Mary Stefaniak showed me that your career is not the only part of your life, or in other words it is important to be well rounded. The friendship of Jeff Stevens, Greg Weber, Scott Mobley, Andy Taylor, Eric Stine, my coworkers at the Arizona Poison and Drug Information Center, and many others made this experience fun, even at the worst of times.

TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATIONS	9
LIST OF TABLES.	11
ABSTRACT.	13
1. INTRODUCTION.	15
Physical Properties of VCH	15
Production and Use of VCH.	15
Exposure of Humans to VCH.	16
Toxicology of VCH.	17
Acute toxicity.	17
Chronic toxicity.	19
Metabolism of VCH.	24
Genetic Toxicology of VCH and VCH Metabolites	26
Ovarian Tumor Induction in Rodents	29
Procedures to induce ovarian tumors	29
Oocyte destruction by ovarian carcinogens.	33
Statement of Problem	35
Objectives	36
2. COMPARISON OF THE DISPOSITION AND <u>IN VITRO</u> METABOLISM OF 4-VINYLCYCLOHEXENE IN THE MOUSE AND RAT	37
Abstract	38
Introduction	40
Materials and Methods.	41
Results.	47
Excretion of [¹⁴ C]VCH-equivalents	47
Tissue distribution: [¹⁴ C]VCH-equivalents	48
Tissue distribution: Parent compound . .	51
Studies of VCH-1,2-epoxide blood concentrations <u>in vivo</u> : Species comparison and time-response.	54
Studies of VCH metabolism <u>in vitro</u>	54
Discussion	57

TABLE OF CONTENTS (Continued)

	Page
3. THE ROLE OF EPOXIDATION IN 4-VINYLCYCLOHEXENE- INDUCED OVARIAN TOXICITY	60
Abstract	61
Introduction	63
Materials and Methods.	65
Results.	70
Ovotoxicity of VCH and VCH epoxides	70
Studies of VCH-1,2-epoxide blood concentrations in mice	73
Inhibition of VCH epoxidation and ovotoxicity.	75
Discussion	78
4. THE BIOCHEMICAL BASIS FOR THE SPECIES DIFFERENCE IN 4-VINYLCYCLOHEXENE EPOXIDATION BETWEEN FEMALE RAT AND MOUSE HEPATIC MICROSOMES.	86
Abstract	87
Introduction	89
Materials and Methods.	91
Results.	95
Effect of chloramphenicol treatment of female B6C3F ₁ mice on several hepatic microsomal testosterone hydroxylase activities	95
Effect of inducers on microsomal VCH-epoxidase and steroid hydroxylations	97
The effect of inhibitory cytochrome P450 form specific antibodies on VCH epoxidation and steroid hydroxylation by hepatic microsomes	99
Testosterone 16 α -hydroxylation and VCH epoxidation by hepatic microsomes from untreated and phenobarbital-treated female 129/J mice.	101
Immunochemical analysis of hepatic microsomes	105
Discussion	109
5. THE METABOLISM OF 4-VINYLCYCLOHEXENE BY HUMAN HEPATIC MICROSOMES	114
Abstract	115

TABLE OF CONTENTS (Continued)

	Page
Introduction	117
Materials and Methods.	118
Results.	120
Validation of assay conditions.	120
Comparison of VCH epoxidation by different human hepatic microsome samples.	123
Discussion	126
6. SUMMARY	129
7. REFERENCES.	135

LIST OF ILLUSTRATIONS

Figure		Page
1.	Cummulative excretion of [^{14}C]4-vinyl-cyclohexene-derived radioactivity in urine and expired air of female mice and rats administered [^{14}C]4-vinyl-cyclohexene (400 mg/kg po)	50
2.	Tissue distribution of 4-vinylcyclohexene (parent compound) in selected tissues of mice and rats administered [^{14}C]4-vinyl-cyclohexene (400 mg/kg po)	53
3.	Time course of appearance of 4-vinyl-cyclohexene-1,2-epoxide in the blood of female mice and rats given 4-vinyl-cyclohexene (800 mg/kg ip)	55
4.	Comparison of the dose-response relationship in the reduction in small oocyte counts in the ovaries of rats and mice treated with VCH or VCH epoxides ip for 30 days.	71
5.	The effect of treatment duration on reduction in small oocyte counts in mice treated with 4-vinylcyclohexene 800 mg/kg/day, ip.	74
6.	Comparison of the 4-vinylcyclohexene-1,2-epoxide blood concentration-time profile in female mice after ip administration of equitoxic doses of 4-vinylcyclohexene of 4-vinylcyclohexene-1,2-epoxide.	76
7.	The dose-response relationship of the reduction of 4-vinylcyclohexene-1,2-epoxide blood levels in female mice preterated with selected doses of chloramphenicol 1 hr prior to administration of 4-vinylcyclohexene 800 mg/kg, ip.	77

LIST OF ILLUSTRATIONS (Continued)

Figure		Page
8.	The effect of saline of chloramphenicol (200 mg/kg, ip) pretreatment on ovarian toxicity induced by 4-vinylcyclohexene (800 mg/kg, ip) in mice treated daily for 15 days.	80
9.	The effect of inducer treatment on female B6C3F ₁ mouse hepatic microsomal VCH epoxidase and testosterone hydroxylase activities. . . .	98
10.	The effect of inhibitory antibodies on female B6C3F ₁ mouse hepatic microsomal VCH epoxidase and testosterone hydroxylase activities	102
11.	Western blot of hepatic microsomes using an anti-P450 _{PB-B} antibody.	107
12.	Western blot of hepatic microsomes using an anti-P450 _{15α} antibody	108
13.	The effect of incubation time on VCH epoxidation by human hepatic microsome sample D10 (donor)	121
14.	The effect of protein concentration on VCH epoxidation by human hepatic microsome sample D10 (donor)	122
15.	Comparison of the rate of formation of VCH-1,2-epoxide from VCH by hepatic microsomes of female mouse, rat, and human samples	127
16.	Schematic diagram illustrating the proposed events which lead to oocyte destruction following VCH administration	133
17.	Schematic diagram illustrating how VCH-induced ovarian toxicity may lead to tumor induction	134

LIST OF TABLES

Table		Page
I.	Incidence of Ovarian Tumors in Female Mice Treated with 4-Vinylcyclohexene For 2 Years.	21
II.	Comparison of the Survival of Female Rats and Mice in a 2 Year 4-Vinylcyclohexene Carcinogenicity Bioassay	23
III.	Excretion of Radioactivity from Female Rats and Mice Following Oral Administration of [¹⁴ C]4-Vinylcyclohexene.	49
IV.	The Distribution of Radioactivity in the Ovaries of Mice and Rats Orally Administered [¹⁴ C]4-Vinylcyclohexene (400 mg/kg).	52
V.	Formation Rates of VCH-1,2-epoxide by Hepatic Microsomes Incubated with 4-Vinylcyclohexene (1 mM).	56
VI.	ED ₅₀ Values for the Reduction in Small Oocyte Counts in Mice and Rats Administered 4-Vinylcyclohexene (VCH) and VCH epoxides ip for 30 Days.	62
VII.	Effect of ip Chloramphenicol (200 mg/kg) Treatment on Female Mouse Hepatic Microsomal VCH Epoxidation.	79
VIII.	The Effect of Chloramphenicol (CAP) Treatment (200 mg/kg) on Female B6C3F ₁ Mouse Hepatic Microsomal Testosterone Hydroxylase Activities	96
IX.	The Effect of Phenobarbital (PB) Treatment on Female F-344 Rat Hepatic Microsomal VCH-epoxidase and Androstenedione Hydroxylase Activities	100

LIST OF TABLES (Continued)

Table		Page
X.	The Effect of Anti-Rat-P450 _{PB-B} IgG on VCH-epoxidase and Androstenedione Hydroxylase Activities in Hepatic Microsomes from Untreated and Phenobarbital-Treated (PB-treated) Female F-344 Rats	103
XI.	The Effect of Anti-Mouse-P450 _{15α} IgG on VCH-epoxidase and Testosterone 15α-hydroxylase Activities in Untreated Female B6C3F ₁ Mouse Hepatic Microsomes	104
XII.	Comparison of VCH-epoxidase and Testosterone 16α-hydroxylase Activities in Untreated and Phenobarbital-treated Female 129/J Mouse Hepatic Microsomes	106
XIII.	Formation Rates of VCH Epoxides by Human Hepatic Microsomes.	124
XIV.	Comparison of the Formation Rates of VCH-1,2-epoxide by Hepatic Microsomes from Male and Female Humans.	125

ABSTRACT

The basis for the species difference between B6C3F₁ mice (susceptible) and Fischer 344 rats (resistant) to 4-vinylcyclohexene (VCH)-induced ovarian tumorigenicity was investigated. Greater than 95% of a single oral 400 mg/kg dose of [¹⁴C]VCH was eliminated in 48 hr by mice and rats. Approximately 50-60% of the administered dose was excreted in the urine, while the remaining 30-40% of the dose was expired as organically soluble radioactivity. VCH-treated mice had dramatically higher blood concentrations of the VCH metabolite VCH-1,2-epoxide compared to VCH-treated rats. Furthermore, mouse hepatic microsomes catalyzed the conversion of VCH to VCH-1,2-epoxide at greater rates than rat hepatic microsomes.

The destruction of oocytes was used as an index of ovarian toxicity to compare the potency of VCH and VCH epoxides in the mouse and rat. VCH markedly reduced the number of small oocytes in mice while no detectable change in oocyte number occurred in rats. Epoxide metabolites of VCH destroyed oocytes in both species at lower doses than VCH. Inhibition of VCH epoxidation reduced VCH-1,2-epoxide blood levels and partially protected mice from VCH-induced ovarian toxicity. Thus, the conversion of VCH to epoxides and the subsequent destruction of oocytes are critical steps in the induction of ovarian tumors by VCH. Rats may be resistant

because the amount of VCH converted to epoxides is insufficient to destroy oocytes.

The biochemical basis for the species difference in the rate of VCH epoxidation by hepatic microsomes from mice and rats was investigated. Studies using inducers and inhibitors of certain cytochrome(s) P450 showed that hepatic microsomes of female mice perform VCH epoxidation at greater rates than rats because of the constitutive expression of P450 IIA and IIB forms. Hepatic microsomes of human females perform VCH epoxidation at lower rates than rats. This suggests that if the rate of epoxidation of VCH by the liver is the most important factor determining susceptibility to VCH toxicity then the rat may better model the response of humans exposed to VCH than mice.

CHAPTER 1

INTRODUCTION

Physical Properties of VCH

4-Vinylcyclohexene (VCH) is a colorless liquid with a density of 0.83 g/ml at 20°C. It has a boiling point of 128.9°C and a vapor pressure of 25.8 mm of mercury at 38°C (Sandmeyer, 1981). VCH will vaporize at room temperature and has a sharp, irritating odor.

Production and Use of VCH

VCH is produced commercially through the dimerization of 1,3-butadiene (IARC, 1986). The most common use of VCH is as an intermediate in the chemical synthesis of other compounds. Examples include the synthesis of vinylcyclohexene diepoxide which itself is used as an epoxy resin diluent. Manufacture of insecticides containing ethylene chlorohydrin or thiocyanate, flame retardants, and ethylcyclohexyl carbinol plasticizers also require VCH as a precursor. The reported quantity of VCH produced in the United States in 1977 ranged from 1.2 and 12.1 million pounds (USEPA, 1980). Unfortunately, VCH is also produced as an unwanted byproduct while curing polybutadiene rubber for passenger tire manufacture (Rappaport and Fraser, 1976). Studies using laboratory models of rubber vulcanization have shown that VCH

is the major product released into the air by curing rubber (Rappaport and Fraser, 1977).

Exposure of Humans to VCH

The exposure of humans to VCH occurs mainly by inhalation in occupations involving 1,3-butadiene polymers. VCH has been measured by combined gas chromatography and mass spectrometry in several Italian rubber goods manufacturing sites (Cocheo et. al., 1983). The highest concentrations (30-210 ug VCH/m³) were found in the vulcanization area of a shoe sole factory. In electrical cable insulation and tire retreading plants the concentration of VCH ranged from 0-10 µg/m³ and 0-3 µg VCH/m³, respectively. In the Soviet Union, Bykov (1968) measured extremely high concentrations of VCH near its site of production and the workers exposed to these concentrations were reported to suffer occupationally related illnesses. In a curing area of a United States passenger tire manufacturing facility VCH concentrations as high as 520 µg/m³, have been reported (Rappaport and Fraser, 1976).

The main concern of the exposure of workers to VCH is not over the acute effects of exposure but rather the consequence of chronic exposure to VCH. Epidemiological studies have determined that workers in the rubber industry have an increased occurrence of certain forms of cancer (IARC, 1982). Unfortunately, only in rare instances have these studies

actually associated exposure of workers to a carcinogenic chemical or even a certain task within a rubber plant with an increase in a specific type of cancer. Identification of the etiological factors of cancer causation in the rubber industry are difficult for several reasons. Today we are studying cancers which are the result of exposures which took place decades ago. Data is often unavailable on the concentrations of chemicals to which the workers were exposed. Exposure to particular chemicals usually occurs only in certain jobs, however, workers in adjacent areas may also be exposed to chemicals not associated with a particular task, making it difficult to assign a chemical exposure to a work area. Furthermore, workers are frequently exposed to mixtures of chemicals. This makes the association of the exposure to a specific chemical and increased cancer risk difficult. Finally, information on risk factors which are not related to the work environment such as smoking and diet are often lacking. In light of this, it is not surprising that the consequences of chronic exposure of humans to VCH is unknown.

Toxicology of 4-Vinylcyclohexene

Acute toxicity

The single dose LD₅₀ values of VCH indicate that it is of low acute toxicity. In the Carworth-Wistar rat the oral LD₅₀ of VCH is 2.6 g/kg (Smyth et al., 1969). Dermal toxicity

studies in the rabbit showed VCH to have an LD₅₀ value of 17 g/kg (Smyth et al., 1969). The LC₅₀ of VCH by inhalation is 47 g/m³ in the rat and 27 g/m³ in the mouse (IARC, 1986).

The most thorough investigation of the acute toxicology of VCH has been conducted by the National Toxicology Program. Fourteen day and 13 week daily gavage studies were performed using male and female B6C3F₁ mice and Fischer 344 rats (Collins and Manus, 1987 and NTP, 1986). These studies were performed to determine if VCH toxicity targeted a certain organ and to select appropriate doses of VCH for use in a 2 year carcinogenicity bioassay.

In the 14 day study, mice and rats were gavaged daily with 0, 300, 600, 1250, 2500, and 5000 mg/kg VCH in corn oil. All rats and most mice died before completion of the study at or above doses of 1250 mg/kg VCH. Necropsies performed on the survivors revealed no compound related gross abnormalities. The stomach was the only tissue which was examined histologically as it was suspected to be the target tissue. No pathological findings were observed in the histology of the stomach of these animals.

The doses of VCH used in the 13 week daily gavage study were 0, 50, 100, 200, 400, and 800 mg/kg in rats and 0, 75, 150, 300, 600, and 1200 mg/kg in mice. Tissues from animals receiving the highest dose of VCH or from any animal which died before the end of the study was evaluated histologically.

In rats the most significant histopathological lesion was hyaline droplet degeneration of the proximal tubule of the kidney. This lesion was only observed in dosed male rats. The nephrotoxic effect of VCH was severe enough to be considered life threatening in a 2 year study. Inflammation of the nonglandular portion of the stomach was only observed in 3 female rats and one male rat of the 800 mg/kg group. Inhibition of body weight gain was less than 10% in all treated rats compared to their respective controls. In VCH-treated mice most males and 50% of the females receiving 1200 mg/kg died before the completion of the study. There was mild inflammation of the stomach in several males and one female. An unexpected compound related effect was observed in the ovaries of female mice receiving 1200 mg/kg VCH. The ovaries of all mice in this group (including those which died before the completion of the study) contained a reduced number of primary and mature follicles. These findings were not quantified by counting oocytes in serially sectioned ovaries and the lower dose groups were not examined for ovarian histological abnormalities. With the exception of one high dose male mouse the inhibition of body weight gain was less than 10% in all treatment groups.

Chronic toxicity

Based on data obtained in the 14 day and 13 week studies daily doses of 0, 200, and 400 mg/kg of VCH were selected for

the 2 year studies (Collins et al., 1987 and NTP, 1986). B6C3F₁ mice and Fischer 344 rats of both sexes were used as the test species. Necropsies were performed on all animals including those dying before the final termination date. Histopathological examination was performed on all major tissues. An undesired problem which interfered with this study was early mortality. Only in the low dose male and female mice was survival comparable to the vehicle controls. In all other groups survival was significantly decreased. In high dose male and female rats, survival was significantly decreased at 5 weeks. This was unexpected since no deaths occurred at this dose and time in the 13 week studies. In addition, these deaths were of an undetermined cause. A further complication of these studies was the lack of a clear carcinogenic response in rats and male mice. Because of these complications the studies in rats and male mice were considered inadequate studies of carcinogenicity.

In female mice VCH was clearly carcinogenic. Both doses of VCH resulted in the induction of ovarian neoplasms of several different types in female mice. Spontaneous ovarian tumors are extremely rare neoplasms in mice. This was shown not only by the lack of ovarian tumors in the vehicle controls but also by the historical controls of other NTP experiments (NTP, 1986). In previous NTP studies only 3/1,028 control mice had ever developed ovarian tumors. Shown in Table I is

Table I.
Incidence of Ovarian Tumors in Female Mice
Treated with 4-Vinylcyclohexene for 2 Years^a

Tumor Type	Vehicle Control	200 mg/kg	400 mg/kg
Mixed Tumor, Benign			
Incidence	0/49	25/48	11/47
% with Tumor Type	0%	52%	23%
Granulosa Cell Tumor			
Incidence	1/49	9/48	11/47
% with Tumor Type	2%	19%	23%
Granulosa Cell Carcinoma or Granulosa Cell Tumor			
Incidence	1/49	10/48	13/47
% with Tumor Type	2%	21%	28%

^a Data taken from NTP, 1986.

the incidence of the major ovarian tumors induced by VCH treatment of female mice. The three major tumor types induced by VCH treatment in female mice were benign mixed tumors, granulosa cell tumors, and granulosa cell carcinomas. Many of these tumors were not observed grossly. However, the ovarian tumors which were observed at necropsy were as large as 1 to 1.5 cm in diameter. This is quite impressive since the normal mouse ovary is only 1 to 2 mm in diameter. Tumors identified as benign mixed tumors were defined as noninvasive neoplasms which were not metastasizing and were comprised of proliferating germinal epithelial cells and granulosa cells. Granulosa cell neoplasms contain proliferating granulosa cells. These neoplasms can range from benign hyperplastic lesions to malignant neoplasms and it is often difficult to make a distinction between them. In these studies the majority of granulosa cell neoplasms were either solid or follicular in nature and a particular pattern was usually evident within a lesion. Neoplasms of Granulosa cells diagnosed as carcinomas had replaced the entire ovary. They were cystic, hemorrhagic, and had metastasized to the lungs.

Despite the fact that the study was considered inadequate to determine the presence or absence of carcinogenicity in female rats, certain comparisons to the mouse study seem reasonable. In Table II the survival between female rats and mice in the VCH bioassay is compared. The survival of

Table II.

Comparison of the Survival of Female Rats and Mice
in a 2 year 4-Vinylcyclohexene Carcinogenicity Bioassay^a

Species and Parameter	Vehicle Control	200 mg/kg	400 mg/kg
Mouse			
Animals in Study	50	50	50
Deaths Before Termination	10	11	31
Killed at Termination	40	39	17
Other	0	0	2
Rat			
Animals in Study	50	50	50
Deaths Before Termination	10	21	35
Killed at Termination	40	28	13
Other	0	1	2

^a Data taken from NTP, 1986.

control animals in this study is identical. This is contrasted with lower survival of dosed rats in both treatment groups. However, in VCH-treated mice the induction of ovarian tumors was evident even when survival was poor (i.e. as in the high dose group). Using this argument it seems likely that if VCH could induce ovarian tumors in rats, enough animals survived this study for this effect to be detected, even if the incidence of ovarian tumor induction was lower in VCH treated rats. Therefore, it is clear that a dramatic species difference exists between the female rat and mouse in the ability of VCH to induce tumors of the ovary.

Metabolism of VCH

The absorption, metabolism, and excretion of VCH in vivo has not been studied in any species. However, the in vitro metabolism of VCH has been investigated in rat and mouse hepatic microsomes. Watabe and coworkers (1981) identified 4-vinylcyclohexene-1,2-epoxide (VCH-1,2-epoxide), 4-vinylcyclohexene-7,8-epoxide (VCH-7,8-epoxide), 4-vinylcyclohexene-1,2-dihydrodiol (VCH-1,2-diol), and 4-vinylcyclohexene-7,8-dihydrodiol (VCH-7,8-diol) as metabolites of VCH generated by male rat hepatic microsomes. VCH-1,2-epoxide and VCH-7,8-epoxide were detected only in the presence of the epoxide hydrolase inhibitor 3,3,3-trichloropropene oxide (TCPO). The diepoxide metabolite of VCH was not

detected under any of the incubation conditions used. The rate of VCH-1,2-epoxide production was three fold higher than the rate of VCH-7,8-epoxide production. The production of these metabolites required the presence of an NADPH generating system and the substrate VCH (2 mM). In the absence of the epoxide hydrolase inhibitor only VCH-1,2-diol and VCH-7,8-diol were detected in microsomes incubated with VCH indicating that the epoxides produced by the reaction had been hydrolyzed to their respective diols. The microsomal metabolism of VCH was studied by Gervasi et. al. (1980) in hepatic microsomes obtained from male mice pretreated with phenobarbital, an inducer of certain cytochrome P450 forms. In their study a time course experiment was performed to allow detection of various microsomal metabolic intermediates of VCH. VCH-1,2-epoxide increased in microsomal incubations with VCH up to 5 minutes. After this time VCH-1,2-diol and VCH-1,2,7,8-tetraol were the predominant metabolites. Interestingly, these authors reported the production of VCH diepoxide as a metabolite of VCH-1,2-epoxide and to a lesser extent VCH. The most consistent detection of this metabolite occurred in the presence of TCPO.

Testi and his colleagues (1982) measured the destruction of the hepatic microsomal mixed function oxidase cytochrome P450 by VCH using microsomes from phenobarbital-treated male mice. Cytochrome P450 loss was monitored by measuring the

amount of spectral heme remaining after incubations with VCH, microsomes, and NADPH. VCH, but not a structurally similar analog cyclohexene, destroyed cytochrome P450. This suggested that the presence of the vinyl group of VCH was necessary for VCH to destroy this enzyme. Furthermore, the studies suggested that metabolism of VCH to a reactive intermediate was required for the heme destruction. Destruction of P450 did not occur in incubations with VCH-7,8-epoxide indicating that the reactive intermediate was not that epoxide.

These studies show that VCH can be metabolized to several oxygenated metabolites in rat and mouse hepatic microsomes. Apparently, the VCH metabolites formed are conserved between the mouse and rat perhaps with the exception of the diepoxide. Most interesting metabolites of VCH are the three epoxy compounds since aliphatic epoxides are often reactive electrophilic compounds capable of alkylating DNA. Many reactive electrophiles are mutagens and carcinogens (Miller and Miller, 1977).

Genetic Toxicology of VCH and VCH Metabolites

A high correlation exists between chemical carcinogens and chemical mutagens. Scientists have exploited this association to develop rapid and sensitive assays to detect mutagenic and potentially carcinogenic chemicals. Both bacterial and mammalian cells have been employed in these

assays. Point mutations and frameshift mutations can be detected in bacterial cells. Mammalian cells have the added advantage that these cells can be used to assess chemicals which produce chromosomal damage. To improve detection of carcinogenic chemicals which require bioactivation liver subcellular fractions can be added to provide a source of biotransforming enzymes (Ames et al., 1975).

The mutagenicity of VCH and VCH metabolites have been investigated using several assays. VCH was not mutagenic when incubated with or without Aroclor 1254 induced male Sprague-Dawley rat or male hamster hepatic S-9 fraction prior to addition to several different *S. typhimurium* strains (NTP, 1986 and Watabe et al., 1981). However, the epoxide metabolites of VCH have shown some activity in mutagenicity assays. VCH-1,2-epoxide was found to be a point mutagen in the Ames/Salmonella assay (Simmon and Baden, 1980). The high cytotoxicity of VCH-1,2-epoxide and VCH-7,8-epoxide has precluded adequate mutagenicity studies by other investigators (Watabe et al., 1981; Turchi et al., 1981; and Gervasi et al, 1981). Turchi et al. (1981) used *S. typhimurium* and V79 chinese hamster cells to assess the ability of VCH metabolites to induce both point mutations and chromosomal aberrations. VCH-1,2-epoxide did not induce point mutations in either cell system. However, this metabolite did produce produce chromosomal damage in the form of anaphase bridges and

micronuclei in V79 cells. Interestingly, VCD is consistently mutagenic when tested by a number of different laboratories in both bacterial and mammalian cells. VCD is capable of producing point mutations but not frameshift mutations in *S. typhimurium* (Watabe et al., 1981; Wade et al., 1979; Turchi et al., 1981; Simmon and Baden, 1980; Mortelmans et al., 1986; Voogd et al., 1981; Murray and Cummins, 1979; and El-Tantawy and Hammock, 1980). Gervasi et al. (1981) and Turchi et al. (1981) both found that point mutations were induced in V79 hamster cells by VCD. Furthermore, Gervasi and coworkers (1981) showed that VCD also produced anaphase bridges and the formation of micronuclei.

The above results suggest that epoxide metabolites of VCH are mutagenic. These studies suggest that the metabolism of VCH to epoxides may be important in VCH-induced ovarian carcinogenicity. VCH may not be mutagenic even when preincubated in the presence of liver S-9 fraction because the rate VCH epoxidation is insufficient to produce the concentrations of epoxides necessary to produce mutations. An alternative explanation is that the epoxides are rapidly hydrolyzed to the dihydrodiols by epoxide hydrolase present in the microsomal fraction. This explanation is supported by studies which showed that the mutagenic activity of three epoxides (allylbenzene oxide, styrene oxide, and 4-chlorophenyl glycidyl ether) was greatly reduced by the

addition of hepatic microsomes (El-Tantawy and Hammock, 1980). Therefore, it is not surprising that VCH is not mutagenic even in the presence of biotransforming enzymes.

Ovarian Tumor Induction in Rodents

The mechanism(s) by which chemicals induce ovarian cancers is/are unknown. However, it is likely that the sequence of events is similar to that of other chemically induced cancers. This would follow the pattern of initiation-promotion-progression. Summarized below are studies which describe the events which occur during these three phases. The destruction of oocytes by chemicals occurs early and is probably part of the the initiation phase. The initiated cells within the ovary are promoted through stimulation by gonadotropins which are secreted by the pituitary at continuously elevated levels in oocyte depleted animals. Eventually neoplasms form and can progress from tumors to carcinomas. Examining the pathogenesis of treatment-induced ovarian tumors can provide insight into the possible mechanism(s) of VCH-induced ovarian neoplasms.

Procedures to induce ovarian tumors

Several methods have been used to study the pathogenesis of ovarian tumor induction including the use of strains of animals susceptible to spontaneous tumors, surgical procedures, alteration of environmental factors, and exposure

to physical and chemical carcinogens (Jull, 1973 and Marchant, 1987). These methods may share common mechanism(s) of tumor induction with VCH and are useful in understanding how VCH may induce ovarian neoplasms.

Mice (C57BL/6J X C3H/HeJ F₁ W^x/W^v) which carry a particular mutant gene have several abnormalities including increased susceptibility to the induction of spontaneous ovarian tumors (Russell and Fekete, 1968). These mice are born with less than 1% of the normal number of oocytes because during fetal life the primordial germ cells fail to divide to produce the large pool of primordial oocytes normally present. Since the oocyte is a nondividing cell type, they are never replenished. In these animals, gonadotropins are elevated and by seven months of age bilateral tubular adenomas develop (Murphy and Beamer, 1973 and Murphy, 1972). These tumors can subsequently form granulosa cell tumors later in life.

Surgical procedures disrupting normal ovarian development or function can result in the ultimate formation of neoplasia. Neonatal thymectomy of mice performed before day four of age leads to arrest in ovarian growth, oocyte depletion, early sterility and development of tubular adenomas and granulosa cell tumors (Nishizuka and Sakakura, 1971 and Nishizuka et al., 1972). This presumably occurs by an autoimmune mechanism (Nishizuka et al., 1979). Ovarian neoplasia also has been induced following surgery of the ovary itself. In rats, a 30

minute interruption of the blood supply to the ovary establishes a state of constant estrous. After 20 months ovarian tumors were observed in these animals (Marchant, 1987). Transplantation of the ovary to the spleen of castrated female animals induces a high incidence of ovarian tumor formation (Furth and Soble, 1947 and Biskind and Biskind, 1949). Splenic venous blood empties into the portal circulation of the liver resulting in inactivation of the estrogen and progestins produced by the ovary. The blood levels of ovarian steroids is then insufficient to produce the normal negative feedback on the pituitary secretion of gonadotropins (Lee et al., 1975). It is thought that the increased circulating gonadotropins acts as a promoting event for ovarian neoplasia. It follows that tumorigenesis in this model can be inhibited by systemic administration of estrogens or androgens or by allowing one ovary to remain in situ (Biskind et al., 1950 and Li and Gardner, 1949).

Exposure of mice to constant bright illumination causes a continuous estrous (Murthy and Russfield, 1970). After 10 months of exposure the ovaries of mice contained cystic follicles, preneoplastic germinal epithelial changes, and occasional tumors. Similar changes have also been observed in rats exposed to constant illumination (Singh, 1969).

By far, the majority of studies have utilized physical or chemical carcinogen exposure to induce ovarian tumors. In

1936, Furth and Butterworth reported the tumorigenic effect of X-rays on the ovary. Furth and Boon (1947) showed that there was a dose-response relationship between the dose of X-rays and incidence of ovarian tumors in mice. Elegant studies have shown that irradiation of the ovary and not the whole animal is required to induce ovarian tumors. Lick et al. (1949) showed that if the ovary was withdrawn from the peritoneal cavity and irradiated while the animal's body was protected by lead shielding ovarian tumors were induced. However, if the opposite experiment was performed and the animal was irradiated and the ovary was protected no ovarian tumors were induced (Deringer et al., 1955).

In 1954 the tumorigenic potential of the polycyclic aromatic hydrocarbon 7,12-dimethylbenzanthracene (DMBA) was reported by Marchant et al. and Howell et al. These investigators found that repeated applications of DMBA to the skin resulted in a high incidence of ovarian granulosa cell tumors. Subsequent studies have shown that the administration of DMBA by oral (Biancifiori et al., 1961; Jull et al., 1966; Krarup, 1967), intraperitoneal (Krarup, 1967), intravenous (Jull, 1969), or subcutaneous routes (Shisa and Nishizuka, 1968) will cause ovarian tumors in mice. Even direct application of DMBA to the ovary has induced tumors (Krarup, 1969). Studies comparing the potency of different polycyclic aromatic hydrocarbons (PAH) to induce ovarian tumors show that

DMBA > 3-methylnanthracene > benzo(a)pyrene (Biancifiori et al., 1961; Mody, 1960).

Oocyte destruction by ovarian carcinogens

A consistent finding among all treatments which induce ovarian tumors is the rapid loss of oocytes. The small or primordial oocytes are the most sensitive cells in the ovary to the ovotoxic effects of ionizing radiation or chemical carcinogens (Jull, 1973). The toxic effects of ionizing radiation on the ovary have been extensively reviewed by Baker (1971 and 1985). The small oocytes of female rats and mice are surprisingly radiosensitive through the entire lifespan (i.e. from fetal development to adulthood). Factors affecting sensitivity of oocytes to radiation include the species, strain, age, stage of development of the oocyte and chromosome configuration. X-irradiation of day 10 fetal mouse ovaries results in a rapid reduction of oogonia. Since oogonia are capable of cell division, many of the destroyed cells can be replaced. However, cell division ceases in fetal mouse oogonia as they progress to oocytes at day 15.5. Exposure to x-rays after this time results in destruction of oocytes with no restoration. The highest sensitivity of mouse oocytes to destruction by x-irradiation occurs between 2-4 weeks of age. Primordial oocytes are more sensitive than the oocytes contained within growing and large follicles to the destructive effects of x-rays and appropriate doses can

completely eliminate primordial oocytes from the ovary by 24 hr after exposure. Mice are more sensitive than rats to the ovotoxic effects of x-rays (Baker, 1971). With increasing age the sensitivity of oocytes to radiation decreases.

Studies with ovarian cancer-causing chemicals have shown that destruction of oocytes also precedes the formation of tumors. The ovotoxicity of PAH have been extensively studied and reviewed by Mattison (1983). Factors affecting oocyte destruction by PAH include the species, strain, and age of the animal, as well as the stage of development of the oocyte and the route of administration of the toxicant. When PAH are injected into mice, oocytes are destroyed in a dose and time dependent fashion (Mattison and Thorgeirsson, 1979). As shown for radiation, primordial follicle destruction occurs at lower doses of PAH than does the destruction of growing or large follicles. Furthermore, mice are usually more sensitive to the effects of ovarian toxicants than are rats. The rank order of potency of DMBA, 3-methylcholanthrene, and benzo(a)pyrene to destroy oocytes is identical to that which induces ovarian tumors. Studies with 3-methylcholanthrene suggest that the intraperitoneal route is the most ovotoxic route of administration for PAH (Dobson and Felton, 1983).

Dobson and Felton (1983) have studied primordial oocyte destruction by a wide variety of chemicals. Of the 77 chemicals tested for ovarian toxicity, 21 were scored as

positive. An interesting finding in their study was that the 21 chemicals which were ovotoxic were also known mutagens or carcinogens. These compounds were either chemically reactive compounds such as epoxides, or compounds that were bioactivated to reactive chemicals by the whole animal.

The long latency period required for tumor induction makes it an inconvenient marker for mechanistic studies. However, since the induction of ovarian tumors by carcinogenic treatments involves the destruction of oocytes early in the pathogenesis of this disease, loss of oocytes can be used to predict the carcinogenic potential of chemicals and factors which affect this potential.

Statement of Problem

The basis for the species difference in ovarian tumorigenicity following chronic VCH treatment in rats and mice is unknown. Understanding the reason for this species difference is important, especially for compounds such as VCH which are carcinogenic in only one of two test species. Clearly, more information is needed to determine which species would best predict the toxicity of VCH in humans. An understanding of how VCH treatment causes ovarian tumors in animals could improve the assessment of the sensitivity of humans to VCH toxicity/carcinogenicity.

Objectives

The objective of this research was to determine why chronic VCH treatment induces ovarian tumors in mice, why the rat is resistant to this effect, and which species may best predict the sensitivity of humans to VCH toxicity. The second chapter of this dissertation compares the disposition of a single oral dose of [^{14}C]VCH (400 mg/kg) in the rat and mouse. These experiments were performed to determine if the disposition of VCH was dramatically different between rats and mice. Chapter three explores the role of oocyte destruction in the induction of ovarian tumors by VCH. Unquestionably, oocyte destruction is a critical event in the pathogenesis of carcinogen induced ovarian tumors. A difference in ability of VCH to destroy oocytes between rats and mice could explain the susceptibility of the mouse and resistance of the rat to VCH-induced ovarian tumors. Additional studies were included to determine if bioactivation of VCH to mutagenic and potentially ovotoxic epoxides was necessary for this compound to be toxic. The fourth chapter discusses the identification of the major hepatic cytochrome P450 forms involved in VCH epoxidation. This would show if the enzymes which metabolize VCH by an ovotoxic epoxidation pathway are low or absent in the rat. Finally, chapter five examines the epoxidation of VCH by hepatic microsomes obtained from humans. These studies will aid in the determination of the susceptibility of humans to VCH toxicity.

CHAPTER 2

COMPARISON OF THE DISPOSITION AND IN VITRO METABOLISM
OF 4-VINYLCYCLOHEXENE IN THE MOUSE AND RAT

ABSTRACT

4-Vinylcyclohexene (VCH) is a chemical to which humans are exposed in the rubber industry. A chronic carcinogenicity bioassay conducted by the National Toxicology Program showed that oral administration of VCH induced tumors in the ovaries of mice but not rats. The hypothesis tested was that the species and organ specificity of VCH toxicity was due to differences in the disposition of VCH between the female rat and mouse. Therefore, the disposition of a single oral dose of 400 mg/kg [^{14}C]VCH was studied in female B6C3F1 mice and Fischer 344 rats. Mice eliminated > 95% of the dose in 24 hr, whereas, rats required 48 hr to eliminate > 95% of the dose. The major routes of excretion of [^{14}C]VCH-derived radioactivity were in the urine (50-60 %) and expired air (30-40 %). No evidence was obtained to indicate that the ovaries of either species retained VCH as parent compound or as radioactive equivalents. A dramatic difference was observed between the rat and mouse in the appearance of the monoepoxides of VCH in blood from 0.5 to 6 hr after VCH administration (800 mg/kg ip). VCH-1,2-epoxide was present in the blood of mice with the highest concentration at 2 hr (41 nmol/ml). The blood concentration of VCH-1,2-epoxide in rats was < 2.5 nmol/ml at all times examined. VCH-7,8-epoxide was not present in the blood of either species at the level

of detection. These findings were supported by in vitro studies of VCH epoxidation in hepatic microsomes. The rate of epoxidation of VCH (1 mM) to VCH-1,2-epoxide was 6.5 fold greater in mouse hepatic microsomes compared to rat hepatic microsomes. The species difference in the rate of epoxide formation by the liver may be an important factor in the species difference in susceptibility to VCH-induced ovarian tumors.

INTRODUCTION

4-Vinylcyclohexene (VCH) is a colorless liquid with a strong characteristic odor. Workers in the rubber industry are exposed to VCH by inhalation (Rappaport and Fraser, 1977). During the production of synthetic rubber, VCH is formed by a dimerization reaction of 1,3-butadiene (Rappaport and Fraser, 1976). This occurs primarily when the rubber is curing. Furthermore, exposure of workers to VCH occurs when VCH is used as an intermediate in the production of VCH diepoxide, a reactive diluent in epoxy resin manufacture (IARC, 1986).

The toxicology of VCH was investigated in B6C3F1 mice and Fischer 344 rats by the National Toxicology Program (Collins and Manus, 1987 and Collins et. al., 1987). Acute (14 day) oral studies revealed no gross or histopathologic effects related to VCH treatment. However, most of the mice and all rats died when the dose was ≥ 1250 mg/kg. Histopathologic effects observed in 13 week oral studies included a dose dependent hyaline droplet degeneration of the proximal tubule of male rats and a reduction in the number of primary and mature follicles in the ovaries of female mice receiving 1200 mg/kg VCH. In the 2 year oral carcinogenicity studies the most significant finding was that female mice receiving 200 mg/kg or 400 mg/kg VCH developed rare ovarian neoplasms.

Although survival was poor in dosed female rats, those surviving the entire 2 years had no apparent ovarian pathology (NTP, 1986).

The following study was undertaken to determine if a species difference in the disposition of VCH (400 mg/kg) existed between the rat and mouse following a single oral dose. A species difference in the disposition of VCH could explain in part the species difference in susceptibility to VCH induced ovarian tumors.

MATERIALS AND METHODS

Animals. Female B6C3F₁ mice (17-23g) and female Fischer 344 rats (175-250g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals were allowed free access to food (Wayne Lab Blox; Chicago, IL) and water. The animals were maintained on a 12 hr light/dark cycle and acclimated to this environment for at least 7 days prior to use in the disposition studies.

Chemicals. [Ethylene ¹⁴C] 4-Vinylcyclohexene, sp act 11.6 mCi/mmol was obtained from New England Nuclear (Boston, MA). The radiochemical purity was 98% as determined by gas-liquid chromatography mass spectrometry. Unlabeled 4-vinylcyclohexene (99%) containing t-butylcatechol as an antioxidant was purchased from Aldrich Chemical Co. (Milwaukee, WI). VCH-7,8-epoxide was synthesized by the

method of Watabe et al. (1981). Nuclear magnetic resonance spectroscopy was used to confirm the structure of VCH-7,8-epoxide.

Disposition studies. Animals were fasted overnight and treated between 0700 and 1100 hr. [^{14}C]VCH dissolved in corn oil was administered by gavage at a dose of 400 mg/kg (4-45 uCi/mouse, 4.8 ml/kg; 4-80 uCi/rat, 4.5 ml/kg). The amount of radioactivity administered to the animals was varied to maximize detection of [^{14}C]VCH-equivalents in the ovary at later time points. After dosing, the animals were transferred to glass metabolism cages for the separate collection of urine, feces, and expired air. At selected times the animals were killed by carbon dioxide asphyxiation. The major tissues were obtained by necropsy immediately after the animals were killed. These tissues were weighed and stored at -20°C until assayed. Duplicate tissue aliquots (50 - 150 mg) were combusted to $^{14}\text{CO}_2$ using a Packard Model 306 Sample Oxidizer (United Technologies; Downers Grove, IL). The $^{14}\text{CO}_2$ collected was counted using a Beckman LS 2800 liquid scintillation spectrophotometer (Irvine, CA). Muscle, blood, skin, and adipose tissue were estimated as 45%, 7.6%, 14.5%, and 9.8% of total body weight in mice (Tuey and Matthews, 1980) and 50%, 9%, 16%, and 7% of total body weight in rats (Matthews and Anderson, 1975), respectively. Aliquots of urine (0.1 ml) were directly counted for radioactivity. Feces were

collected, weighed, and digested with 5 ml/g 1 M potassium hydroxide. Samples (0.5 ml) of the fecal digest were dried, combusted, and counted for radioactivity as described for tissues. Expired organically soluble [^{14}C]VCH-equivalents were collected by drawing cage air through a bubble trap containing 2-methoxyethylether. The bubble trap was maintained in an ice slush. Radioactivity present in the organic trap was determined by directly counting 1.0 ml of the solvent. In addition, traps containing 2:1 carbosorb:ethylene glycol were connected in tandem after the organic traps to collect expired $^{14}\text{CO}_2$.

Determination of unmetabolized VCH in selected tissues of mice and rats dosed with unlabeled VCH (400 mg/kg, po). Animals were dosed orally with VCH (400 mg/kg) and experiments performed as described for the radiolabeled compound. An exception was that excreta was not collected in these studies. Tissues were chosen based on the findings of the radiolabeled studies. Tissue samples (blood, muscle, skin, adipose, and ovary) were immediately frozen in liquid nitrogen and stored on dry ice until processing. This was to prevent the loss of volatile parent compound. Tissue aliquots (0.3 - 1.0 g) were added to polypropylene tubes containing 2 ml of a 1:1 methanol:potassium phosphate buffer (40 mM, pH 7.4) at 4°C. The mixture was homogenized using a Polytron Homogenizer (Brinkmann Instruments; Westbury, NY) with a speed control

setting of 5. There were some exceptions to this protocol. Blood was not homogenized. Skin was placed in a stainless steel cylinder containing 30 ml of liquid nitrogen. After the liquid nitrogen evaporated, a fitted stainless steel piston was placed inside the cylinder and the sample pulverized by striking the piston with a sledge hammer. The frozen skin powder was then homogenized as described above. The ovaries were placed in a microcentrifuge tube containing 0.2 ml of the methanol:buffer mixture. The tissue was homogenized using a fitted pestle.

Two milliliters (0.2 ml for ovarian homogenates) of hexane containing decane as an internal standard was added to the homogenates. The tubes were shaken for 5 min and the phases separated by centrifugation (1500 x g for 10 min). The organic phase was transferred to glass crimp top vials and stored at -20°C until assayed. Tissue homogenates spiked with [¹⁴C]VCH and extracted with hexane indicated that the extraction efficiency for blood, muscle, adipose, and skin was 82%, 84%, 89%, and 80%, respectively. The coefficient of variation was $\leq 5\%$.

Analysis of hexane extracts for VCH. VCH present in the hexane extracts was quantified by capillary gas-liquid chromatography using a Hewlett-Packard HP 5890A gas chromatograph (Palo Alto, CA) fitted with a 0.32 mm x 10 M RSL-300 capillary column (Altec Associates; Deerfield, IL) and

a flame ionization detector (FID). The N₂ carrier gas flow rate was 1 ml/min with a split vent flow rate of 10 ml/min. The FID gas flow rates for H₂, N₂, and air were 30 ml/min, 30 ml/min, and 240 ml/min, respectively. The respective injector, oven, and detector temperatures were 100°C, 40°C, and 300°C. The retention time of VCH was 2.7 min while decane eluted at 6.0 min. VCH was quantified by comparing the peak area ratio of VCH and decane to a standard curve prepared by analyzing hexane extracts from tissue homogenates spiked with known amounts of VCH.

Studies of VCH-1,2-epoxide blood concentrations in vivo:
Species comparison and time-response. Animals were given a single intraperitoneal dose of VCH (800 mg/kg) in corn oil (2.5 ml/kg). The animals were killed by CO₂ asphyxiation at 0.5, 1, 2, 4, and 6 hr after VCH administration. Blood was drawn into heparinized plastic syringes by cardiac puncture. Blood (0.5 ml) was dispensed into a screw-top glass vial and extracted with 0.25 ml hexane containing cis-cyclodecene as the internal standard. Vials were shaken for 5 min and the phases separated by centrifugation (1500 X g for 10 min). The hexane phase was removed and sealed in a 300 ul crimp-top vial. The hexane extract was analyzed for VCH-1,2-epoxide and VCH-7,8-epoxide by capillary gas liquid chromatography.

Capillary gas-liquid chromatographic conditions for VCH-1,2-epoxide analysis. Analyses were performed on a Hewlett-

Packard HP 5890 A gas chromatograph equipped with a 0.32 mm X 25 M RSL-300 capillary column (Altec Associates, Dearfield, IL) and a flame ionization detector. The nitrogen carrier gas flow rate was 2 ml/min with a split flow rate of 30 ml/min. The detector gas flow rates were as stated above for VCH analysis. The injection temperature was 200°C with a detector temperature of 300°C. The oven temperature for epoxide analysis was held isothermally at 100°C. The retention times of VCH-1,2-epoxide, VCH-7,8-epoxide and cis-cyclodecene were 4 min, 5 min and 5.5 min, respectively. VCH-1,2-epoxide and VCH-7,8-epoxide were quantified by comparing the peak area ratio of VCH-1,2-epoxide or VCH-7,8-epoxide and cis-cyclodecene to a standard curve prepared by analyzing hexane extracts from blood samples spiked with known amounts of the respective VCH epoxides.

Studies of VCH metabolism in vitro: Preparation of microsomes. Livers were removed and microsomes prepared by ultracentrifugation as described by Halpert et al. (1983). Protein concentrations were determined by the by the method of Lowry et al. (1951). Microsomal cytochrome P-450 content was determined by the method of Omura and Sato (1964).

Studies of VCH metabolism in vitro: Microsomal incubation conditions. Microsomal incubations contained microsomal protein (0.5 mg/ml), NADP⁺ (0.5 mM), glucose-6-phosphate dehydrogenase (2 units), glucose-6-phosphate (10

mM), MgCl_2 (15 mM), EDTA (0.1 mM), VCH (1 mM) in methanol 1% v/v for the total incubation, HEPES (0.05 M, pH 7.5), to a final volume of 2 ml. Studies were performed to define the assay conditions such that metabolite production was linear with protein concentration and time. Incubations were performed in the presence of 3,3,3-trichloropropene oxide (2 mM) a potent inhibitor of microsomal epoxide hydrolase catalyzed VCH-1,2-epoxide hydrolysis (Watabe et al., 1981). Screw cap vials containing the microsomal suspension were preincubated at 37°C for 3 min and the reaction started by the addition of glucose-6-phosphate. Glucose-6-phosphate was absent from blank incubations. The reaction proceeded for 5 min and was terminated by the addition of 0.2 volumes of 5 M NaOH. Incubations were extracted with 0.2 volumes of 19:1 hexane:ethanol containing cis-cyclodecene as the internal standard, vortexed, shaken for 5 min, and the phases separated by centrifugation. The organic layer was removed and analyzed for VCH-1,2-epoxide by capillary gas-liquid chromatography using the conditions as described for blood.

Statistical analysis. Two sample inference was made using Student's t-test. Means were considered significantly different at $p < 0.05$.

RESULTS

Excretion of [^{14}C]VCH-equivalents. The fate of [^{14}C]VCH-

derived radioactivity after oral administration of VCH to mice and rats is shown in Table III. By 24 hr mice and rats eliminated 97% and 88% of the dose, respectively. By 48 hr rats had eliminated 100% of the dose. In both species urine was the major route of excretion with 50-60% of the administered dose eliminated by this route. An important secondary route of elimination of VCH-derived radioactivity was by expiration. Rats and mice expired approximately one-third of the administered dose collected as expired organics. This was most likely parent compound due to the high volatility of VCH compared to epoxide metabolites. In both species negligible amounts of radioactivity were collected in the CO₂ trap indicating that [¹⁴C]VCH was not metabolized to ¹⁴CO₂. Excretion of [¹⁴C]VCH-derived radioactivity in the feces was a minor route of excretion in both species. Figure 1 shows the cumulative excretion of [¹⁴C]VCH-derived radioactivity in the urine and expired air. The majority of [¹⁴C]VCH-derived radioactivity excreted in the urine of mice and rats occurred by 24 hr. Radioactivity collected in the expired organic trap was essentially all collected by 8 hr after administration.

Tissue distribution: [¹⁴C]VCH-equivalents. By 24 hr after [¹⁴C]VCH administration to mice no tissue contained more than 1% of the dose. In rats, adipose, muscle, and skin contained 3.4%, 1.1%, and 1.1% of the dose at 24 hr after dosing. These

Table III.
Excretion of Radioactivity from Female Rats and Mice
Following Oral Administration of [^{14}C]4-Vinylcyclohexene

	Percentage total dose ^a	
	Rat	Mouse
Time (hr)	48	24
Urine	52.1 \pm 1.1 ^b	57.7 \pm 7.8
Expired Organics	36.0 \pm 2.1	31.4 \pm 1.8
Feces	9.6 \pm 5.1	3.1 \pm 2.9
Tissues	2.4 \pm 0.9	1.8 \pm 1.4
Cage Wash	0.6 \pm 0.1	2.9 \pm 2.6
Recovery	100.7 \pm 6.4	96.9 \pm 3.8

^a 400 mg/kg.

^b Mean \pm standard deviation; N = 3.

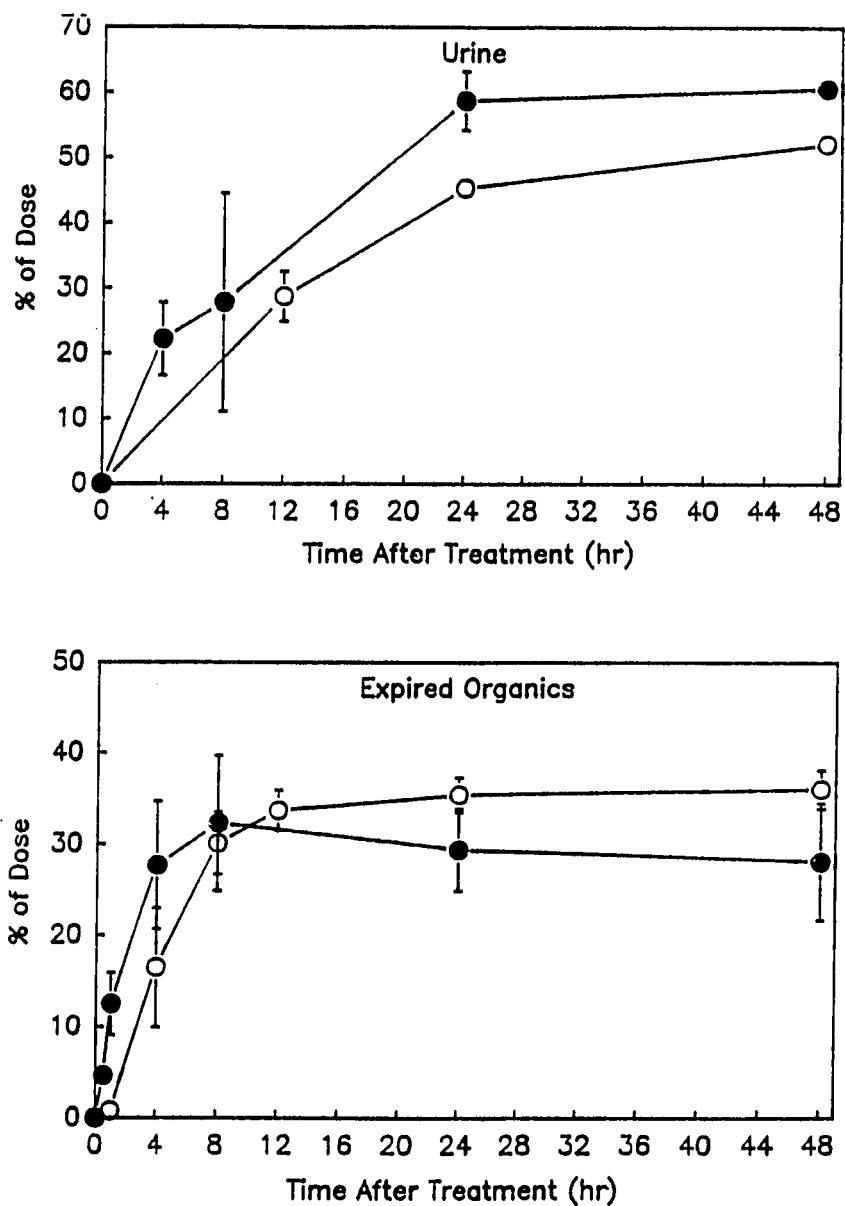


Figure 1. Cumulative excretion of [^{14}C]VCH-derived radioactivity in the urine and expired air of female mice and rats administered [^{14}C]VCH (400 mg/kg po). Rats and mice are represented by open and closed circles, respectively. Each point represents the mean and standard deviation of at least 3 animals.

tissues also contained higher concentrations of [^{14}C] VCH-equivalents per mg of tissue than did the tissues of mice (data not shown). The disposition of [^{14}C]VCH-equivalents in the ovary is presented in Table IV. There were no dramatic differences between the rat and mouse in the disposition of [^{14}C]VCH-equivalents in the ovary. As expected when the data were expressed as the percentage of total radioactivity administered, the amount distributed to the ovaries was negligible (total ^{14}C < 0.05% of the administered dose). However, when the data were expressed as the concentration (nmol [^{14}C]VCH-equivalents/mg tissue) of radioactivity present in the ovaries these values were comparable to liver (eg. 1 hr mouse liver 1.57 nmol [^{14}C]VCH-equivalents/mg tissue; 1 hr mouse ovary 1.10 [^{14}C]VCH-equivalents/mg tissue).

Tissue distribution: Parent compound. The distribution of VCH as parent compound was studied at 1, 2, 4, and 8 hr in the rat and at 1, 2, 4, and 6 hr in the mouse after oral administration. Results are presented in Figure 2. Using the gas chromatographic assay it was not possible to measure VCH in most tissues of mice after 6 hr. The tissue with the highest concentration of VCH in both species was adipose tissue. Interestingly, a different profile of VCH distribution into adipose tissue was observed between the rat and mouse. The highest concentration of VCH in the adipose tissue of mice was between 1 to 2 hr after VCH treatment.

Table IV.
The Distribution of Radioactivity in the Ovaries
of Mice and Rats Orally Administered
[¹⁴C]4-Vinylcyclohexene (400 mg/kg)

Time (hr)	Rat		Mouse	
	% of Dose ^a	nmol/mg ^b	% of Dose	nmol/mg
1	0.010 ± 0.002	0.72 ± 0.09	0.030 ± 0.004	1.10 ± 0.35
4	0.018 ± 0.010	1.28 ± 0.62	0.025 ± 0.014	1.18 ± 0.58
8	0.015 ± 0.003	1.00 ± 0.12	0.008 ± 0.004	0.34 ± 0.22
24	0.003 ± 0.003	0.21 ± 0.09	0.001 ± 0.000	0.08 ± 0.03
48	0.001 ± 0.000	0.07 ± 0.03	0.001 ± 0.000	0.04 ± 0.01

^a Data presented as mean ± standard deviation (N = 3-4).

^b Concentration of radioactivity in nmol [¹⁴C] VCH-equivalents/mg ovarian tissue.

Tissue Distribution of VCH (400 mg/kg p.o.)

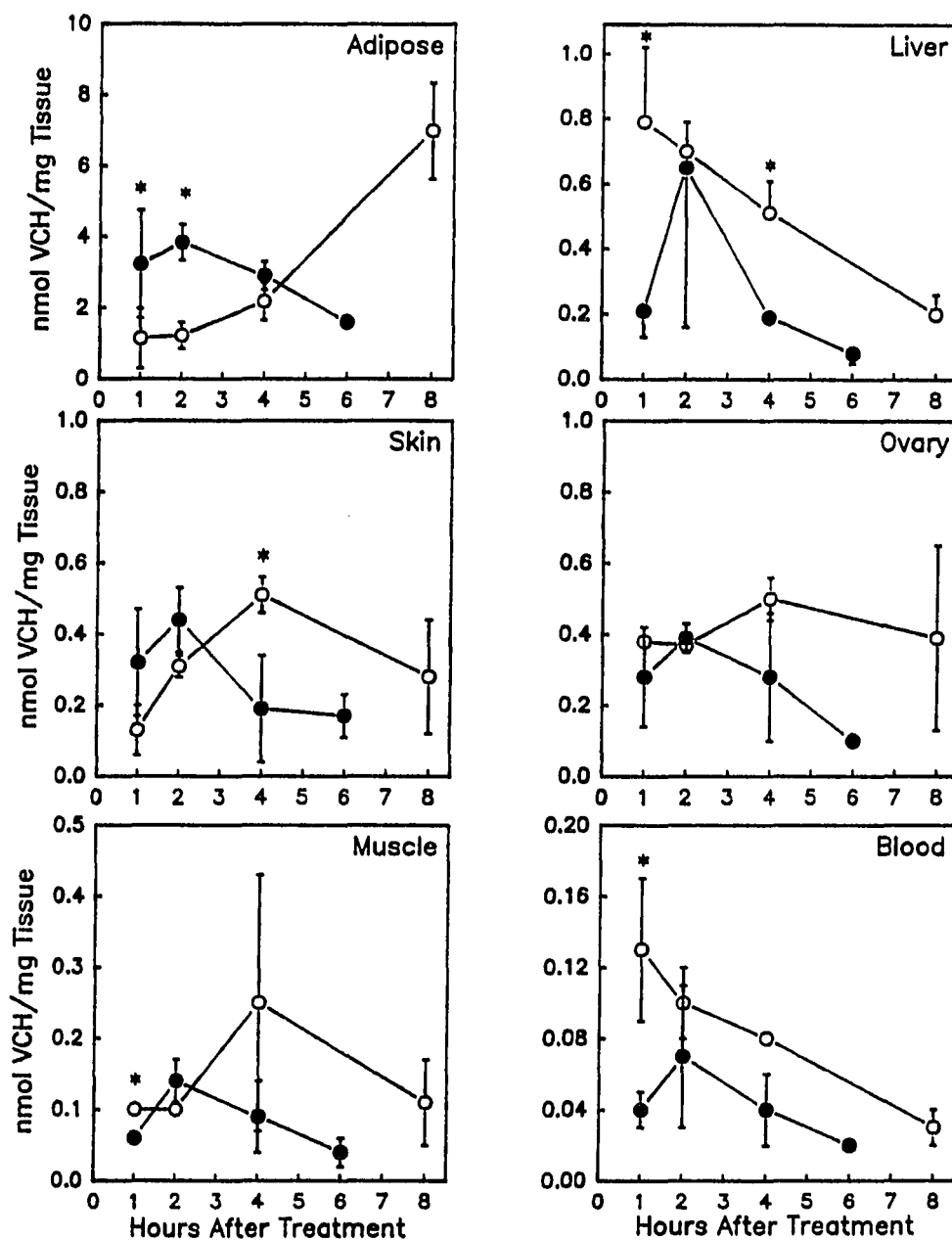


Figure 2. Tissue distribution of VCH (parent compound) in selected tissues of female mice and rats administered VCH (400 mg/kg po). Rats and mice are represented by open and closed circles, respectively. Each point represents the mean and standard deviation of at least 3 animals. An asterisk denotes significant differences ($p < 0.05$) from the complementary time point in the other species.

Rat adipose tissue continued to accumulate VCH until at least 8 hr after dosing. In other tissues examined the trend of the data indicated that the tissue concentrations of VCH were slightly higher in the tissues of rats than the corresponding values in mouse tissues over the times studied. However, because of variability this was confirmed statistically at only a few time points. In the ovary there were no statistical differences in the tissue concentrations of VCH.

Studies of VCH-1,2-epoxide blood concentrations in vivo:
Species comparison and time-response. The concentration-time profile for the appearance of VCH-1,2-epoxide in the blood of mice and rats treated with VCH (800 mg/kg ip) is presented in Figure 3. The highest concentration in mice was 40.7 ± 12.6 nmol/ml of blood, which occurred 2 hr after dosing. VCH-7,8-epoxide was below our level of sensitivity (2.5 nmol/ml) in the blood of mice over the entire 6 hr period. In rats, neither of these metabolites could be measured over this 6 hr period.

Studies of VCH metabolism in vitro. The basis of the species difference in the blood concentration of VCH-1,2-epoxide after VCH treatment was investigated by comparing the in vitro oxidation of VCH to VCH-1,2-epoxide by the hepatic microsomes of female mice and rats. As shown in Table V the rate of VCH epoxidation by mouse hepatic microsomes is 6.5 fold greater than the rate in rat hepatic microsomes. When

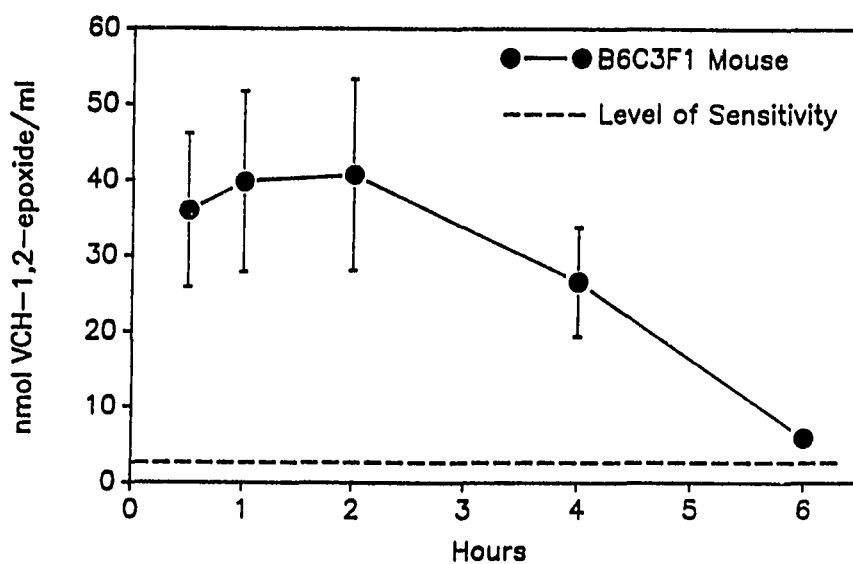


Figure 3. Time course of appearance of VCH-1,2-epoxide in the blood of female mice and rats given VCH (800 mg/kg ip). Each point represents the mean and standard deviation of four animals. VCH-7,8-epoxide was below the level of sensitivity in the blood of mice. Neither epoxide was above the level of sensitivity in the blood of rats.

Table V.
Formation Rates of VCH-1,2-epoxide by Hepatic Microsomes
Incubated with 4-Vinylcyclohexene (1 mM)

Species	nmol P_{450} /mg ^a	nmol/min/mg ^b	nmol/min/nmol P_{450}
Rat	0.87 \pm 0.03 ^c	1.4 \pm 0.04	1.6 \pm 0.07
Mouse	1.38 \pm 0.11 [*]	9.1 \pm 0.70 ^{**}	6.6 \pm 0.25 ^{**}

^a nmol cytochrome P_{450} /mg of microsomal protein.

^b nmol VCH-1,2-epoxide formed/min/mg of microsomal protein.

^c Mean \pm standard deviation (N = 4-5).

Significantly different from rat; * p < 0.05, ** p < 0.002.

the data are expressed as nmole of VCH-1,2-epoxide formed per nmole P450, mouse hepatic microsomes formed 4 times more of this metabolite.

DISCUSSION

The results of the present study indicate that there are no dramatic differences in the disposition of a single oral dose of [^{14}C]VCH (400 mg/kg) between the rat and mouse. Of interest was to compare the disposition of [^{14}C]VCH in the ovary, the target tissue for VCH toxicity. These studies showed that neither [^{14}C]VCH-derived radioactivity nor VCH as parent compound was selectively distributed to the ovaries of either species.

Subtle differences suggested that mice were metabolizing VCH to hydrophilic metabolites more rapidly than rats. For instance, mice eliminated essentially the same percent of dose in the urine in 4-8 hr as did rats in 12 hr (Figure 1). Furthermore, VCH was being eliminated from the adipose tissue of mice at the same time that rat adipose tissue was accumulating VCH (Figure 2). Studies in which rats and mice were administered VCH 800 mg/kg ip showed that mice have an enhanced ability to oxidize VCH to VCH-1,2-epoxide in vivo compared to rats. These studies provided the rationale for directly studying the metabolism of VCH in hepatic microsomes from either species. Other investigators have shown that VCH

is oxidized to epoxides by rat (Watabe et al., 1981) and mouse hepatic microsomes (Gervasi et al., 1980). The results of these investigations indicated that hepatic microsomes obtained from female mice oxidized VCH to VCH-1,2-epoxide more rapidly than hepatic microsomes obtained from female rats. Therefore, the enhanced epoxidation of VCH in vivo in mice is due in part to an increased rate of formation in the liver compared to rats. In mice apparently VCH-1,2-epoxide is formed in the liver and is stable enough to circulate in blood and reach extrahepatic tissues such as the ovary.

The enhanced ability of the mouse to bioactivate VCH may be an important factor in the susceptibility of this species to VCH induced ovarian tumors. Simmon and Baden (1980) showed that VCH-1,2-epoxide was mutagenic in *S. typhimurium*, however, the high cytotoxicity of this epoxide has precluded adequate mutagenicity testing by other investigators (Watabe et al., 1981; Turchi et al., 1981; and Gervasi et al., 1980). Turchi et al. (1981) showed that VCH-1,2-epoxide could produce chromosomal aberrations in chinese hamster V79 cells. If this mutagenic epoxide can reach the ovary in vivo deleterious effects would be expected. Dobson and Felton (1983) have shown that of 77 chemicals tested for oocyte destruction the 21 which were scored as positive were all known mutagens or carcinogens. Many treatments which destroy oocytes also induce ovarian tumors (Jull, 1973). Presented chapter 3 is

evidence which suggests the importance of the species difference in the formation of VCH-1,2-epoxide in vivo. Both VCH and VCH-1,2-epoxide destroy oocytes in mouse ovaries, whereas, only the epoxide appears to be ovotoxic to rats. The dose of VCH-1,2-epoxide required to destroy oocytes is lower than the parent compound which indicates that the biotransformation of VCH to VCH-1,2-epoxide results in a compound of greater ovotoxic potency.

In summary, following a single oral dose of VCH (400 mg/kg) female mice and rats rapidly eliminate VCH by urinary excretion and expiration. VCH is lipophilic and hence achieves the highest tissue concentration in the adipose tissue of both species. It does not appear that the ovary of either species selectively concentrates VCH-equivalents or parent compound after oral administration. In vitro and in vivo studies revealed that female mice have an enhanced ability to metabolize VCH to the more potent ovotoxicant, VCH-1,2-epoxide. This explains, at least in part, the basis of the susceptibility of mice to VCH induced ovarian tumors.

CHAPTER 3

THE ROLE OF EPOXIDATION IN 4-VINYLCYCLOHEXENE-INDUCED
OVARIAN TOXICITY

ABSTRACT

4-Vinylcyclohexene (VCH) is present in gases discharged during synthetic rubber production. Chronic treatment of B6C3F₁ mice and F-344 rats with VCH by gavage has been shown to induce ovarian tumors in mice but not rats. Our objective was to understand the mechanism of the species difference in VCH-induced ovarian tumors. Since a critical step in the induction of ovarian tumors is destruction of small oocytes, small oocyte counts obtained from serially sectioned ovaries were used as an index of toxicity. VCH or its epoxide metabolites [VCH-diepoxide, VCH-1,2-epoxide and VCH-7,8-epoxide (in mice only)] were given to 28 day old female mice and rats in corn oil, ip, at doses ranging from 0.07 to 7.4 mmol/kg body weight/day for 30 days. The dose which reduced the small oocyte count to 50% of control was defined as the ED₅₀. In mice, the ED₅₀ for the reduction in small oocytes by VCH was 2.7 mmol/kg, whereas, no detectable oocyte loss occurred in rats at the highest dose of VCH (7.4 mmol/kg). The potency of the epoxides of VCH was greater than VCH in both species. The ED₅₀ for oocyte loss by VCH-1,2-epoxide in mice and rats was 0.49 and 1.4 mmol/kg, respectively. In mice, VCH-7,8-epoxide had comparable potency to VCH-1,2-epoxide (ED₅₀ 0.72). VCH diepoxide was even more potent with ED₅₀ values of 0.15 and 0.42 mmol/kg, in mice and rats

respectively. The dose-response of the blood concentration of VCH-1,2-epoxide in mice after VCH showed that doses of VCH which caused minimal toxicity had the lowest blood level of this ovotoxic epoxide. Pretreatment of mice with the cytochrome P450 inhibitor chloramphenicol (200 mg/kg, ip) inhibited VCH epoxidation in vivo and in vitro and partially protected mice from VCH toxicity. Thus it appears that metabolism of VCH to epoxides and their subsequent destruction of oocytes are critical steps in VCH induced ovarian tumors. Rats may be resistant to ovarian tumor induction by VCH because the amount of VCH converted to epoxides is insufficient to produce oocyte destruction.

INTRODUCTION

4-Vinylcyclohexene (VCH) induces ovarian tumors when administered by gavage for 2 years to female B6C3F₁ mice (Collins et al., 1987). Interestingly, female Fischer 344 rats surviving identical VCH treatment appear to be highly resistant to tumor induction (NTP, 1986). The mechanism of this species difference is unknown.

Spontaneous ovarian tumors in rodents are rare (NTP, 1986). However, ovarian tumors can be induced by several different techniques including genetic deletion of germ cells, neonatal thymectomy, transplantation of the ovaries to the spleen, exposure to constant bright illumination, and exposure to physical or chemical carcinogens (Marchant, 1987). An early finding which is common among these methods of ovarian tumor induction is ovarian failure brought about by the loss of primordial or small oocytes (Murphy and Beamer, 1973 and Jull, 1973). In rodents with ovaries devoid of oocytes, gonadotropin secretion is increased which promotes ovarian tumor development (Marchant, 1961 and Murphy and Beamer, 1973). Therefore, destruction of oocytes appears to be a critical event in the mechanism of ovarian tumor induction. Dobson and Felton (1983) showed that of 77 compounds tested for ovarian toxicity, the 21 compounds which destroyed mouse oocytes were all known mutagens and carcinogens.

In the preceding chapter the disposition and hepatic microsomal metabolism of VCH were compared in female B6C3F₁ mice and Fischer 344 rats. The results indicated that the major difference in disposition of VCH between these species was the elevated blood concentration of VCH-1,2-epoxide in mice after VCH treatment. This correlated well with increased hepatic microsomal rates of VCH epoxidation in mouse compared to rat microsomes. The metabolism of VCH to epoxides is supported by other investigators who have detected VCH-1,2-epoxide, VCH-7,8-epoxide, and VCH diepoxide (VCD) as metabolites in incubations with rat and mouse hepatic microsomes (Watabe et al., 1981 and Gervasi et al., 1981). The enhanced ability of female mice compared to female rats to convert VCH to epoxides may be of critical importance in determining the species difference in ovarian carcinogenicity. Additional evidence suggesting the importance of biotransformation is that VCH does not appear to be a mutagen (NTP, 1986 and Watabe et al., 1981), whereas, VCH epoxides are mutagenic as determined using a battery of assays (El-Tantawy and Hammock, 1980; Gervasi et al., 1981; Mortelmans et al., 1986; Murray and Cummins, 1979; Simmon and Baden, 1980; Turchi et al., 1981; Voogd et al., 1981; Wade et al., 1979; and Watabe et al., 1981).

The hypothesis tested in these investigations was that the species difference in the incidence of VCH induced ovarian

tumors was related to a species difference in the ability of VCH to destroy oocytes. Due to their mutagenic nature, the ovotoxic potency of the epoxide metabolites of VCH was suspected to be greater than the parent compound. Several studies were performed to prove these hypotheses. First, the ovotoxic potency of VCH and VCH epoxides in the rat and mouse was compared. Second, the blood concentration of VCH-1,2-epoxide was determined in mice after toxic and minimally toxic doses of VCH. Third, the blood-concentration profile of VCH-1,2-epoxide was compared in mice following equitoxic doses of VCH and VCH-1,2-epoxide. Finally, the effect of inhibiting VCH-1,2-epoxide formation in vivo was evaluated as a means of reducing VCH-induced ovarian toxicity in mice.

MATERIALS AND METHODS

Animals. Female B6C3F₁ and Fischer 344 rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) at 21 days of age. The animals were housed in cages with sawdust bedding and had free access to food (Teklad^R, 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 use in toxicity or metabolism studies.

Ovarian toxicity studies: Species comparison. Twenty eight day old female mice and rats were given daily

intraperitoneal (ip) treatments of the test compound dissolved in corn oil (2.5 ml/kg) for 30 days. Doses of 100, 400, and 800 mg/kg (0.9, 3.70, and 7.4 mmol/kg) were selected for VCH based on ovarian toxicity observed in the subchronic NTP study (NTP, 1986 and Collins and Manus, 1987). Since many mutagenic compounds are known to destroy oocytes (Dobson and Felton, 1983), the ovotoxic potency of VCD was suspected to be greater than VCH. Doses of 10, 40, and 80 mg/kg (0.07, 0.29, and 0.57 mmol/kg) of VCD were selected. It was unknown if the ovotoxic potency of the monoepoxides of VCH would be similar to VCH or VCD. Therefore, intermediate doses of 42.5, 170 and 340 mg/kg (0.34, 1.37, and 2.74 mmol/kg) were chosen. The doses expressed as mg/kg were converted to mmol/kg to allow comparison of the potency of VCH and VCH epoxides on an equal molar basis. On day 31 the animals were killed by CO₂ asphyxiation. Their ovaries were removed and fixed in Bouin's solution for 24 hours, transferred to 70% ethanol, and processed for sectioning. Serial sections were prepared at 6 μ m and the sections then stained in hematoxylin and eosin. The oocytes were identified by the method of Pedersen and Peters (1968) and counted in every 20th or 40th section in one randomly selected ovary from mice and rats, respectively. The oocyte counts from individual sections were summed to calculate the total oocyte count for each ovary.

Ovarian toxicity studies: Time-response of VCH in female mice. Female mice were given corn oil 2.5 ml/kg or VCH (800 mg/kg, ip), daily, for 5, 10, 15 or 30 days. Treated and control animals were killed at each time point on the same day and the ovaries removed, processed, and oocytes counted as stated above.

Ovarian toxicity studies: The effect of chloramphenicol pretreatment. Female mice were divided into 4 groups. Each group received two daily intraperitoneal treatments 1 hour apart for 15 days. Group 1 received saline (2.5 ml/kg) followed by corn oil (2.5 ml/kg) 1 hour later. Group 2 received chloramphenicol (200 mg/kg) in saline vehicle (2.5 ml/kg) followed by corn oil 1 hour later. Group 3 received saline followed by VCH (800 mg/kg) 1 hour later. Group 4 received chloramphenicol 200 mg/kg followed by VCH (800 mg/kg) 1 hour later. On day 16 the animals were killed, the ovaries removed, processed, and the oocyte count determined.

Studies of VCH-1,2-epoxide blood concentrations in vivo:
Dose-response. Female mice were given 100, 400, and 800 mg/kg VCH dissolved in corn oil by ip administration. Two hours later the animals were killed, blood drawn by cardiac puncture and analyzed for VCH-1,2-epoxide as described in chapter 2.

Studies of VCH-1,2-epoxide blood concentrations in vivo:
Blood concentration-time profile after administration of

equipotent doses of VCH and VCH-1,2-epoxide. Female mice were given either VCH (2.7 mmol/kg) or VCH-1,2-epoxide (0.49 mmol/kg) by ip administration. The animals were killed at the times indicated in the figure legend, blood drawn by cardiac puncture and analyzed for VCH-1,2-epoxide. The area under the curve (AUC) was estimated graphically using Sigma Scan (Corte Madera, CA).

Studies of VCH-1,2-epoxide blood concentrations in vivo:
The effect of chloramphenicol pretreatment. Female mice were pretreated with saline (2.5 ml/kg) or chloramphenicol (50, 100, 200, 300 mg/kg) ip one hour prior to VCH (800 mg/kg) ip. Two hours later the animals were killed, blood drawn by cardiac puncture and analyzed for VCH-1,2-epoxide.

Studies of VCH metabolism in vitro: **The effect of chloramphenicol pretreatment.** Female mice were administered saline (2.5 ml/kg) or chloramphenicol (200 mg/kg) ip. The animals were killed one hour later, the liver removed, and microsomes prepared (Halpert et al., 1983). Microsomal protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Microsomal cytochrome P450 content was determined spectrally using the method of Omura and Sato (1964). The rate of metabolism of VCH to VCH-1,2-epoxide was determined as described in chapter 2.

Chemicals. 4-Vinylcyclohexene, vinylcyclohexane-1,2-epoxide, vinylcyclohexene diepoxide, and cis-cyclodecene were obtained from Aldrich Chemical Co. (Milwaukee, WI). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, EDTA, bovine serum albumin, and trizma base were purchased from Sigma Chemical Co. (St. Louis, MO). HEPES and magnesium chloride were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Mallinckrodt Inc. (Paris, KY), respectively. VCH-7,8-epoxide was synthesized by the method of Watabe et al. (1981). Nuclear magnetic resonance spectroscopy was used to confirm the structure of VCH-7,8-epoxide. Chloramphenicol sodium succinate for sterile injection was obtained from Park-Davis (Morris Plains, NJ). The doses given were normalized to the mg/kg of chloramphenicol base administered.

Data analysis. The dose-response curves for the various compounds were fitted to a 4 parameter logistic function by nonlinear regression using PC nonlin (Statistical Consultants Inc; Lexington, KY). The equation was as adapted from that described by DeLean et al. (1978).

$$Y = \frac{a - d}{1 + (X/c)^b} + d$$

where

Y = Oocyte #
 X = Dose (mmol/kg/day)
 a = Oocyte # at zero dose
 d = Oocyte # at infinite dose
 c = ED₅₀ (dose reducing the oocyte # to 50% of controls)
 b = Slope

Significant differences between dose-response curves were determined by analyzing the sum of squares of the two data sets separately and as a single pool to calculate an F value (Motulsky and Ransnas, 1987). A students t-test was used to determine significant differences between two group means. Multiple comparisons were made using one-way analysis of variance (ANOVA). When significant differences were detected within the ANOVA individual groups were compared using a Newman-Kuel's range test. The level of significance for all tests was $p < 0.05$.

RESULTS

Ovotoxicity of VCH and VCH epoxides. A comparison of the dose-response relationship of the reduction in small oocyte counts in the ovaries of mice and rats following 30 days of treatment with VCH and the epoxide metabolites of VCH is presented in Figure 4. A dramatic difference in toxicity was observed in the ovaries of mice treated with VCH compared to rats. VCH treatment destroyed small oocytes in the ovaries of mice with dose-dependency. However, this VCH treatment regimen produced no detectable change in oocyte number in the ovaries of rats. Clearly, the epoxides of VCH were much more potent than the parent compound at destroying small oocytes in both species. In both species the dose-response curves of the monoepoxides were significantly different from both VCH

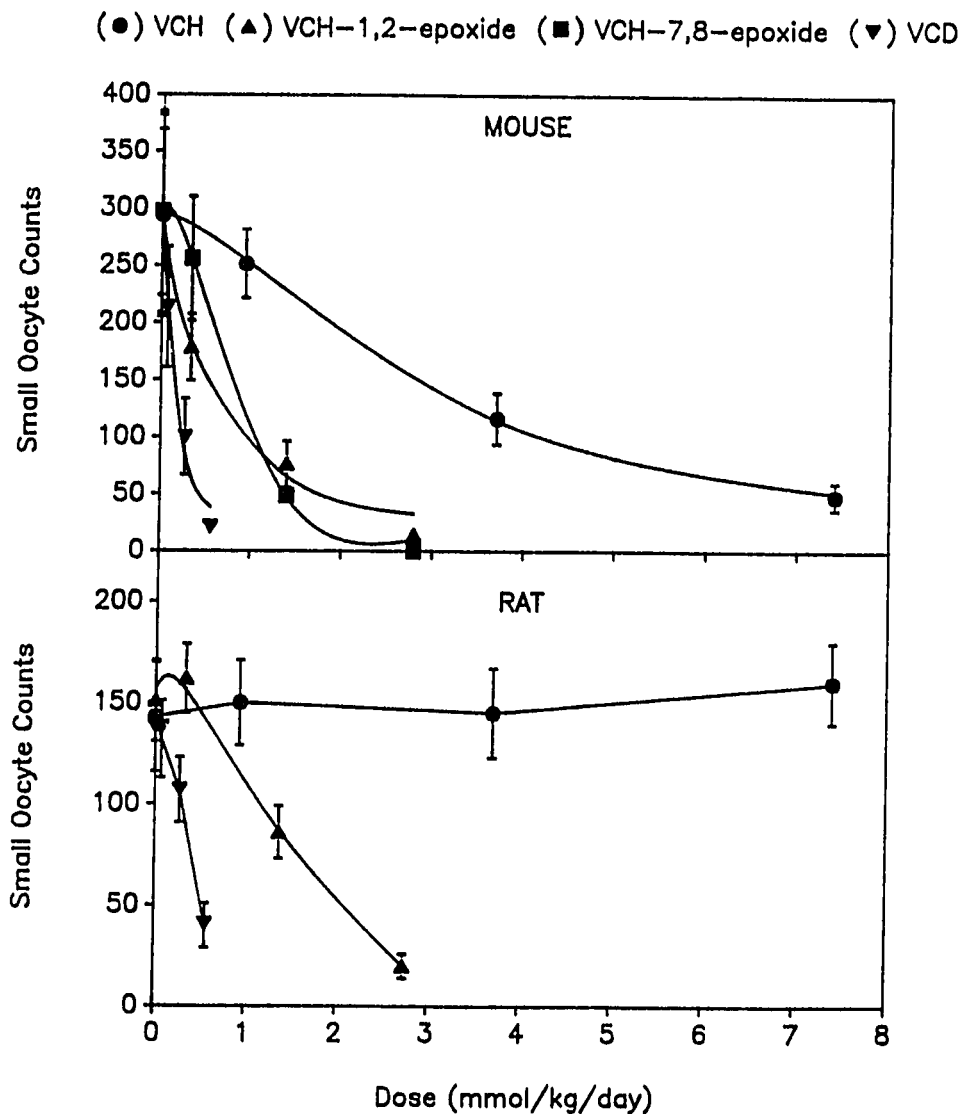


Figure 4. Comparison of the dose-response relationship in the reduction in small oocyte counts in the ovaries of rats and mice treated with VCH or VCH epoxides ip for 30 days. Each point represents the mean of 4-10 animals. The standard deviation is indicated by the bars.

Table VI.

ED₅₀^a Values for the Reduction in Small Oocyte Counts
in Mice and Rats Administered 4-Vinylcyclohexene (VCH)
and VCH epoxides ip for 30 Days

Species	VCH	VCH-1,2-epoxide	VCH-7,8-epoxide	VCD
Mouse	2.73	0.49	0.72	0.15
Rat	> 7.41 ^b	1.41	ND ^c	0.42

^a Dose in mmol/kg/day which reduces the small oocyte count to 50% of that observed in control animals.

^b Highest dose given.

^c Not done.

and VCD as determined by the non-linear regression analysis. The doses of the various compounds which reduced the number of small oocytes to 50% of that observed in control animals (ED_{50}) is presented in Table VI. The ED_{50} of VCH in the mouse was 2.73 mmol/kg (295 mg/kg), a dose 4 and 5 fold greater than the ED_{50} calculated for VCH-7,8-epoxide and VCH-1,2-epoxide, respectively. Even more potent than the monoepoxides was VCD, which had an ED_{50} value 18 fold lower than VCH. Importantly, these data revealed that the rat was sensitive to ovarian toxicity by both VCH-1,2-epoxide and VCD. VCH-7,8-epoxide was not administered to rats because only limited quantities were available.

The destruction of small oocytes of mice by VCH was dependent not only on the dose administered but also on the duration of treatment. The time-response for VCH induced oocyte destruction in female mice is presented in Figure 5. When mice are administered 800 mg/kg of VCH ip daily there was no significant reduction in the number of small oocytes until after 15 days of treatment. The number of small oocytes continued to decline in animals in which VCH treatment continued through day 30.

Studies of VCH-1,2-epoxide blood concentrations in mice.

Two studies were performed in mice to show bioactivation of VCH to VCH-1,2-epoxide in vivo was required to produce ovarian toxicity. The concentration of VCH-1,2-epoxide in the blood

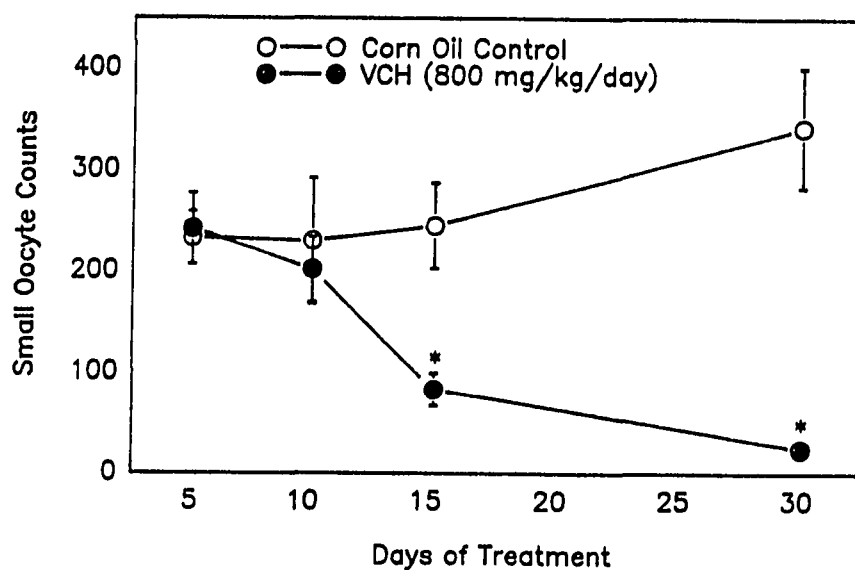


Figure 5. The effect of treatment duration on the reduction in small oocyte counts in mice treated with VCH 800 mg/kg/day, ip. Animals were treated for the number of days indicated in the figure. Each point represents the mean of 5 animals. The standard deviation is indicated by the bars. The asterisk indicates groups which were significantly different from their respective controls ($p < 0.05$).

2 hr after ip treatment of VCH at doses of 100, 400, and 800 mg/kg was 3.5 ± 1.6 , 27.1 ± 3.1 and 42.1 ± 7.6 nmol/ml blood (mean \pm SD; n = 4-5), respectively. Thus, increasing the dose of VCH increased the blood concentration of this ovotoxic epoxide metabolite of VCH. Another approach used was to compare the blood concentration-time profile of VCH-1,2-epoxide after administration of equitoxic doses of either VCH or VCH-1,2-epoxide. As shown in Figure 6 administration of the ED₅₀ doses of VCH or VCH-1,2-epoxide resulted in dramatically different blood concentration-time profiles. From 5 to 15 min after dosing the blood concentration of VCH-1,2-epoxide is much higher in VCH-1,2-epoxide treated mice, whereas, from 30 min to 2 hr after dosing the blood concentration is higher in VCH treated mice. Comparison of the AUC of the blood concentration of VCH-1,2-epoxide for the two treatments as determined from the graph were similar, with 26 nmol/ml * hr and 50 nmol/ml * hr for VCH-1,2-epoxide and VCH treated mice, respectively.

Inhibition of VCH epoxidation and ovotoxicity. Shown in Figure 7 is the effect of a single dose 1 hr ip pretreatment of chloramphenicol pretreatment on the blood concentration of VCH-1,2-epoxide in mice 2 hr after VCH (800 mg/kg, ip). At doses of chloramphenicol greater than 50 mg/kg the blood concentration of VCH-1,2-epoxide decreased. Since the maximal reduction in VCH-1,2-epoxide blood concentration occurred with

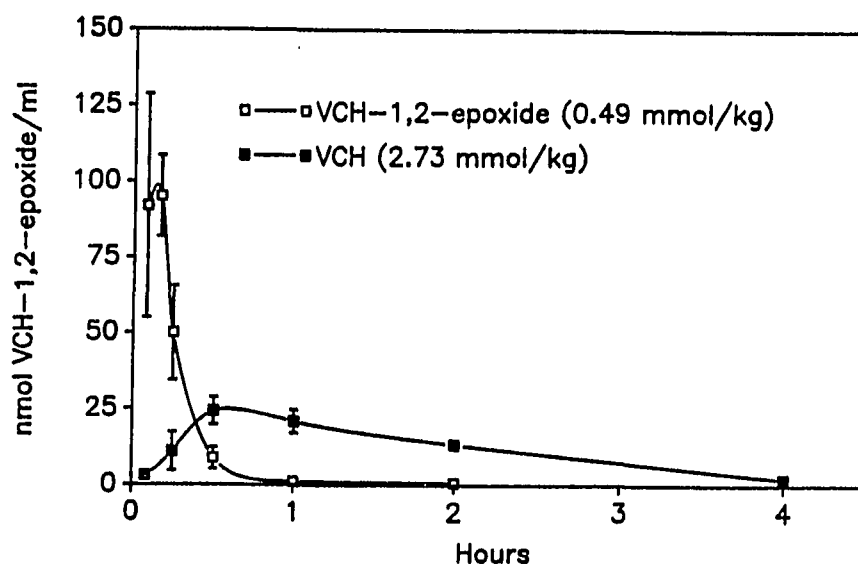


Figure 6. Comparison of the VCH-1,2-epoxide blood concentration-time profile in female mice after ip administration of equitoxic doses of VCH or VCH-1,2-epoxide. Each point represents the mean of 3-4 mice. The standard deviation is indicated by the bars.

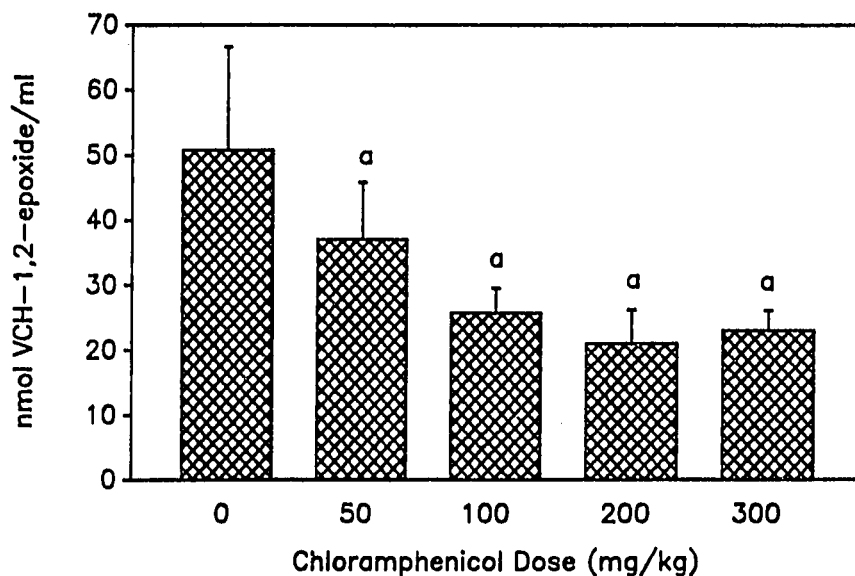


Figure 7. The dose-response relationship of the reduction of VCH-1,2-epoxide blood levels in female mice pretreated ip with selected doses of chloramphenicol 1 hr prior to administration of VCH (800 mg/kg ip). Blood was drawn and analyzed for VCH-1,2-epoxide 2 hours after VCH. Shown is the mean of 4 animals. The standard deviation is represented by the bar. Groups with ^a are not significantly different as determined by ANOVA and a Newman-Kuel's range test ($p < 0.05$).

a one hr ip chloramphenicol pretreatment of 100 to 300 mg/kg, a dose of 200 mg/kg was administered to mice 1 hr prior to the preparation of hepatic microsomes. This pretreatment resulted in a 69% reduction in the rate of metabolism of VCH to VCH-1,2-epoxide (Table VII). Only a slight decrease was observed in the microsomal cytochrome P450 content. A 15 day toxicity study was performed to determine if chloramphenicol treatment could also modulate the ovarian toxicity of VCH 800 mg/kg. The oocyte loss induced by VCH was partially overcome in mice treated with chloramphenicol (Figure 8).

DISCUSSION

The evidence supporting the relationship between oocyte destruction induced by chemical or physical agents and ovarian tumor formation is substantial (Krarup, 1970a,b,c and Jull, 1973). Many known mutagens and carcinogens will destroy primordial oocytes following ip administration (Dobson and Felton, 1983). When VCH is administered to mice ip, daily, for 30 days, small oocytes are destroyed in a dose-dependent fashion. Furthermore, VCH destroyed the small oocytes of mice with time-dependency. Rats, which do not develop ovarian tumors following chronic VCH administration were resistant to oocyte destruction by VCH at the doses and time examined. Thus, there is a strong correlation between VCH-induced oocyte destruction and the subsequent development of ovarian

Table VII.
Effect of ip Chloramphenicol (200 mg/kg) Treatment
on Female Mouse Hepatic Microsomal VCH Epoxidation

Parameter	Saline	Chloramphenicol	
nmol P450/mg ^a	0.88 ± 0.06	0.75 ± 0.06*	(85%) ^c
nmol/min/mg ^b	8.8 ± 1.4	2.7 ± 0.72**	(31%)

^a nmol cytochrome P450/mg microsomal protein.

^b nmol VCH-1,2-epoxide formed/mg microsomal protein/min.

^c Percent of saline control.

Significantly different from saline group;

* p < 0.05, ** p < 0.001.

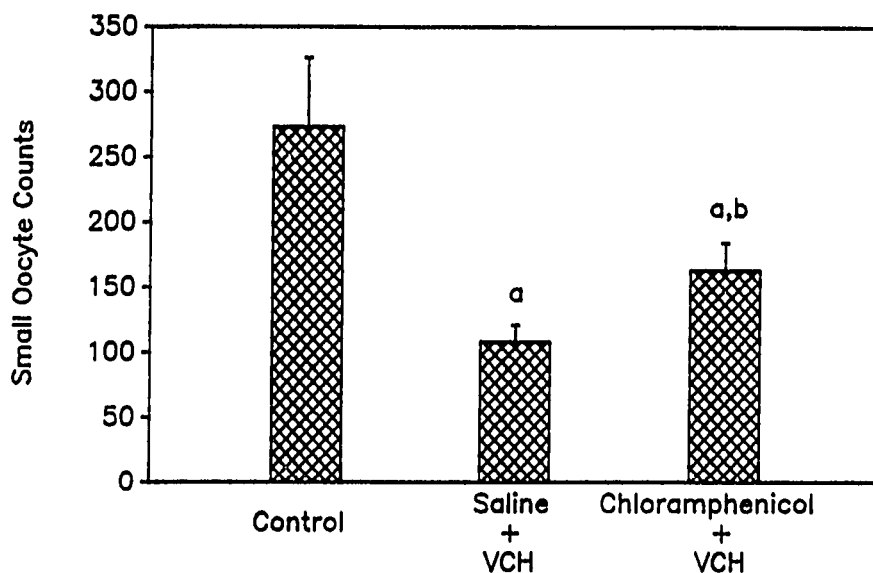


Figure 8. The effect of saline or chloramphenicol (200 mg/kg ip) pretreatment on ovarian toxicity induced by VCH (800 mg/kg ip) in mice (n = 5-6) treated daily for 15 days. The oocyte counts in the saline or chloramphenicol pretreated corn oil controls were 242 ± 59 or 302 ± 22 (n = 5 each), respectively. A Students t-test showed these groups did not differ significantly and hence they were combined to form a common group (n = 10) indicated on the figure as control. The means and standard deviations are shown in the figure. The chloramphenicol pretreated VCH treated group was significantly different from the control^a and the saline pretreated VCH treated^b groups as determined by ANOVA and a Newman-Kuel's range test ($p < 0.05$).

neoplasms.

A series of experiments by Mattison and coworkers clearly show that for the polycyclic aromatic hydrocarbons to be ovotoxic, bioactivation of the parent compound to reactive epoxides must occur (Mattison and Thorgeirsson, 1979; Shiromizu and Mattison, 1984; Shiromizu and Mattison, 1985; and Takizawa et al., 1985). Several studies were conducted to determine if bioactivation of VCH to epoxides is required for this chemical to produce oocyte destruction. The dose-response experiments showed that the potency of the epoxide metabolites of VCH to destroy small oocytes is much greater than VCH. Therefore, a critical step in VCH induced ovarian toxicity may be conversion of the parent compound to ovotoxic VCH epoxides. In the preceding chapter a species difference was observed in the blood concentration of VCH-1,2-epoxide after administration of VCH (800 mg/kg, ip) to rats and mice. Female mice had dramatically higher blood concentrations of VCH-1,2-epoxide. It was also shown that hepatic microsomes from female mice converted VCH to VCH-1,2-epoxide at a rate 6.5 fold greater than rat microsomes. The blood concentration of VCH-1,2-epoxide measured 2 hr after administration of VCH to mice correlated well with the toxicity results. As the dose of VCH increased, the blood concentration of VCH-1,2-epoxide increased as did the loss of oocytes from the ovary. When equitoxic doses of VCH and VCH-1,2-epoxide are given, the

VCH-1,2-epoxide blood concentration-time profile is clearly different. At a 5 fold lower dose of VCH-1,2-epoxide compared to the dose of VCH, the area under the VCH-1,2-epoxide blood concentration curve was only 2 fold lower. Explanations could include higher peak levels of VCH-1,2-epoxide in the ovary because of delivery through the systemic circulation and absorption by the ovary of VCH-1,2-epoxide from the peritoneal cavity (Takizawa et al., 1985) or inaccuracies in the determination of the ED₅₀ since only 3 dose levels were used.

Shiromizu and Mattison (1985) have shown that it is possible to protect mice from polycyclic aromatic hydrocarbon-induced ovotoxicity by pretreating animals with the arylhydrocarbon hydroxylase inhibitor α -naphthoflavone. Therefore, if bioactivation of VCH is required for this compound to be ovotoxic then inhibition of VCH metabolism should result in protection from ovarian injury. This was the basis of the chloramphenicol studies. Dixon and Fouts (1962) showed that chloramphenicol inhibits drug metabolism in mice both in vivo and in vitro. Later studies by Halpert et al. (1983) showed that the mechanism of chloramphenicol mediated inhibition of drug metabolism was due to mechanism-based inactivation of cytochrome P450. Because of the mechanism of enzyme inhibition by chloramphenicol, it does not induce microsomal drug metabolism after multiple administration, as do other classical chemical inhibitors of cytochromes P450

such as SKF 525A (Halpert et al., 1988 and Snyder and Remmer, 1982). The results from these investigations showed that chloramphenicol inhibited the metabolism of VCH by hepatic microsomes. This inhibition also occurred in vivo because the concentration of VCH-1,2-epoxide in the blood after VCH treatment was lower in chloramphenicol treated mice. The inhibition of VCH epoxidation was partially effective in protecting mice from VCH-induced ovarian injury. Complete protection from toxicity was not expected since the blood concentration of VCH-1,2-epoxide in chloramphenicol pretreated mice was not reduced to subtoxic levels. The blood level of the epoxide in chloramphenicol treated mice after 800 mg/kg of VCH is similar to that produced by 400 mg/kg of VCH in untreated mice. A 400 mg/kg dose of VCH is known to produce oocyte loss after repeated administration. Thus, when taken together, the chloramphenicol studies support the hypothesis that VCH requires bioactivation to VCH-1,2-epoxide by the liver to produce ovarian toxicity. However, this does not preclude the possibility that additional bioactivation of VCH-1,2-epoxide to VCD could occur in the ovary, as the ovary has been shown to contain xenobiotic biotransforming enzymes (Mattison and Thorgeirsson, 1978 and Mukhtar et al., 1978a,b).

These studies show that when VCH is bioactivated oocyte destruction occurs. Premature oocyte loss is a critical step in the pathogenesis of ovarian carcinogenesis. Once VCH

destroys oocytes ovarian failure will result (Gosden et al., 1983). The ovary devoid of oocytes synthesizes only small quantities of estrogens and progestins insufficient to inhibit the pituitary secretion of gonadotropins (FSH and LH). This would expose the ovary to continuous elevated serum concentrations of gonadotropins which are thought to promote ovarian tumor development. Therefore, it follows that VCH-induced ovarian toxicity would ultimately cause an endocrine imbalance favorable to ovarian tumor formation.

The metabolism of VCH to epoxides by rats is insufficient to produce oocyte destruction. The poor ability of the rat compared to the mouse to bioactivate VCH could be due to a slow rate of bioactivation or an enhanced rate of detoxication. Studies with hepatic microsomes indicate that mice bioactivate VCH more rapidly than rats. The molecular basis for this difference could reside in species differences in the microsomal content of cytochrome P450 forms which bioactivate VCH. Studies are underway to determine if such differences exist. In addition, a species difference in the detoxication of VCH-1,2-epoxide by glutathione-S-transferase or epoxide hydrolase pathways seems plausible. Data presented in the preceding chapter support this possibility since the species difference in epoxidation rates are not as dramatic as the difference in VCH-1,2-epoxide blood levels observed in vivo. Hepatic microsomes obtained from rats hydrolyze VCH-

1,2-epoxide to VCH-1,2-dihydrodiol more rapidly than mouse hepatic microsomes (unpublished observations). Therefore, the lack of a toxic response by VCH in rats could be due to slow rates of bioactivation and rapid rates of detoxication.

In summary, the data presented support the hypothesis that VCH induces ovarian tumors in mice but not rats because of a difference in the ability of VCH to destroy oocytes. VCH can be metabolized to epoxides which are more ovotoxic than the parent compound. It appears from these studies that VCH is bioactivated to epoxides in the liver which circulate in blood, and produce damage in the ovary ultimately leading to tumor formation.

CHAPTER 4

THE BIOCHEMICAL BASIS FOR THE SPECIES DIFFERENCE IN
HEPATIC MICROSOMAL 4-VINYLCYCLOHEXENE EPOXIDATION
BETWEEN FEMALE MICE AND RATS

ABSTRACT

Mice but not rats are susceptible to 4-vinylcyclohexene (VCH) induced ovarian toxicity and carcinogenicity. This is due in part to a 4-6 fold greater rate of hepatic microsomal bioactivation of VCH to the ovotoxicant VCH-1,2-epoxide. The biochemical basis for this difference was investigated in microsomes using enzyme induction, enzyme inhibition with chloramphenicol or specific inhibitory antibodies, and correlation with marker steroid hydroxylase activities to associate VCH epoxidation with particular cytochrome P450 forms¹. VCH epoxidation by hepatic microsomes was increased in female mice and rats by phenobarbital treatment and was inhibited by approximately one-third by anti-rat-P450_{PB-B} IgG in microsomes from untreated animals of both species. Furthermore, microsomal VCH epoxidase and testosterone 16 α -hydroxylase activities were lower (34%) in female 129/J mice (deficient in constitutive expression of P450IIB forms) than in B6C3F₁ mice. These results suggested partial involvement of P450IIB forms in the microsomal epoxidation of VCH. In microsomes from untreated female mice, VCH epoxidase activity was inhibited 48% by anti-mouse-P450_{15 α} IgG at a concentration that inhibited testosterone 15 α -hydroxylase activity by 86%. No protein immunochemically related to mouse P450_{15 α} was detected in female rat hepatic microsomes. These data show that P450IIA forms in mice can also catalyze VCH epoxidation.

In contrast, no evidence was found supporting the involvement of P450IIIA forms in VCH epoxidation by female mouse hepatic microsomes. Therefore, P450 forms IIA and IIB account for the majority of VCH bioactivation in female mouse liver which explains in part the susceptibility of mice to VCH-induced ovarian toxicity and carcinogenicity.

¹ Nomenclature: In accordance with the recent nomenclature of Nebert et al. (1989) cytochromes P450 are categorized by gene families or subfamilies. Where appropriate, trivial nomenclature for individual cytochromes P450 are used based on the nomenclature used by the laboratory in question. Mouse cytochromes P450_{15α} (Harada and Negishi, 1984) and I-P450_{16α} (Devore et al., 1985) are encoded by the P450IIA3 (Squires et al., 1988) and P450IIB9 (Noshiro et al., 1988) genes, respectively. Rat cytochrome P450_{pg-B} (Guengerich et al., 1982) is encoded by the P450IIB1 (Fuji-Kuriyama et al., 1982) gene. Rat cytochrome P450_{PCNB} (Graves et al., 1987 and Halpert, 1988) has an identical N-terminal amino acid sequence to the putative P450IIIA2 (Gonzalez et al., 1986) gene product.

INTRODUCTION

Workers in the rubber industry are exposed to 4-vinylcyclohexene (VCH) during the production of passenger tires (Rappaport and Fraser, 1977). The effects of chronic VCH exposure in humans are unknown. However, VCH is considered a carcinogen in female B6C3F₁ mice since chronic treatment with this chemical results in the induction of rare ovarian neoplasms (Collins et al., 1987). In contrast, female Fischer 344 rats are resistant to ovarian tumor induction by VCH (NTP, 1986). In previous studies aimed at determining the mechanism of this species difference, repeated administration of VCH to mice but not rats reduced the number of small oocytes in the ovaries with both dose-and time-dependency (Chapter 3). This is important since it is well established that the destruction of oocytes is a critical step in the pathogenesis of chemically-induced ovarian carcinogenesis (Jull, 1973). Mice treated with VCH also have dramatically higher blood concentrations of the VCH metabolite, VCH-1,2-epoxide, compared with VCH-treated rats (Chapter 2). Furthermore, VCH-1,2-epoxide destroyed oocytes of mice and rats at doses much lower than that required for oocyte destruction by VCH. Chloramphenicol is a mechanism based inhibitor of several rat cytochrome P450 forms (Halpert et al., 1985). Administration of chloramphenicol reduces the

concentration of VCH-1,2-epoxide in the blood of VCH-treated female mice and partially protected mice from VCH-induced ovarian toxicity (Chapter 3). Taken together, these data indicate that metabolism of VCH to epoxides, and the subsequent destruction of oocytes are critical steps in the induction of ovarian tumors by VCH. The resistance of the rat to ovarian tumor induction by VCH is likely due to the inability of rats to produce sufficient amounts of VCH-1,2-epoxide to cause oocyte destruction.

The enhanced ability of mice to bioactivate VCH likely resides in the liver, since the conversion of VCH to VCH-1,2-epoxide is catalyzed at a rate 6.5 fold greater in hepatic microsomes obtained from female mice compared to female rats (Chapter 2). The present studies were designed to determine the biochemical basis for the species difference in hepatic microsomal VCH epoxidation. The approach used was to associate VCH epoxidation with certain cytochrome P450 forms in mice, then examine the involvement of the orthologous P450 form in the rat. Regio- and stereospecific hydroxylation of steroids has previously been shown to be performed by distinct P450 forms in hepatic microsomes from mice and rats (Harada and Negishi, 1984 and Waxman et al., 1985). Therefore, the microsomal metabolism of steroids was used to monitor the effect of the P450 inducers phenobarbital and dexamethasone and the inhibitors chloramphenicol or P450 form specific

antibodies on the activity of individual P450 forms. This would allow the changes observed in VCH epoxidase activity after various treatments to be correlated with particular P450 forms.

MATERIALS AND METHODS

Animals. Adult female B6C3F₁ and Fischer 344 rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Female 129/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were housed in cages with sawdust bedding and had free access to food (Teklad^R, 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 use in metabolism studies.

Chemicals. [4-¹⁴C]Androstenedione (52 mCi/mmol) and [4-¹⁴C]testosterone (50 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 4-Vinylcyclohexene and 4-vinylcyclohexene-1,2-epoxide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Unlabeled androstenedione, 6 β -OH androstenedione, 7 α -OH testosterone, and 6 β -OH testosterone were purchased from Steraloids (Wilton, NH). Unlabeled testosterone and 16 α -OH testosterone were purchased from Sigma Chemical Co. (St. Louis, MO). 15 α -OH testosterone and 7 α -OH androstenedione were gifts from by Dr. D.F. Johnson (National

Institutes of Health, Bethesda, MD) and Dr. David Waxman (Harvard Medical School, Boston, MA), respectively.

Animal treatments. Microsomes used in the induction studies were obtained from animals treated with phenobarbital or dexamethasone. Female mice (B6C3F₁ and 129/J) and rats (control N = 3; treated N = 4) received 0.1% (w/v) phenobarbital in the drinking water for 6 days. Female B6C3F₁ mice received 100 mg/kg dexamethasone in corn oil ip for 4 days. The following day the animals were killed by cervical dislocation, livers removed and microsomes prepared as described by Halpert (1983). For the studies with mice, hepatic microsomes were prepared with 2 livers in each pool, a total of 4 individual pools for each treatment. Rat hepatic microsomes were prepared from individual livers. Microsomal protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Microsomal cytochrome P450 was determined spectrally by the method of Omura and Sato (1964). For the chloramphenicol studies female B6C3F₁ mice were treated with saline (2.5 ml/kg) or chloramphenicol sodium succinate 200 mg/kg (as chloramphenicol base). One hr later the animals were killed and microsomes prepared (Chapter 3).

Metabolic Assays. Microsomal androstenedione hydroxylase activity was determined by the method of Waxman et al. (1983) as modified by Graves et al. (1987). Testosterone hydroxylase

was assayed in mouse hepatic microsomes by a modification of the method of Harada and Negishi (1984). Incubations contained: 200 μ M testosterone, 0.25-0.50 mg/ml microsomal protein, 50 mM HEPES buffer (pH 7.6), 15 mM MgCl_2 , 0.1 mM EDTA, and 1 mM NADPH. After a 2 min preincubation at 37°C the reaction was started by the addition of NADPH and terminated after 5 min with 50 μ l of tetrahydrofuran. The reaction mixtures were spotted on silica gel TLC plates [Baker silica gel, 250 μ m, Si250F (19c)] and developed twice in chloroform:ethylacetate (1:2 v/v) or dichloromethane:acetone (4:1 v/v) for separation of androstenedione or testosterone metabolites, respectively. The metabolites were localized by autoradiography, identified by comparison with unlabeled standard compounds, and quantified by liquid scintillation counting. VCH epoxidase activity was based on the amount of VCH-1,2-epoxide formed from VCH as determined by capillary gas-liquid chromatography by the method described in Chapter 2. Briefly, VCH (1mM) was incubated at 37°C for 5 min with microsomal protein (0.1 - 0.5 mg/ml), and a NADPH generating system. Under these conditions the addition of 3,3,3-trichloropropene oxide was not required to prevent microsomal VCH-1,2-epoxide hydrolysis. Steroid hydroxylations were performed with pooled microsomes containing equal amounts of microsomal protein from the individual samples. VCH epoxidation was performed on the individual microsome samples.

Antibody inhibition studies. Anti-rat-P450_{PB-B} IgG (Duignan et al., 1987), anti-rat-P450_{PCNB} IgG (Graves et al., 1987), and control antibody were available from previous experiments. Anti-mouse-P450_{15 α} was a generous gift from Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Titration experiments using from 5-15 mg anti-P450_{PB-B} IgG/nmol P450 showed that 10 mg IgG/nmol P450 maximally inhibited testosterone 16 α -hydroxylase activity (58%) in untreated female B6C3F₁ mouse microsomes. Therefore, anti-P450_{PCNB} IgG was also used at a concentration of 10 mg IgG/nmol P450 which inhibited 76% of testosterone 6 β -hydroxylase activity by untreated female B6C3F₁ mouse microsomes. Eight mg IgG/nmol P450 of anti-P450_{PB-B} inhibited 84% of androstenedione 16 β -hydroxylase activity in microsomes from phenobarbital-treated male rats. Affinity purified anti-mouse-P450_{15 α} was used at a fixed concentration of 27 μ g/mg microsomal protein. The antibody inhibition studies were performed in duplicate using pooled microsomes.

Immunochemical methods. Separation of the proteins present in microsomes was performed by SDS-PAGE (7.5% polyacrylamide gels) by the method of Laemmli (1970). Proteins were transferred electrophoretically to nitrocellulose membranes using a Bio-Rad Trans-Blot cell according to the method of Tobin et al. (1979). Following

incubations with the primary and secondary antibodies the immunoreactive proteins were identified with a horseradish peroxidase Immuno-Blot assay kit (Bio-Rad, Richmond, CA).

Data analysis. Where necessary Student's t-test was used to compare means of 2 different samples using $P < 0.05$ as the level of significance.

RESULTS

Effect of chloramphenicol treatment of female B6C3F₁ mice on several hepatic microsomal testosterone hydroxylase activities. Previous studies with hepatic microsomes from chloramphenicol-treated female mice showed that VCH epoxidation was reduced by 69% (Chapter 3). Since chloramphenicol is somewhat selective in the inactivation of rat hepatic P450 forms (Halpert et al., 1985) the selectivity of chloramphenicol for mouse hepatic P450 forms was assessed by comparing the rates of regio- and stereospecific testosterone hydroxylations by microsomes from saline or chloramphenicol-treated mice. Chloramphenicol treatment caused statistically significant reductions in the microsomal hydroxylation of testosterone in the 15 α and 6 β positions (Table VIII). In female mice testosterone hydroxylation in the 15 α , 16 α , and 6 β positions mainly reflect the microsomal content of P450IIA (Harada and Negishi, 1984), P450IIB and/or D (Devore et al., 1985 and Harada and Negishi, 1984), and

Table VIII.
The Effect of Chloramphenicol (CAP) Treatment (200 mg/kg) on
Female B6C3F₁ Mouse Hepatic Microsomal
Testosterone hydroxylase Activities^a

	Testosterone Metabolite			
	15 α -OH	16 α -OH	7 α -OH	6 β -OH
Saline	0.26 \pm 0.03	0.47 \pm 0.08	0.96 \pm 0.08	1.87 \pm 0.32
CAP	0.14 \pm 0.02 ^b (54%) ^c	0.38 \pm 0.07 (81%)	1.21 \pm 0.21 ^b (126%)	0.70 \pm 0.09 ^b (37%)

^a Activities expressed as nmol metabolite formed/min/mg microsomal protein. Assays performed using 0.5 mg microsomal protein/ml.

^b Significantly different from saline control ($P < 0.05$).

^c Numbers in parentheses represent the percent of saline control values.

P450IIIA (Waxman et al., 1985 and Wrighton et al., 1985), forms, respectively. Therefore, the chloramphenicol experiments are consistent with the involvement of P450 IIA or IIIA forms in VCH epoxidation in uninduced female mice.

Effect of inducers on microsomal VCH epoxidation and steroid hydroxylations. Female B6C3F₁ mice were treated with phenobarbital or dexamethasone to determine the effect of increasing the microsomal content of P450IIB [phenobarbital (Devore et al., 1985 and Waxman et al., 1985)] and P450IIIA [dexamethasone (Waxman et al., 1985; Wrighton et al., 1985; and Meehan et al., 1988)] forms on the metabolism of VCH and testosterone. In hepatic microsomes isolated from phenobarbital pretreated mice VCH epoxidation was increased 5.7-fold (Figure 9). Increases of 5.1, 4.0, and 2.9 fold were observed in the rate of microsomal testosterone hydroxylation in the 15 α , 16 α , and 6 β positions, respectively. In dexamethasone-treated mice the rate of microsomal VCH epoxidation increased 2.6-fold and testosterone hydroxylation in the 16 α and 6 β positions increased 2.2 and 3.8 fold, respectively. These data suggest that induction of P450IIA, P450IIB, or P450IIIA forms in female mice may account for the increase VCH epoxidase activity in hepatic microsomes.

The effect of phenobarbital treatment of female rats on the rate of hepatic microsomal VCH epoxidation and androstenedione hydroxylation is shown in Table IX. Treatment

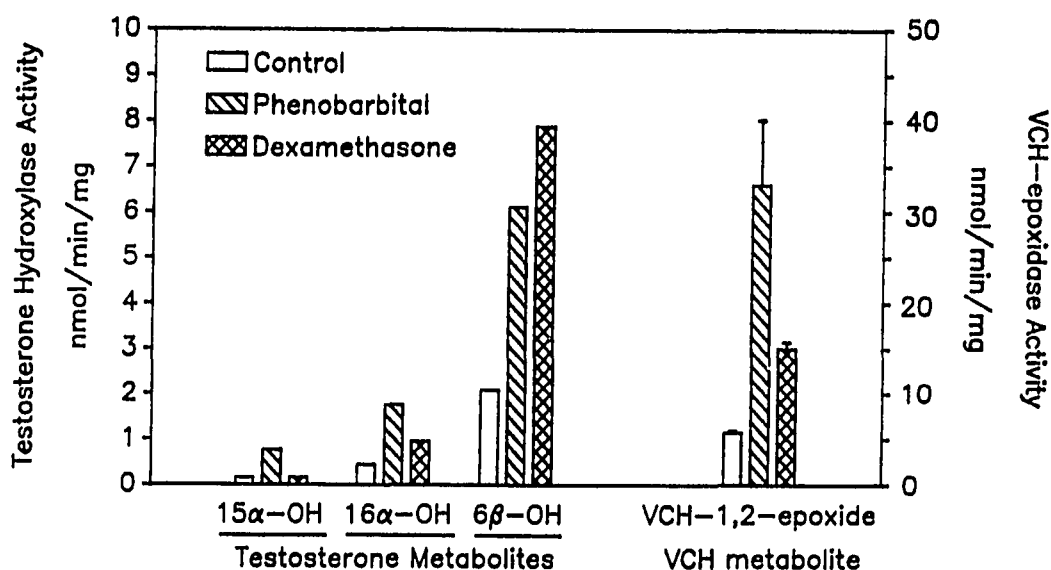


Figure 9. The effect of inducer treatment on female B6C3F₁ hepatic microsomal VCH epoxidase and several testosterone hydroxylase activities. VCH epoxidase assays were performed in duplicate on microsomes from individual pools (microsomal protein concentration 0.25 mg/ml and 0.1 mg/ml for control and induced samples, respectively). The mean and standard deviation (N = 4) for these data are represented by the bars. Testosterone hydroxylase assays were performed in duplicate on pooled microsomes formed from the individual microsome samples (microsomal protein concentration 0.5 mg/ml). The mean of duplicate samples is represented by the bars.

of female rats with phenobarbital resulted in a 9-fold increase in hepatic microsomal VCH epoxidase activity and was accompanied by a 47-fold increase in the rate of androstenedione hydroxylation in the 16β position. In phenobarbital-treated rats androstenedione hydroxylations in the 16α and 16β positions mainly reflect the microsomal activities of P450IIB forms (Waxman et al., 1983 and Waxman et al., 1985). This suggests that induction of P450IIB forms in female rats will increase VCH epoxidase activity.

The effect of inhibitory cytochrome P450 form specific antibodies on VCH epoxidation and steroid hydroxylation by hepatic microsomes. The effect of inhibitory antibodies on VCH epoxidation and steroid hydroxylations was also used to characterize the P450 forms involved in VCH metabolism. Preincubation of hepatic microsomes from untreated female mice with anti-rat-P450_{PB,B} IgG resulted in a 34% inhibition of VCH epoxidase activity (Figure 10). This antibody also produced a 48% decrease in testosterone hydroxylation in the 16α position, but had minimal effects on the rate of hydroxylation of testosterone at other positions. Preincubation of hepatic microsomes from untreated female mice with anti-rat-P450_{PCNB} IgG had no effect on VCH epoxidation, whereas, testosterone 6β -hydroxylase activity was inhibited by 68%. The results in Table X show that anti-P450_{15 α} antibody at a concentration that inhibited 86% of the hydroxylation of testosterone in the 15α

Table IX.

The Effect of Phenobarbital (PB) Treatment on Female F-344 Rat
Hepatic Microsomal Androstenedione hydroxylase
and VCH-epoxidase Activities^a

Treatment	Androstenedione Metabolite		VCH Metabolite
	16 β -OH	16 α -OH	VCH 1,2-epoxide
Untreated	0.27	0.22	2.36 \pm 0.17
PB-treated	12.59 (47) ^b	1.73 (7.9)	21.2 \pm 4.6 (9.0)

^a Activities expressed as nmol metabolite formed/min/mg microsomal protein. The microsomal protein concentrations for VCH epoxidase assays were 0.5 mg/ml for untreated microsomes and 0.1 mg/ml for phenobarbital-treated microsomes. The microsomal protein concentration used for androstenedione hydroxylase assays was 0.25 mg/ml.

^b Numbers in parentheses represent the fold increase in metabolism rates after phenobarbital treatment.

position caused a 48% decrease in VCH epoxidation by hepatic microsomes from untreated female mice. The antibody inhibition experiments suggested that in untreated female mice P450 forms IIA and IIB but not P450IIIA forms catalyze VCH epoxidation.

In hepatic microsomes obtained from untreated female rats anti-P450_{PB-B} IgG inhibited 33% and 38% of VCH epoxidation and androstenedione 16 β -hydroxylation, respectively (Table XI). The same concentration of anti-P450_{PB-B} IgG inhibited 89% and 93% of VCH epoxidation and androstenedione 16 β -hydroxylation by microsomes from phenobarbital-treated rats, respectively. These findings are consistent with a minor role for P450IIB forms in the epoxidation of VCH in untreated female rats. However, in microsomes of phenobarbital-treated female rats P450IIB forms catalyze the majority of VCH epoxidation.

Testosterone 16 α -hydroxylation and VCH epoxidation by hepatic microsomes from untreated and phenobarbital-treated female 129/J mice. Female 129/J mice are deficient in their constitutive expression of hepatic microsomal testosterone 16 α -hydroxylase activity (Ford et al., 1979 and Noshiro et al., 1986). Since, it appeared that treatments of mice that affect testosterone 16 α -hydroxylase also affect VCH epoxidase activity, the 129/J strain was evaluated to determine if this strain also had lower rates of microsomal VCH epoxidation. As shown in Table XII, the rate of VCH epoxidation in

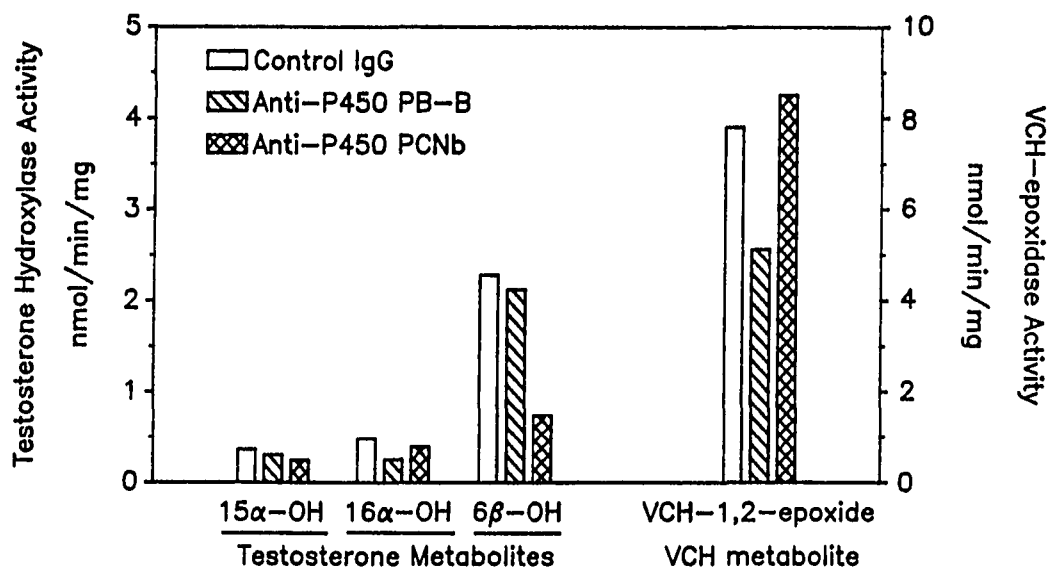


Figure 10. The effect of inhibitory antibodies on female B6C3F₁ hepatic microsomal VCH epoxidase and several testosterone hydroxylase activities. Each assay was performed in duplicate on pooled microsomes formed from the individual microsome samples using 10 mg IgG/nmol P450 (microsomal protein concentration 0.25 mg/ml and 0.5 mg/ml for VCH epoxidase and testosterone hydroxylase assays, respectively). The mean of duplicates is represented by the vertical bars.

Table X.

The Effect of Anti-Mouse-P450_{15 α} IgG on
 Testosterone 15 α -hydroxylase and VCH-epoxidase Activities in
 Untreated Female B6C3F1 Mouse Hepatic Microsomes^a

Incubation Conditions	Testosterone Metabolite	VCH Metabolite
	15 α -OH	VCH-1,2-epoxide
(-) Antibody	0.29	7.1
(+) Antibody	0.04	3.7
	(14%) ^b	(52%)

^a Activities expressed as nmol metabolite formed/min/mg microsomal protein. The microsomal protein concentrations for VCH epoxidase assays were 0.25 mg/ml for untreated microsomes. The microsomal protein concentration used for testosterone hydroxylase assays was 0.5 mg/ml. Anti-P450_{15 α} IgG used at a concentration of 27 μ g IgG/mg microsomal protein.

^b Numbers in parentheses represent the percent of the activity in incubations without antibody.

Table XI.

The Effect of Anti-Rat-P450_{PB-B} IgG
on Androstenedione hydroxylase and VCH-epoxidase Activities
in Hepatic Microsomes From Untreated and Phenobarbital-Treated
(PB-treated) Female F-344 Rats^a

Treatment	Androstenedione Metabolite		VCH Metabolite
	16 β -OH	16 α -OH	VCH 1,2-epoxide
Untreated			
Control Antibody	0.34	0.19	2.76
P450 _{PB-B} antibody	0.21	0.22	1.84
	(62%)	(116%)	(67%)
PB-treated			
Control antibody	10.77	1.34	35.47
P450 _{PB-B} antibody	0.78	0.16	3.85
	(7%)	(12%)	(11%)

^a Activities expressed as nmol metabolite formed/min/mg microsomal protein. The microsomal protein concentrations for VCH epoxidase assays were 0.5 mg/ml for untreated microsomes and 0.1 mg/ml for phenobarbital-treated microsomes. The microsomal protein concentration used for androstenedione hydroxylase assays was 0.25 mg/ml. Control of anti-P450_{PB-B} IgG was used at a concentration of 10 mg IgG/nmol P450.

^b Numbers in parentheses represent the percent of control antibody.

microsomes from female 129/J was 66% of that observed with microsomes of B6C3F₁ mice. The rate of testosterone hydroxylation in the 16 α position was also lower (62% of control) in hepatic microsomes of female 129/J mice compared with B6C3F₁ mice. As observed with female B6C3F₁ mice, microsomes from phenobarbital-treated 129/J mice had increased rates of VCH epoxidation and testosterone 16 α -hydroxylation compared with controls.

Immunochemical analysis of hepatic microsomes.

Immunoblots were performed to demonstrate that the differences in hepatic microsomal VCH epoxidase and steroid hydroxylase activities observed in mice and rats following various treatments were due to different microsomal levels of P450 IIA and IIB forms. Immunoblots of various microsomes using anti-rat-P450_{PB-B} IgG were performed to detect differences in P450IIB content (Figure 11). Differences in the staining intensity of the upper band indicate that constitutive microsomal P450IIB content was higher in female B6C3F₁ mice (lane 1) than female 129/J mouse microsomes (lane 2). This is consistent with the lower VCH epoxidase and testosterone 16 α -hydroxylase activities observed in 129/J mouse microsomes. Phenobarbital treatment increased P450IIB content in hepatic microsomes of phenobarbital-treated mice and rats (lanes 3-8), as it did microsomal VCH epoxidase and the appropriate P450IIB steroid hydroxylase activities. The results obtained

Table XII.

Comparison of Testosterone 16 α -hydroxylase and VCH-epoxidase
Activities in Untreated and Phenobarbital-treated
Female 129/J Mouse Hepatic Microsomes^a

Treatment and Mouse Strain	Testosterone Metabolite	VCH Metabolite
	16 α -OH	VCH-1,2-epoxide
Control B6C3F ₁	0.53	8.8 \pm 1.4
Untreated 129/J	0.33	5.8 \pm 1.1
	[62%] ^b	[66%]
PB-treated 129/J	2.79	47.1 \pm 4.5
	(8.5) ^c	(8.1)

^a Activities expressed as nmol metabolite formed/min/mg microsomal protein. The microsomal protein concentrations for VCH epoxidase assays were 0.25 mg/ml for untreated microsomes and 0.1 mg/ml for phenobarbital-treated microsomes. The microsomal protein concentration used for testosterone hydroxylase assays was 0.5 mg/ml.

^b Numbers in brackets indicate percent of control B6C3F₁ metabolism rates by 129/J mice.

^c Numbers in parentheses indicate fold increase of metabolism rates in PB-treated 129/J mice compared with untreated 129/J mice.

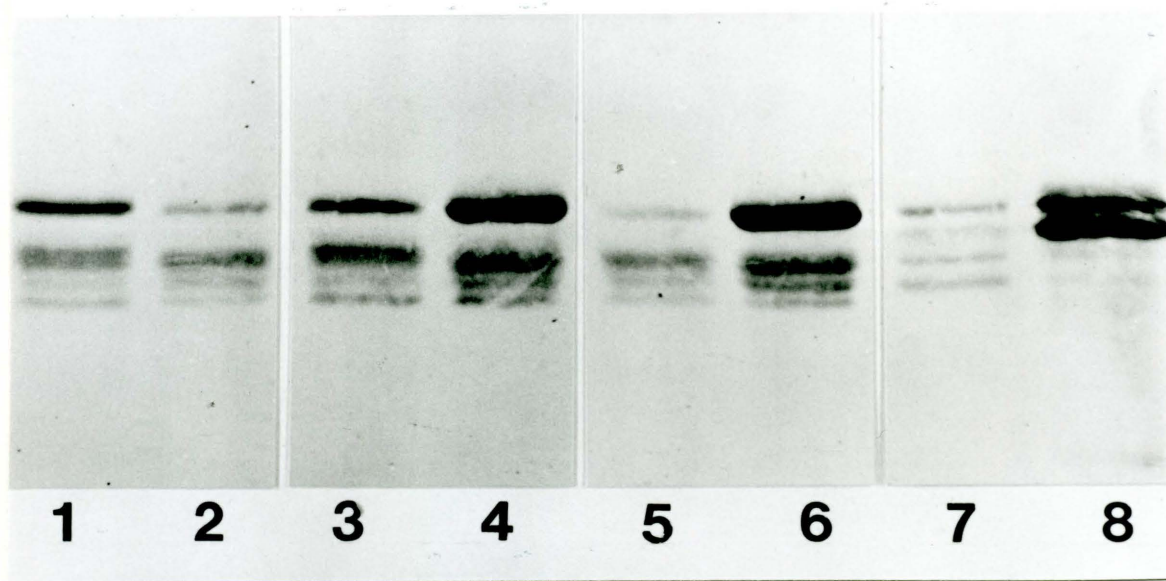


Figure 11. **Western blot of hepatic microsomes using an anti-P450_{PB-B} antibody.** The amount of microsomal protein per lane was 5µg and 2µg for female mouse and rat microsomes, respectively. Lane 1, B6C3F₁ mouse; lane 2, 129/J mouse; lane 3, untreated B6C3F₁ mouse; lane 4, phenobarbital-treated B6C3F₁ mouse; lane 5, untreated 129/J mouse; lane 6 phenobarbital-treated 129/J mouse; lane 7 untreated F-344 rat; lane 8, phenobarbital-treated F-344 rat.

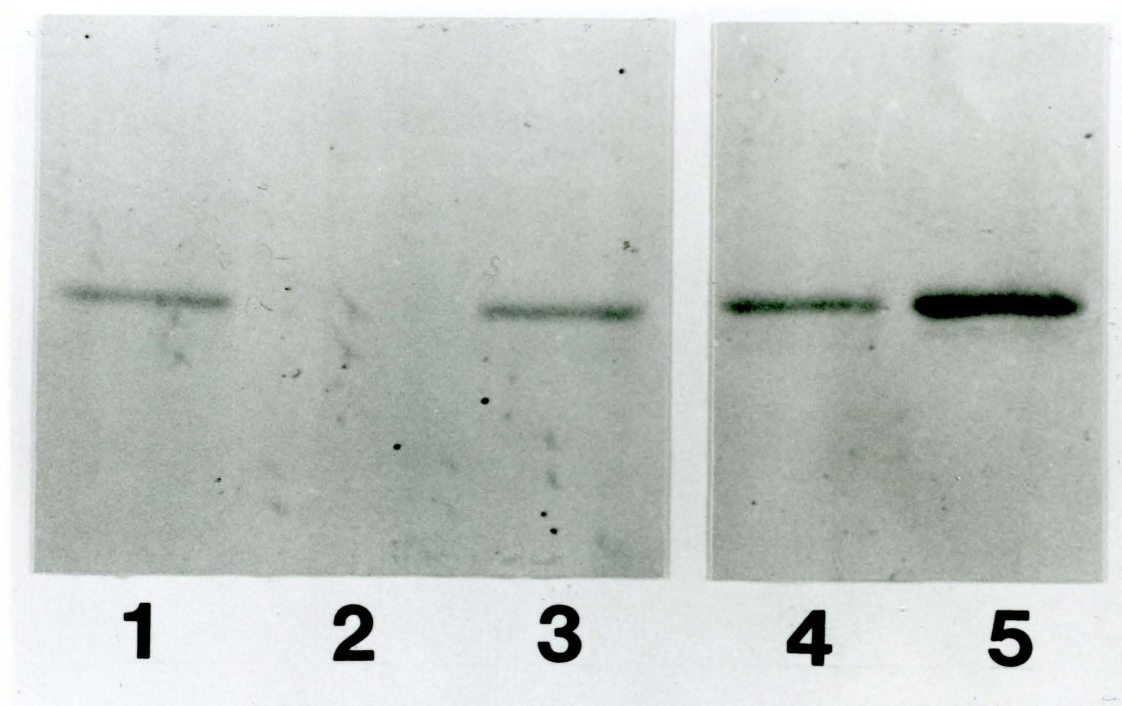


Figure 12. **Western blot of hepatic microsomes using an anti-P450_{15α} antibody.** The amount of microsomal protein per lane was 2 μ g and 5 μ g for female mouse and rat microsomes, respectively. Lanes 1 and 3, untreated B6C3F₁ mouse; lane 2, untreated F-344 rat; lane 4, untreated female B6C3F₁ mouse; lane 5 phenobarbital-treated female B6C3F₁ mouse.

from immunoblots performed using anti-mouse P450_{15 α} IgG to estimate mouse P450IIA content are shown in Figure 12. This antibody recognized a single protein band in female B6C3F₁ (lanes 1 and 3) mouse hepatic microsomes, however, no immunochemically related band was observed in hepatic microsomes of female rats (lane 2). Treatment of mice with phenobarbital increased hepatic microsomal P450IIA content (lanes 4 and 5), which is consistent with the increase in VCH epoxidase and testosterone 15 α -hydroxylase activities following phenobarbital treatment.

DISCUSSION

The data presented in this report suggests that the majority of VCH epoxidation by hepatic microsomes of untreated female mice can be accounted for by the constitutive expression of P450 IIA and IIB forms. VCH epoxidation by the liver is clearly an important step in VCH-induced ovarian toxicity and carcinogenicity. Therefore, the presence of P450 IIA and IIB forms in mouse liver partially explains the susceptibility of mice to VCH-induced ovarian carcinogenicity.

Several experiments suggested involvement of P450IIA forms in hepatic microsomal VCH epoxidation by female mice. Inhibition of microsomal VCH epoxidation in chloramphenicol-treated female mice was also correlated with lower testosterone 15 α -hydroxylation, a marker activity for mouse

P450IIA forms. Similar results were obtained using an antibody specific for mouse P450IIA forms in which inhibition of both VCH epoxidase and testosterone 15 α -hydroxylase activities was observed using untreated female mouse microsomes. Phenobarbital treatment of female mice increased VCH epoxidase activity. This is partially due to induction of P450IIA forms by phenobarbital as shown by increases in testosterone 15 α -hydroxylase activity and increased microsomal content of P450IIA forms by immunoblotting. Rat P450IIA forms are not immunochemically related to mouse P450IIA forms, have different regiospecificity for steroid hydroxylation [rat P450IIA catalyze 7 α -hydroxylation (Waxman et al., 1985); mouse P450IIA catalyze 15 α -hydroxylation (Harada and Negishi, 1984)], and probably have limited involvement in VCH epoxidation. Thus, we conclude that hepatic microsomal P450IIA forms of untreated female mice provide an important contribution to VCH epoxidation.

Evidence was obtained which suggested the involvement of P450IIB forms in VCH epoxidation. In female B6C3F₁ mice, VCH epoxidase and testosterone 16 α -hydroxylase activities increased to a greater extent following treatment with phenobarbital compared to dexamethasone. This suggests that the level of induction of an activity specific to P450IIB forms is closely related to the level of VCH epoxidase activity in these microsomes. Immunoblots using anti-P450_{pg}.

8 showed that the increase in these activities following phenobarbital treatment of female B6C3F₁ mice was due to increased microsomal content of P450IIB forms. Anti-P450_{PB-B} IgG partially inhibited VCH epoxidase and testosterone 16 α -hydroxylase activities of hepatic microsomes of untreated B6C3F₁ mice. Additional evidence suggesting the involvement of P450IIB forms in VCH epoxidation was provided by the studies with female 129/J mice. Female 129/J mice are deficient in constitutive expression of P450IIB forms and hence have low rates of microsomal testosterone 16 α -hydroxylation (Ford et al., 1979 and Noshiro et al., 1986). VCH epoxidation was lower in 129/J mice compared to B6C3F₁ mice suggesting that deficient expression of P450IIB forms is also associated with lower rates of VCH epoxidation. Thus, evidence obtained using several different experimental approaches are all consistent with the partial involvement of P450IIB forms in the epoxidation of VCH by hepatic microsomes of untreated female B6C3F₁ mice.

Studies were performed to determine the relative contribution of P450IIB forms in the microsomal epoxidation of VCH by female rats. As observed with mice, hepatic microsomal VCH epoxidase activity increased with phenobarbital treatment of female rats. Phenobarbital treatment of rats also increased hepatic microsomal androstenedione 16 β -hydroxylase activity and content of P450IIB forms, consistent

with the findings of Waxman et al. (1985). VCH epoxidase and androstenedione 16 β -hydroxylase activities were inhibited to a similar degree by anti-P450_{PB-B} IgG in hepatic microsomes of untreated female rats. It is important to note that the overall rate of microsomal VCH epoxidase activity inhibited by this antibody was greater in untreated female B6C3F₁ mice (2.7 nmol/mg/min) compared to untreated female rats (0.9 nmol/mg/min). Therefore, P450IIB forms in both species catalyze microsomal VCH epoxidation, however, the contribution of these forms appears to be greater in untreated female mice than in untreated female rats. Noshiro et al. reported that microsomes from female BALB/cJ mice contained about 0.26 nmol P450IIB/mg microsomal protein (Noshiro et al., 1986), while Waxman et al. showed that female Sprague-Dawley rats had only 0.07 nmol P450IIB/mg microsomal protein (Waxman et al., 1985). Thus, lower expression of P450IIB forms in female rat compared to female mouse liver could partially account for the lower rates of VCH epoxidation by female rats. Clearly, other cytochromes P450 are involved in VCH epoxidation in untreated female rat hepatic microsomes. However, these unidentified rat liver VCH epoxidases are probably expressed at very low levels and/or have poor catalytic activity toward VCH.

In untreated female mouse hepatic microsomes P450IIIA forms were found not to be involved VCH epoxidation. Phenobarbital and dexamethasone treatment of female mice

increased testosterone 6 β -hydroxylation, a marker activity for P450IIIA forms. However, phenobarbital was not as effective at inducing testosterone 6 β -hydroxylation than was dexamethasone, whereas, the opposite effect of these inducers was shown for VCH epoxidation. Antibody inhibition experiments showed that in microsomes from untreated female B6C3F₁ mice anti-P450_{PCNB} inhibited testosterone 6 β -hydroxylase activity by 68%, while having no effect on VCH epoxidation. These data do not support a role for P450IIIA forms in VCH epoxidation by untreated female mouse hepatic microsomes.

The high rate of VCH epoxidation by hepatic microsomes of phenobarbital-treated female rats suggests that phenobarbital treatment could increase the blood level of VCH-1,2-epoxide in VCH-treated rats and increase susceptibility to VCH-induced ovarian carcinogenicity. Interestingly, phenobarbital treatment has no effect on the blood level of VCH-1,2-epoxide in VCH-treated rats (data not shown). Thus, it appears that an additional component protecting rats from VCH-induced ovarian carcinogenicity may be the rapid degradation of VCH-1,2-epoxide in vivo.

CHAPTER 5

METABOLISM OF 4-VINYLCYCLOHEXENE BY HUMAN HEPATIC MICROSOMES

ABSTRACT

Carcinogenicity studies have shown that chronic administration of VCH will induce ovarian tumors in B6C3F₁ mice but not F-344 rats. This occurs because the blood level of the ovotoxic VCH metabolite, VCH-1,2-epoxide, is dramatically higher in VCH-treated female mice compared with rats. This species difference in VCH epoxidation is also reflected in the rate of VCH metabolism by hepatic microsomes (female mouse >> female rat). Since humans are exposed to VCH in certain occupational settings, the objectives of these studies were to assess the ability of microsomes obtained from human liver to metabolize VCH to epoxides. The production of VCH-1,2-epoxide and VCH-7,8-epoxide from VCH (1 mM) by human hepatic microsomes was linear with respect to microsomal protein concentration (0.25 - 1.0 mg/ml) and incubation time (5 - 20 min). VCH-1,2-epoxide was the major metabolite, while VCH-7,8-epoxide formation was about 6 fold lower and in some cases was below the limit of detection. There was no substantial difference in the rates of VCH epoxidation by hepatic microsomes obtained from males and females. The rate of VCH-1,2-epoxide formation by female human hepatic microsomes was 0.69 ± 0.40 nmol/mg microsomal protein/min ($n = 4$). This is 13 and 2 fold lower than the rate of VCH-1,2-epoxide formation by female mouse and rat hepatic microsomes, respectively. Therefore, if the rate of hepatic VCH

epoxidation is the main factor which determines the ovotoxicity of VCH then the results of these studies provide initial evidence that humans would be less susceptible to VCH-induced ovarian toxicity.

INTRODUCTION

Human exposure to 4-vinylcyclohexene (VCH) occurs in the rubber industry when workers inhale gases discharged while the rubber is curing. This is of concern because in a chronic cancer bioassay female B6C3F1 mice develop rare ovarian neoplasms when treated with VCH (NTP, 1986). Interestingly, female Fischer 344 rats did not develop ovarian tumors in this bioassay. A major focus of our previous work was to understand both the mechanism by which ovarian tumors are induced in VCH-treated mice and the reason that the rat is not susceptible to VCH-induced ovarian tumors. The ultimate goal is to apply this information to the human situation so that the risk of human exposure to VCH can be determined.

Mechanistic toxicology studies suggest that VCH induces ovarian tumors in mice because this chemical is metabolized to epoxides which destroy small oocytes. Several lines of evidence support this view. First, VCH treatment will destroy oocytes of mice but not rats when administered for 30 days, whereas, VCH epoxides destroy oocytes in both species at much lower doses than VCH. Second, the blood concentration of an ovotoxic epoxide metabolite of VCH (VCH-1,2-epoxide) is dramatically higher in VCH-treated mice compared to VCH-treated rats. Finally, inhibition of VCH epoxidation in vivo reduces the blood concentration of VCH-1,2-epoxide and

partially protects mice from VCH-induced ovarian toxicity.

The epoxidation of VCH by hepatic microsomes correlates well with toxicity since the rate of formation of the ovotoxic metabolite, VCH-1,2-epoxide, is much greater by microsomes from mice (susceptible to VCH-induced ovarian tumors) compared to microsomes from rats (resistant to VCH-induced ovarian tumors). Therefore, the rate of epoxidation of VCH by human hepatic microsomes could provide information needed to assess the risk if any to humans exposed to VCH.

MATERIALS AND METHODS

Sources of Human Liver. Two sources of human liver were used in these studies. The primary source of liver tissue was from organ donors dying in traumatic accidents. Only tissues unsuitable for transplantation were used in these studies. A secondary source of human liver tissue was from patients undergoing surgical resection of liver tumors. Normal tissue resected during the removal of the tumor was available for use in our studies. Following removal from the body the liver was stored in ice-cold modified Sack's buffer for a period not greater than 6 hours before microsome preparation.

Preparation of Microsomes. The liver was cut into small cubes and homogenized in 8 g aliquots. Microsomes were prepared using the calcium precipitation method of Schenkman and Cinti (1984). The microsomal pellet was resuspended in

a storage buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20% v/v glycerol, and 100 μ M phenylmethylsulfonyl fluoride, a protease inhibitor. Microsomes were stored for future use at -80°C . Preliminary studies with microsomes prepared using this procedure indicated that in some samples extensive VCH epoxidation occurred in the absence of NADPH. Therefore, an additional wash of the microsomes was performed before use by diluting approximately 2 ml of stored microsomes in approximately 20 ml of storage buffer. This mixture was centrifuged at 100,000 X g for 60 min. The supernatant was discarded and the final microsomal pellet was resuspended in 1 ml of storage buffer. The protein concentration of all microsomes used was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Microsomal Incubation Conditions for VCH epoxidation.

The method used was essentially the same as was used to measure VCH epoxidation by mouse and rat hepatic microsomes (see chapter 2). Microsomal incubations contained microsomal protein, NADP^{+} (0.5 mM), glucose-6-phosphate dehydrogenase (2 units), glucose-6-phosphate (10 mM), MgCl_2 (15 mM), EDTA (0.1 mM), VCH (1 mM) in methanol 1% v/v for the total incubation, HEPES (0.05 M, pH 7.5), to a final volume of 2 ml. Incubations were performed using microsomes from D10 to define the assay conditions such that metabolite production was linear with protein concentration and incubation time.

Preliminary incubations of human microsomes with VCH (1 mM) for 20 min in the presence and absence of the epoxide hydrolase inhibitor 3,3,3-trichloropropene oxide [TCPO (2 mM)]. Under these conditions the epoxides of VCH were undetectable in the absence of TCPO. Screw cap vials containing the microsomal suspension were preincubated at 37°C for 3 minutes and the reaction started by the addition of glucose-6-phosphate. Glucose-6-phosphate was absent from blank incubations. The reaction was terminated by the addition of 0.2 volumes of 5 M NaOH. Incubations were extracted with 0.2 volumes hexane containing cis-cyclodecene as the internal standard, vortexed, shaken for 5 minutes, and the phases separated by centrifugation. The organic layer was removed and analyzed for VCH-1,2-epoxide by capillary gas-liquid chromatography.

RESULTS

Validation of assay conditions. Microsomes prepared from human liver D10 were used to define the assay conditions needed such that product formation would be linear with respect to the concentration of microsomal protein and incubation time. Figure 13 shows that when VCH was incubated with 1.0 mg microsomal protein/ml product, formation was linear for at least 20 min of incubation. As illustrated in Figure 14 the formation of both monoepoxides of VCH was linear with protein concentration over a range of 0.25 to 1.0 mg/ml.

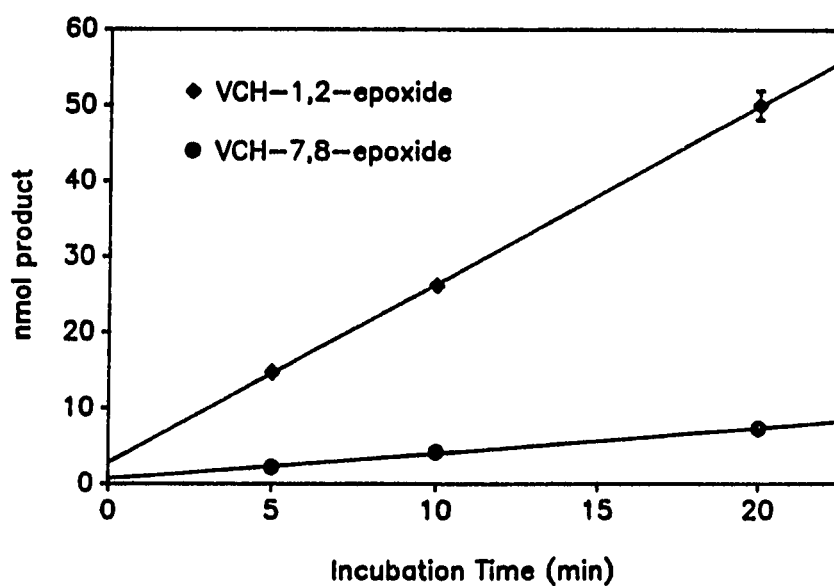


Figure 13. The effect of incubation time on VCH epoxidation by human hepatic microsome sample D10 (donor). Microsomal protein (1.0 mg/ml) was incubated in for 5, 10, and 20 min under conditions specified in MATERIALS AND METHODS. Each point represents the mean of duplicate incubations. The range/2 is indicated by the bars. Where the bar is not visible the range/2 is contained between the symbol.

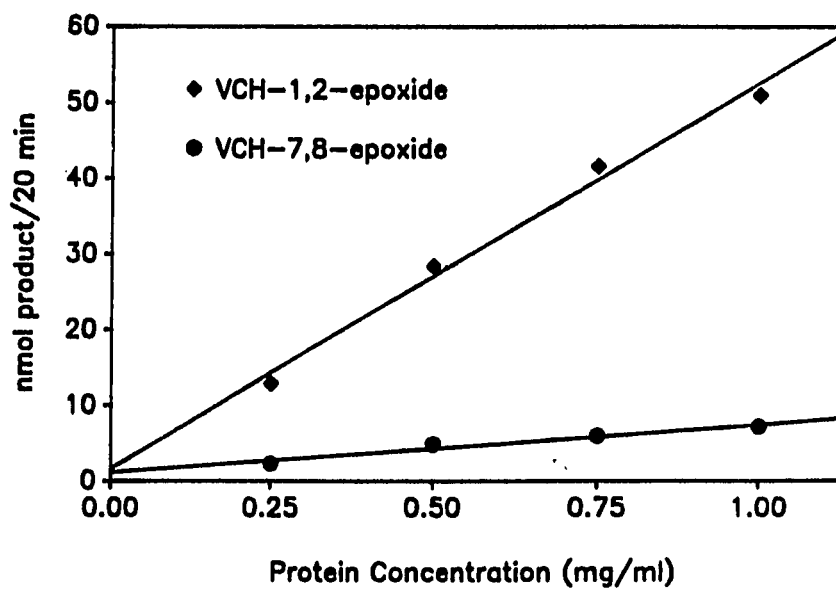


Figure 14. The effect of protein concentration on VCH epoxidation by human hepatic microsome sample D10 (donor). Microsomal protein concentrations of 0.25, 0.50, 0.75, and 1.0 mg/ml were incubated 20 min under conditions specified in MATERIALS AND METHODS. Each point represents the mean of duplicate incubations. The range/2 is indicated by the bars. Where the bar is not visible the range/2 is contained between the symbol.

One problem encountered during these experiments was extensive metabolite formation of in the absence of glucose-6-phosphate (-NADPH). For example using hepatic microsomes from D10 (1.0 mg microsomal protein/ml; 2ml incubation volume), 68.1 nmol and 18.1 nmol of VCH-1,2-epoxide were formed in 20 min in the presence and absence of glucose-6-phosphate, respectively. The samples without cofactor generation were subtracted from samples with cofactor generation to determine the amount of product formed which required NADPH. Thus, D10 microsomes without NADPH catalyzed 27% of the activity with NADPH under these conditions. Microsomal VCH-1,2-epoxide formation in the absence of NADPH was reduced to an average of 6% of the total amount of metabolite formed by washing the stored microsomes. This involved dilution of the microsomal suspension in storage buffer, centrifugation of at 100,000 x g for 60 minutes, and resuspension of the washed microsomal pellet in fresh storage buffer. All of the microsomal samples which appear in Table XIII were washed using this procedure.

Comparison of VCH epoxidation by different human hepatic microsome samples. The rate of metabolism of VCH was assayed in 12 human liver microsomal samples by incubating VCH with 1.0 mg/ml microsomal protein for 20 minutes is presented in Table XIV. The major microsomal metabolite of VCH was VCH-1,2-epoxide. The rate of production of this metabolite ranged from 0.23 to 1.25 nmol/mg microsomal protein/min. VCH-7,8-

Table XIII.
Formation Rates of VCH Epoxides by Human Hepatic Microsomes^a

Sample	Sex	VCH-1,2-epoxide nmol/min/mg	VCH-7,8-epoxide nmol/min/mg
D07	F	0.45	0.06
D08	M	0.23	< 0.01
D09	M	0.68	0.11
D10	M	0.85	0.11
D11	M	0.53	< 0.01
D13	M	0.56	0.08
D14	M	0.54	< 0.01
D20	F	0.36	0.07
R09	F	0.82	0.15
R10 ^b	-	1.14	0.20
R12	F	0.68	0.11
R13	F	1.25	0.21

^a Human hepatic microsomes (1.0 mg/ml) were incubated at 37°C for 20 min with VCH (1 mM), 3,3,3-trichloropropene oxide (2 mM), and a NADPH generating system. D and R denotes human liver obtained from donors and resection patients, respectively. The rates of metabolite production are expressed as nmol metabolite formed/mg microsomal protein/min.

^b Details concerning patient information were not available.

Table XIV.

Comparison of the Formation Rates of VCH-1,2-epoxide
by Hepatic Microsomes from Male and Female Humans^a

Parameter	All	Female	Male
nmol/min/mg	0.67 ± 0.30	0.71 ± 0.35	0.57 ± 0.20
Number of Samples	12	5	6

^a Data as mean and standard deviation from Table XIII.

epoxide was formed at rates approximately 6 fold lower than the 1,2-epoxide. The rate of production of the 7,8-epoxide ranged from < 0.01 to 0.21 nmol/mg microsomal protein/min. Four of the samples were obtained from females, 6 from males, and the sex of one resection patient was unknown. The results in Table XIV show that the rate of hepatic microsomal VCH-1,2-epoxide formation between males and females is similar. A comparison of the rate of production of VCH-1,2-epoxide by microsomes prepared from female mouse, rat, and human liver is presented in Figure 15. It appears that the rate of VCH epoxidation by hepatic microsomes from humans is more comparable to rats than mice.

DISCUSSION

These studies indicate that VCH-1,2-epoxide is the major metabolite of VCH by hepatic microsomes of humans. VCH-1,2-epoxide was also the major metabolite of VCH by hepatic microsomes from female mice and rats. However, the rate of metabolism of VCH proceeds at a lower rate by human microsomes compared with those obtained from either rat or mouse. It has been shown that the production of VCH-1,2-epoxide is intimately involved in the ovarian toxicity by VCH. The rate of production of VCH-1,2-epoxide, and ovotoxic metabolite of VCH, is much greater by hepatic microsomes from female mice (sensitive to VCH-induced ovarian toxicity) compared to female

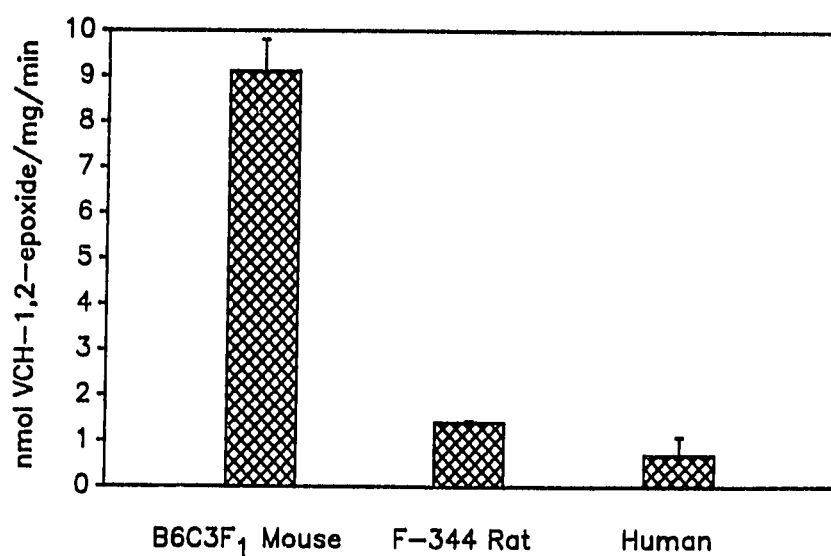


Figure 15. Comparison of the rate of formation of VCH-1,2-epoxide from VCH by hepatic microsomes of female mouse, rat, and human samples. Data for VCH epoxidation by female mouse and rat hepatic microsomes is from Chapter 2. The mean and standard deviation is represented by the bars.

rats (resistant to VCH-induced ovarian toxicity). The sensitivity of the human ovary to VCH or VCH epoxides is unknown, however, animal data suggest that the conversion of VCH to epoxides is a critical step in VCH-induced toxicity. Therefore, the rate of VCH epoxidation by hepatic microsomes provides some information on the susceptibility of species to VCH toxicity. The rate of metabolism of VCH by human hepatic microsomes is even lower than that by rat hepatic microsomes, a species resistant to VCH-induced ovarian tumors. Therefore, if the rate of VCH epoxidation by the liver is the most important factor which determines the sensitivity of a species to VCH-induced ovarian tumors then these studies provide initial evidence that humans would be less susceptible to ovarian toxicity by VCH. However, this view is probably oversimplistic since the rate of detoxification of VCH epoxides by glutathione conjugation and/or hydrolysis by epoxide hydrolase is probably also important in the determination of species susceptibility to VCH-induced ovarian toxicity.

SUMMARY

The goal of this dissertation was to understand the basis for the susceptibility of B6C3F₁ mice and resistance of Fischer 344 rats to ovarian tumor induction following chronic administration of VCH. These studies showed that this occurs because VCH treatment of mice but not rats causes a dramatic reduction in the number of small oocytes. The destruction of oocytes is a critical event which occurs early in the pathogenesis of chemically-induced ovarian tumors. Subsequent studies were performed to determine the basis for the species difference in the ovotoxicity of VCH. These experiments showed that VCH-treated mice metabolize VCH to an ovotoxic compound, VCH-1,2-epoxide, to a much greater degree than rats. The results of several experiments supported this conclusion. First, the blood concentration of VCH-1,2-epoxide is dramatically higher in VCH-treated mice compared to VCH-treated rats. Second, VCH-1,2-epoxide is more potent than VCH and this epoxide is ovotoxic to both mice and rats. Third, the blood concentration of VCH-1,2-epoxide in VCH-treated mice correlates well with the toxicity results since a dose of VCH which causes only a slight reduction in the number of small oocytes produces very low blood concentrations of the epoxide. Fourth, studies with mice showed that inhibition of VCH epoxidation in vivo reduced the blood level

of VCH-1,2-epoxide and partially protected against VCH-induced ovarian toxicity. Studies were also performed to determine if the blood level of VCH-1,2-epoxide was higher in VCH-treated mice because of increased rates of VCH epoxidation by the liver. Experiments with hepatic microsomes supported this view since VCH epoxidation was catalyzed at greater rates by female mouse microsomes compared with female rat microsomes. Investigations into the biochemical basis for this species difference revealed that two mouse hepatic cytochrome P450 forms catalyzed the majority of VCH epoxidation and account for the species difference. Mouse P450 IIA and IIB forms catalyze VCH epoxidation. In the rat, P450IIB forms also catalyze VCH epoxidation although not to the same extent as observed with mice. High rates of VCH epoxidation by microsomes appeared to correlate with susceptibility to VCH-induced ovarian toxicity. Therefore, studies were performed to determine if human hepatic microsomes metabolized VCH at rates comparable to mice or rats. The results showed that hepatic microsomes obtained from human females metabolize VCH at rate slightly lower than rats. Thus, humans like rats may also be resistant to VCH-induced ovarian injury.

The proposed events which lead to oocyte destruction following VCH administration to mice are shown schematically in Figure 16. VCH is distributed to the liver and metabolized by P450IIA and P450IIB forms to the ovotoxicant VCH-1,2-

epoxide. This metabolite circulates in the blood and is delivered to the ovary where oocyte destruction occurs. The events which occur after VCH destroys oocytes which lead to tumor formation are unknown. However, information collected by other methods of ovarian tumor induction support the proposed theory shown schematically in Figure 17. The ovarian failure that results following VCH treatment causes a hormonal imbalance which exposes the ovary to continuously elevated levels of gonadotropins. Elevated gonadotropins are thought to promote ovarian tumor development.

The findings from this research raise a number of interesting questions. The role of ovarian metabolism in VCH toxicity is unknown. The target organ for VCH toxicity could contribute to its own intoxication if the ovary could metabolize VCH to epoxides. Furthermore, VCH-1,2-epoxide may be metabolized by the ovary to the more ovotoxic diepoxide compound. The reason for the increased sensitivity of small oocytes compared to other ovarian cell types to cell killing by VCH is also unknown. An understanding of this area could also clarify the events which occur that cause oocytes to die following exposure to VCH epoxides. Finally, many chemicals in our society contain cyclohexene or vinyl groups that comprise the VCH molecule. Understanding the structural features of VCH which are required for toxicity could help us predict which structurally related chemicals would be ovotoxic.

If the presence of these structural groups can increase the ovotoxicity of chemicals then perhaps we should be concerned about human exposure to any chemical with cyclohexene or vinyl moieties.

Relationship of VCH Epoxidation with Ovarian Toxicity

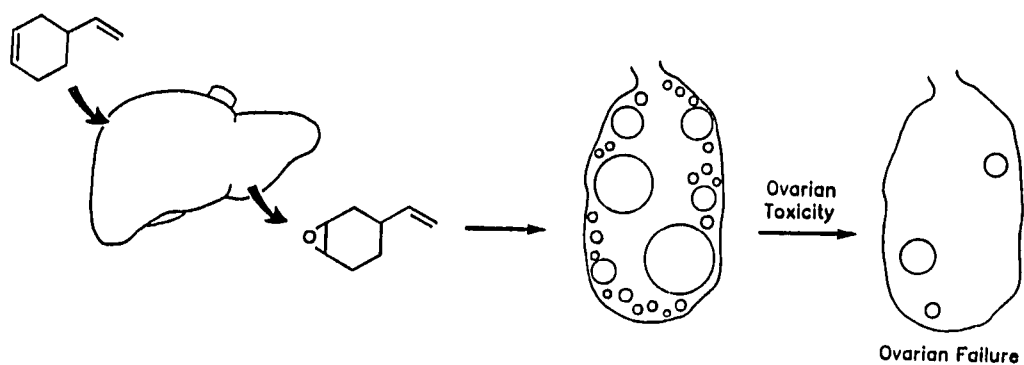


Figure 16. Schematic diagram illustrating the proposed events which lead to oocyte destruction following VCH administration.

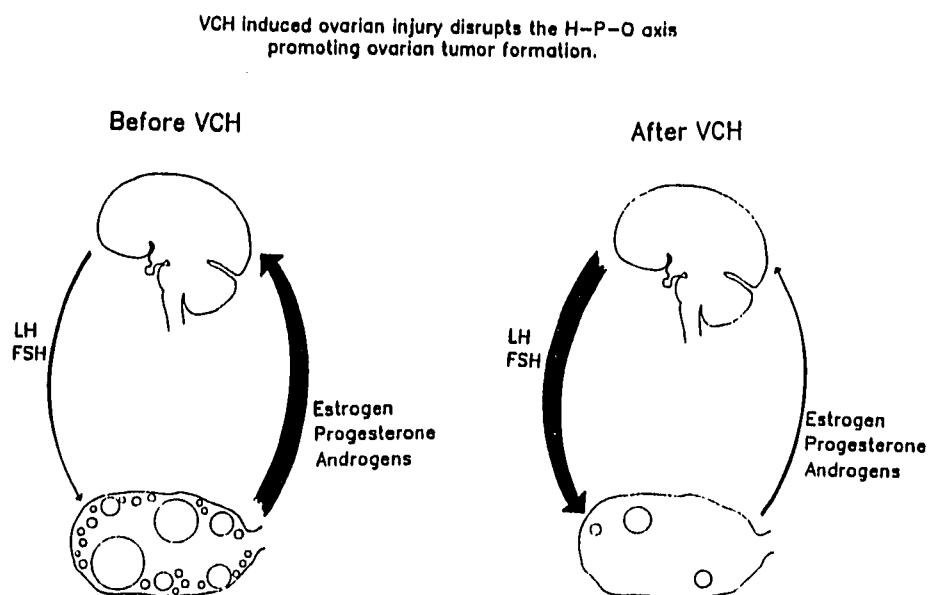


Figure 17. Schematic diagram illustrating how VCH-induced ovarian toxicity may lead to ovarian tumor formation.

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