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4-Vinylcyclohexene

CAS Registry Number: 100-40-3

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TEXAS COMMISSION ON ENVIRONMENTAL QUALITY

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Acronyms and Abbreviations

Acronyms and Abbreviations	Definitions
BD	1,3-butadiene
BMC	benchmark concentration
BMCL	benchmark concentration 95% lower confidence limit
C	concentration or Celsius
CNS	central nervous system
CYP	cytochrome
D	exposure duration, hours per day
DAF	dosimetric adjustment factor
DEB	Diepoxide of BD
DSD	development support document
E	exposure level or concentration
EC	effective concentration
ESL	Effects Screening Level
$^{acute}ESL$	acute health-based Effects Screening Level for chemicals meeting minimum database requirements
$^{acute}ESL_{odor}$	acute odor-based Effects Screening Level
$^{acute}ESL_{veg}$	acute vegetation-based Effects Screening Level
$^{chronic}ESL_{linear(c)}$	chronic health-based Effects Screening Level for linear dose response cancer effect
$^{chronic}ESL_{linear(nc)}$	chronic health-based Effects Screening Level for linear dose response noncancer effects
$^{chronic}ESL_{nonlinear(c)}$	chronic health-based Effects Screening Level for nonlinear dose response cancer effects
$^{chronic}ESL_{nonlinear(nc)}$	chronic health-based Effects Screening Level for nonlinear dose response noncancer effects
$^{chronic}ESL_{veg}$	chronic vegetation-based Effects Screening Level
F	exposure frequency, days per week

Acronyms and Abbreviations	Definitions
FSH	follicle stimulating hormone
g	gram
g/mol	gram per mole
GSH	glutathione
i.p.	intraperitoneal
h or hr	hour
HEC	human equivalent concentration
HQ	hazard quotient
Hg	mercury
HSDB	Hazardous Substances Data Bank
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
g/m^3	gram per cubic meter
K	constant level or severity of response
kg	kilogram
Kow	octanol water partition coefficient
LC_{50}	concentration producing lethality in 50% of experimental animals
LOAEL	lowest-observed-adverse-effect-level
m	meter
mRNA	messenger RNA
µg	microgram
$µg/m^3$	microgram per cubic meter
mg/m^3	milligram per cubic meter
mg	milligram
mg/L	milligram per liter
mm	millimeter

Acronyms and Abbreviations	Definitions
mM	millimole
mmol/kg	millimole per kilogram
MW	molecular weight
min	minute
MOA	mode of action
NADPH	nicotinamide adenine dinucleotide phosphate
NIOSH	National Institute for Occupational Safety and Health
nmol/mL	nanomole per mililiter
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect-level
NOEL	no-observed-effect-level
OSHA	Occupational Safety and Health Administration
P or p	probability
PBPK	physiologically-based pharmacokinetic
POD	point of departure
POD_{ADJ}	point of departure adjusted for exposure duration
POD_{HEC}	point of departure adjusted for human equivalent concentration
ppb	parts per billion
ppm	parts per million
ReV	Reference Value
RGDR	regional gas dose ratio
T	time or exposure duration
TCEQ	Texas Commission on Environmental Quality
TD	Toxicology Division
TWA	Time-Weighted Average
TWA-TLV	Time-Weighted Average Threshold Limit Value

Acronyms and Abbreviations	Definitions
UF	uncertainty factor
UF_H	interindividual or intraspecies human uncertainty factor
UF_A	animal to human uncertainty factor
UF_{Sub}	subchronic to chronic exposure uncertainty factor
UF_L	LOAEL to NOAEL uncertainty factor
UF_D	incomplete database uncertainty factor
URF	unit risk factor
USEPA	United States Environmental Protection Agency
VCD	vinylcyclohexene diepoxide
VCH	4-vinylcyclohexene
VCHE	vinylcyclohexene epoxide
VCME	vinylcyclohexene monoepoxide
wk	week

Chapter 1 Summary Tables

Table 1 for air monitoring and Table 2 for air permitting provide a summary of health- and welfare-based values from an acute and chronic evaluation of 4-vinylcyclohexene (VCH). Please refer to the Air Monitoring Comparison Values Document (AMCV Document) and Fact Sheet available at AMCVs at TCEQ for an explanation of values used for review of ambient air monitoring data and air permitting. Table 3 provides summary information on VCH's physical/chemical data.

Table 1. Air Monitoring Comparison Values (AMCVs) for Ambient Air

Short-Term Values	**Concentration**	**Notes**
Acute ReV	5800 µg/m^3 (1300 ppb) **Short-Term Health**	**Critical Effect(s):** Central Nervous System (CNS) effects observed in Sprague/Dawley rats and B6C3F1 mice
$^{acute}ESL_{odor}$	510 µg/m^3 **Odor**	Pungent, strong odor; 1,3-butadiene odor-based value used as a surrogate
$^{acute}ESL_{veg}$	--- **Short-Term Vegetation**	No data found
Long-Term Values	**Concentration**	**Notes**
Chronic ReV nonlinear(nc)	330 µg/m^3 (74 ppb)	**Critical Effect(s):** Lethargy/tremor/lethality/ovarian atrophy in B6C3F$_1$ mice
Chronic ReV nonlinear(c)	330 µg/m^3 (74 ppb) **Long-Term Health**	**Critical Effect(s):** ovarian atrophy leading to ovarian tumors in mice
$^{chronic}ESL_{linear(c)}$	---	Inadequate information to assess carcinogenic potential via inhalation
$^{chronic}ESL_{veg}$	--- **Long-Term Vegetation**	No data found

Abbreviations for Tables 1 and 2: **HQ**, hazard quotient; **ppb**, parts per billion; **µg/m^3**, micrograms per cubic meter; **h**, hour; **AMCV**, air monitoring comparison value; **ESL**, Effects Screening Level; **ReV**, Reference Value; **^{acute}ESL**, acute health-based ESL; **$^{acute}ESL_{odor}$**, acute odor-based ESL; **$^{acute}ESL_{veg}$**, acute vegetation-based ESL; **$^{chronic}ESL_{nonlinear(nc)}$**, chronic health-based ESL for nonlinear dose-response noncancer effects; **$^{chronic}ESL_{linear(nc)}$**, chronic health-based ESL for linear dose-response noncancer effects; **$^{chronic}ESL_{linear(c)}$**, chronic health-based ESL for linear dose-response cancer effect; **$^{chronic}ESL_{nonlinear(c)}$**, chronic health-based ESL for nonlinear dose-response cancer effect; **$^{chronic}ESL_{veg}$**, chronic vegetation-based ESL

Table 2. Air Permitting Effects Screening Levels (ESLs)

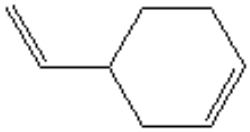
Short-Term Values	**Concentration**	**Notes**
$^{acute}ESL$ [1 h] (HQ = 0.3)	1700 µg/m^3 (390 ppb) [a]	**Critical Effect(s):** CNS effects observed in Sprague/Dawley rats and B6C3F$_1$ mice
$^{acute}ESL_{odor}$	510 µg/m^3 **Short-Term ESL for Air Permit Reviews**	Pungent, strong odor; 1,3-butadiene odor-based value used as a surrogate
$^{acute}ESL_{veg}$	---	No data found
Long-Term Values	**Concentration**	**Notes**
$^{chronic}ESL_{nonlinear(nc)}$ (HQ = 0.3)	97 µg/m^3 (22 ppb)[b]	**Critical Effect(s):** Lethargy/ tremor/lethality/ovarian atrophy in B6C3F$_1$ mice
$^{chronic}ESL_{nonlinear(c)}$ (HQ = 0.3)	97 µg/m^3 (22 ppb)[c] **Long-Term ESL for Air Permit Reviews**	**Critical Effect(s):** ovarian tumors in animals -
$^{chronic}ESL_{veg}$	---	No data found

[a] Based on the acute ReV of 5800 µg/m^3 (1300 ppb) multiplied by 0.3 (i.e., HQ = 0.3) to account for cumulative and aggregate risk during the air permit review.

[b] Based on the nonlinear noncarcinogenic chronic ReV of 330 µg/m^3 (74 ppb) multiplied by 0.3 (i.e., HQ = 0.3) to account for cumulative and aggregate risk during the air permit review.

[c] Based on the nonlinear carcinogenic chronic ReV of 330 µg/m^3 (74 ppb) multiplied by 0.3 (i.e., HQ = 0.3) to account for cumulative and aggregate risk during the air permit review.

Table 3. Chemical and Physical Data

Parameter	**Value**	**Reference**
Molecular Formula	C_8H_{12}	ChemFinder (2010)
Chemical Structure		ChemFinder (2010)
Molecular Weight	108.2 g/mole	(NIOSH 1995)
Physical State	Liquid	(NIOSH 1995)
Color	Colorless	(NIOSH 1995)
Odor	Pungent Strong Sweet aromatic	(MSDS 1989) (USHSED 2002) (AIHA 1991).
CAS Registry Number	100-40-3	(NIOSH 1995)
Synonyms	4-Ethynyl-1-cyclohexene; 4-ethenylcyclohexene, cyclohexenylethylene; butadiene dimer	(NIOSH 1995)
Solubility in water	50 mg/L	(USEPA 1996)
Log K_{ow}	3.93	(HCN 2008)
Vapor Pressure	15.7 mm Hg @ 25ºC	(IUCLID 2006)
Vapor Density (air = 1)	3.7	(NIOSH 1995)
Density (water = 1)	0.829	(NIOSH 1995)
Melting Point	-109 ºC	(NIOSH 1995)
Boiling Point	130ºC	(NIOSH 1995)
Conversion Factors	1 ppm = 4.43 mg/m^3 1 mg/m^3 = 0.23 ppm	Toxicology Division

Chapter 2 Major Sources and Uses

4-Vinylcyclohexene (VCH) is mainly used in organic synthesis of polymers and as an intermediate for the production of vinylcyclohexene dioxide, which is used as a reactive diluent in epoxy resins. It is a dimer of 1,3-butadiene (BD). It is used as a precursor for ethyl cyclohexyl carbinol plasticizers, as an intermediate for thiocyamate insecticides and as an antioxidant (HSDB 2005). VCH is a byproduct released during the production of styrene-butadiene rubber, styrene-butadiene latex, and polybutadiene rubber products and then subsequently recovered along with styrene for recycling and reuse in the process (IUCLID 2006).

VCH may be released to the environment from: volatilization from various waste streams or waste water treatment plants from organics and plastics plants and from rubber processing plants (IUCLID 2006); released into the air as fugitive emissions during downstream processing as a chemical intermediate; and may be present in styrene/butadiene/acrylonitrile copolymers used as a coating for food packaging. United States (US) production in 1975 was greater than 4.54×10^5 grams (HSDB 2005) or approximately 1000 pounds.

Chapter 3 Acute Evaluation

3.1 Health-Based Acute ReV and ^{acute}ESL

3.1.1 Physical/Chemical Properties

VCH is a clear colorless liquid with a pungent smell (MSDS 1989). Since VCH's vapor pressure is 15.7 mm Hg at 25^{o}C and it has a low molecular weight, it will readily volatilize and be present in the atmosphere as a vapor. It is denser than air. It is soluble in ether, benzene, and petroleum ether and miscible with methanol (HSDB 2005). It is slightly soluble in water. Other physical/ chemical properties can be found in Table 3.

3.1.2 Key Studies

3.1.2.1 Human Study

The only information concerning humans exposed to VCH was reported by American Congress of Governmental and Industrial Hygiene (ACGIH 1991, as reported in HSDB 2005). After inhaling mean VCH concentrations ranging from 271 to 542 parts per million (ppm) (with peak VCH concentrations to 677 ppm), Russian rubber workers were reported to suffer from keratitis, rhinitis, headache, and hypotonia. In addition, leukopenia, neutrophilia, lymphocytosis, and "impairment of pigment and carbohydrate metabolism" occurred.

3.1.2.2 Animal Studies

3.1.2.2.1 Key Animal Studies

3.1.2.2.1.1 Two-Day Inhalation Study (Bentley 1992; Bevan, Keller et al. 2001)

Bevan et al. (2001) reported on an inhalation study in rats and mice after 6-hour (h) exposures for 2 days [conducted by Bentley (1992)]. The purpose of the study was to investigate the effect of VCH on micronucleus formation in the bone marrow of rats and mice and was not a standard toxicity study. However, animals were observed daily during and after the exposure period for clinical signs of toxicity and data on body weight changes were reported. The purity of VCH was 99.6% and analytical concentrations were reported. There were no exposure-related deaths during the study.

The lowest-observed adverse-effect level (LOAEL) for central nervous system (CNS) effects (i.e., decreased responsiveness to sound stimulus, inactivity, and narcosis/sleep induction) was the lowest concentration of 500 ppm for a single exposure duration of 6 h and was observed in rats. A no-observed-adverse-effect level (NOAEL) was not identified. Decreases in body weight gain occurred only at 1000 ppm in mice and 2000 ppm in rats. Additional information is provided in the following sections.

Mice

Male and female B6C3F_1/CrBR mice (10/exposure group) were exposed to analytical mean chamber concentrations of 0 ppm (clean air) or 250, 490, and 1000 ppm VCH. Mice were also exposed to 1000 ppm BD, for comparison purposes, since VCH is a dimer of BD. Clinical signs of toxicity were not noted in mice. Body weight gain for the 1000 ppm VCH-exposed male mice were significantly less than controls 24-h post-exposure time (-1.1 ± 0.6 gram (g) vs. 0.7 ± 0.8 g, respectively; $p \leq 0.05$). Decreases in body weight gain were also observed in BD-exposed mice compared to controls (-0.2 ± 0.3 g vs 0.4 ± 0.8 g, respectively; $p \leq 0.05$). The no-observed adverse-effect level (NOAEL) for decreases in body weight gain was 490 ppm.

Rats

Male and female Cr1:CD BR (Sprague-Dawley) rats (10/exposure group) were exposed to mean chamber analytical concentrations of 0 ppm (clean air) or 500, 1000, and 2000 ppm VCH. In rats, body weights for the 2000 ppm VCH-exposed group were significantly lowered at both the 24- and 48-h post-exposure time points compared to controls. At 24-h, body weight gain in the 2000 ppm VCH-exposed rats compared to the controls was -0.6 ± 2.2 g vs. 20.7 ± 1.0 g ($p \leq 0.05$), respectively. At 48-h, body weight gain in the 2000 ppm VCH-exposed rats compared to the controls was 8.3 ± 2.7 g vs. 27.9 ± 1.3 g ($p \leq 0.05$), respectively.

Clinical signs were noted in rats and included decreased responsiveness to sound stimulus, inactivity, and narcosis/sleep induction during both exposures in each VCH treatment group. Animal arousal occurred within approximately 10 minutes after cessation of exposure. No clinical signs of toxicity were noted in rats prior to each exposure or during the recovery period.

3.1.2.2.1.2 Two-Week Inhalation Study (Stadler 1994a)

Rats and mice were exposed by inhalation to VCH 6 h/day, 5 days/wk for 2 wks, with one day of rest between each wk. There was a 3-day post-exposure period. The industry-sponsored study was conducted according to good laboratory practices according to test method EPA OTS 798.2450, purity of the substance was > 99.6%, and analytical concentrations were reported (Stadler 1994a). The TD identified a NOAEL of 240 ppm for both rats and mice for reversible lethargy (i.e., CNS effects). Lethargy was considered an adverse effect because tremors and other CNS effects were associated with mortality observed in the 2-wk study and both 13-wk inhalation and oral gavage studies which are discussed in Section 4.1.

Mice

B6C3F1 mice (5/sex/dose level) were exposed by inhalation to 0 ppm (clean air) or analytical concentrations of 240, 720, or 1500 ppm VCH. All groups of mice, including controls, lost weight over study days 1-3. This effect was particularly marked in both sexes of the 1500 ppm exposed (high dose) animals. Mice lost 18-20% of their initial body weight (statistically significant) during this time. Mice from the control, low and mid dose groups exhibited inconsistent increases in body weight.

All males exposed to 1500 ppm, and 4/5 high dose females, were found dead on study day 4. The remaining high dose females were sacrificed in extremis on study day 4. Tremor was present in 7/10 mice on study day 3, and was considered by the report as a significant feature preceding death. The NOAEL for body weight loss, tremor, and death was 720 ppm for males and females. Reversible lethargy was seen in all mice exposed to 720 and 1500 ppm after removal from the exposure chambers. The NOAEL for reversible lethargy, considered to be a CNS effect, was 240 ppm.

Rats

Male and female Sprague-Dawley rats (5/sex/dose level) were exposed to VCH by inhalation to 0 ppm (clean air), or analytical concentrations of 240, 720 and 1500 ppm VCH. Mean body weight gain over study days 1-11 was significantly decreased in high-dose males exposed to 1500 ppm relative to controls, but not significantly decreased in high-dose females. Final body weights were not significantly decreased in any exposure groups. One mid-dose female exposed to 720 ppm VCH was found dead on study day 2 (presumed unrelated to treatment) and replaced. All other animals survived to the end of the recovery period. Based on body weight gain over study days 1-11, a NOAEL of 720 ppm was derived for male rats and 1500 ppm for females.

Reversible lethargy was noted in all rats exposed to 720 and 1500 ppm (mid- and high-dose groups) following removal from the exposure chambers. Tremor affecting 1/10 rats exposed to 240 ppm and 3/10 rats exposed to 1500 ppm was observed on study day 3 only but was absent on other occasions. A NOAEL of 240 ppm based on lethargy (i.e., CNS effects) was derived for male and female rats.

3.1.2.2.2 Other Studies

3.1.2.2.2.1 Acute Inhalation Lethality Study (Smyth 1962, 1969)

Smyth (1962; 1969) as cited in IUCLID (2006) conducted acute inhalation lethality tests for over 200 compounds, including VCH, for screening purposes only. Therefore, the methods used were not well documented, although according to IUCLID (2006), the results are acceptable for assessment. Groups of six male or female albino rats (specific gender not provided) were exposed for 4 h to a nominal concentration of 8000 ppm VCH then observed for a 14-day follow-up period. A specific concentration producing 50% lethality in rats (LC_{50}) was not provided, but VCH exposure killed four of the six exposed rats.

3.1.2.2.2.2 Fourteen-Day Oral Gavage Study (NTP 1986)

Necropsies and histological examinations were not conducted in the Bevan et al. (2001) or Stadler (1994a) inhalation studies. However, in a 14-day oral gavage study (NTP 1986), necropsies were performed on all animals (macroscopic observations only) and histological examinations of the stomach were reported, which is the reason the results from this oral study are included in this section. *No compound-related gross changes were noted at necropsy.* No microscopic lesions were detected in the stomach, the only organ in which histologic examinations were performed.

- Groups of 5 male and 5 female $B6C3F_1$ mice were administered VCH (>99% pure) in corn oil by gavage at doses of 0, 300, 600, 1250, 2500 or 5000 mg/kg body weight/day.
- Groups of 5 male and 5 female F344 rats were administered VCH (>99% pure) in corn oil by gavage at doses of 0, 300,600, 1250, 2500 or 5000 mg/kg body weight/day.

All rats that were exposed to 1250, 2500, or 5000 mg/kg died before the end of the studies whereas 3/5 male mice that received 1250 mg/kg and all mice that received 2500 or 5000 mg/kg died before the end of the studies. Rats who died were inactive, wet in the perianal region, and had tremors, soft stools, and an unsteady gait. In mice that died, tremors and inactivity were observed.

3.1.2.2.2.3 Reproductive/Developmental Studies

No short-term inhalation developmental studies are available after exposure to VCH. Refer to Section 4.1.2.2 for information on an oral gavage study that assessed reproductive effects in Swiss (CD-1) mice using a continuous breeding protocol (conducted by NTP (1989a; NTP 1991)

and reported by Grizzle et al. (1994)). No adverse effects were reported on pregnancy or pre- and post-natal development following exposure via oral gavage to two generations of pregnant female $B6C3F_1$ mice to VCH, at doses up to 500 mg/kg body weight/day. These results provide data that indicate VCH may not be fetotoxic or teratogenic in the mouse.

VCH exposure reduced the number of primordial, growing and antral follicles in the ovaries of females and slightly reduced spermatid count in males after repeated exposures as discussed in greater detail in Section 4.1. Refer to Appendix A for a summary of the reproductive effects of VCH (Table 5.8 from USEPA 2002). USEPA (2002) reviewed and summarized VCH's reproductive studies when they conducted their Health Assessment of 1,3-Butadiene (USEPA 2002). Most of these studies were repeat intraperitoneal (i.p.) injection or oral studies.

3.1.3 Mode-of-Action (MOA) Analysis and Dose Metric

Effects occurring at the lowest concentration are CNS effects (i.e., decreased responsiveness to sound stimulus, inactivity, and narcosis/sleep induction). The MOA for CNS effects has not been clearly established but may be related to solvent effects on neurological membranes.

In the 2-day and 2-wk studies selected as the key studies (Bentley 1992; Stadler 1994a; Bevan, Keller et al. 2001), data on the exposure concentration of the parent chemical are available. Since the MOA of the toxic response is not fully understood and data on other more specific dose metrics more closely related to the critical effects are not available, the exposure concentration of the parent chemical was used as the default dose metric.

Narcosis and/or neurological effects are assumed to have a threshold or nonlinear dose-response relationship and to be relevant to humans. There is not enough data to determine whether duration plays an important role in producing CNS effects in addition to concentration.

3.1.4 Point of Departure (POD) for Key Study and Dosimetric Adjustments

The critical effect is lethargy, which is indicative of CNS effects. Rats were more sensitive than mice to the CNS effects of VCH after an acute 2-day exposure (LOAEL of 500 ppm, NOAEL not identified) (Bevan et al. 2001). CNS effects were not observed in rats or mice at 240 ppm after exposure for two wks (LOAEL of 720 ppm) (Stadler 1994a).

If the LOAEL of 500 ppm from the 2-day Bevan et al. (2001) study was used as the POD, it would be divided by 3 or 10 to estimate a NOAEL of either 170 or 50 ppm. Both these values would be too conservative, as demonstrated by the observed NOAEL of 240 ppm determined from the 2-wk Stadler (1994a) study. The most appropriate POD is the NOAEL of 240 ppm (Stadler 1994a) with the critical effect being lethargy, even though it is based on a 2-wk study. Benchmark dose modeling was not conducted for this endpoint since incidence of lethargy was 0% at the NOAEL of 240 ppm but was 100% at the mid- and high-dose groups (i.e., data are not amenable to dose-response modeling).

3.1.4.1 Default Exposure Duration Adjustments

Since there is not enough data to determine whether concentration and duration both play a role in the CNS effects caused by VCH, the POD of 240 ppm at a 1-h exposure duration is assumed to be equal to the 6-h exposure duration POD of 240 ppm (i.e., the POD_{ADJ} for 1 h is assumed to be 240 ppm).

3.1.4.2 Default Dosimetry Adjustments from Animal-to-Human Exposure

VCH causes systemic CNS effects rather than point-of-entry respiratory effects. Thus, VCH is considered a Category 3 vapor (USEPA 1994). For Category 3 vapors, the default dosimetric adjustment from animal-to-human exposure is conducted using the following equation:

$$POD_{HEC} = POD_{ADJ} \times [(H_{b/g})_A / (H_{b/g})_H]$$

where:

$H_{b/g}$ = ratio of the blood:gas partition coefficient

A = animal

H = human

The blood:gas partition coefficient in animals ($(H_{b/g})_A$) divided by the blood:gas partition coefficient in humans ($(H_{b/g})_H$) is the regional gas dose ratio (RGDR) (USEPA 1994). For VCH, the $(H_{b/g})_A$ for rat is 16.7 and for mice is 20.1 (Keller 1993) but the blood:gas partition coefficient for humans ($(H_{b/g})_H$) is unknown. Therefore, a default value of one is used for $(H_{b/g})_A / (H_{b/g})_H$.

$$POD_{HEC} = POD_{ADJ} \times RGDR$$
$$= 240 \text{ ppm} \times 1 = 240 \text{ ppm}$$

3.1.5 Critical Effect and Adjustments of the POD_{HEC}

The critical effect is CNS effects in rats and mice and is considered to be relevant to humans and to have a threshold with a nonlinear dose-response relationship. The following uncertainty factors (UFs) were applied to the POD_{HEC} of 240 ppm to derive a reference value (ReV): 10 for intraspecies variability (UF_H); 3 for extrapolation from animals to humans (UF_A); and 6 for database uncertainty (UF_D), for a total UF of 180:

- A UF_H of 10 was used to account for variation in sensitivity among members of the human population.
- A UF_A of 3 was used for extrapolation from animals to humans because default dosimetric adjustments from animal-to-human exposure were conducted, which account for toxicokinetic differences but not toxicodynamic differences.
- Two-day and 2-wk inhalation studies in rats and mice were available but these studies did not examine a wide range of toxicity endpoints after inhalation exposure to VCH, although oral gavage studies provide additional data on endpoints that were not evaluated in the inhalation

studies. An oral gavage chronic reproductive study in mice indicated that VCH does not significantly affect reproductive capability and does not appear to be fetotoxic or teratogenic. However, short-term inhalation developmental studies that examined a wide range of developmental effects were not available. Therefore, a UF_D of 6 was used. The confidence in the acute database is medium.

acute ReV = $POD_{HEC} / (UF_H \times UF_A \times UF_D)$
= 240 ppm / (10 x 3 x 6)
= 1.333 ppm
= 1333 ppb

3.1.6 Health-Based Acute ReV and ^{acute}ESL

The resulting 1-h acute ReV is 1300 ppb (5800 µg/m^3), rounded to two significant figures at the end of all calculations. The rounded acute ReV was then multiplied by 0.3 to calculate the 1-h ^{acute}ESL. At the target hazard quotient of 0.3, the 1-h ^{acute}ESL is 390 ppb (1700 µg/m^3) (Table 4).

Table 4. Derivation of the Acute ReV and ^{acute}ESL

Parameter	**Summary**
Study	Bevan et al. (2001), Bentley (1992), and Stadler (1994a)
Study population	Sprague/Dawley rats and B6C3F$_1$ mice
Study quality	Medium
Exposure methods	Inhalation exposure to 240, 720 or 1500 ppm for 6 h/day for two wks (Stadler 1994a) Inhalation exposure to 500, 1000, and 2000 ppm for 6 h/day for two days (Bevan et al. 2001; Bentley 1992)
Critical effect	CNS effects (lethargy, decreased responsiveness to sound stimulus, inactivity, and narcosis/sleep induction)
LOAEL	500-720 ppm
POD	240 ppm (NOAEL)
Exposure duration	6 h
Extrapolation to 1 h	No adjustment
POD_{ADJ}	240 ppm
POD_{HEC}	240 ppm
Total uncertainty factors (UFs)	180
Intraspecies UF	10
Interspecies UF	3
LOAEL UF	Not applicable
Incomplete Database UF *Database Quality*	6 Medium
acute ReV [1 h] (HQ = 1)	**5800 µg/m3 (1300 ppb)**
acuteESL [1 h] (HQ = 0.3)	**1700 µg/m3 (390 ppb)**

3.2 Welfare-Based Acute ESLs

3.2.1 Odor Perception

The National Institute for Occupational Safety and Health International Chemical Safety Card (NIOSH 1995) states that VCH has a pungent odor. The United Steelworkers Health, Safety &

Environment Department states that VCH has a strong odor (USHSED 2002). The Workplace Environmental Exposure Level Guide states that VCH has a sweet aromatic odor that is very evident at 500 ppb (AIHA 1991). Since VCH has a pungent, disagreeable odor, an $^{acute}ESL_{odor}$ of 510 µg/m^3 was set for VCH based on the $^{acute}ESL_{odor}$ for 1,3-butadiene (TCEQ 2008), a structurally similar diene (TCEQ 2015).

3.2.2 Vegetation Effects

There is no available data evaluating the effects of VCH in vegetation.

3.3 Short-Term ESL

The acute evaluation resulted in the derivation of the following values:

- acute ReV = 5800 µg/m^3 (1300 ppb)
- $^{acute}ESL$ = 1700 µg/m^3 (390 ppb)
- $^{acute}ESL_{odor}$ = 510 µg/m^3

The short-term ESL for air permit evaluations is the $^{acute}ESL_{odor}$ of 510 µg/m^3 since it is lower than the health-based $^{acute}ESL$ of 1700 µg/m^3 (390 ppb) (Table 2).

Chapter 4 Chronic Evaluation

4.1 Noncarcinogenic Potential

4.1.1 Physical/Chemical Properties

For physical/chemical properties, refer to Section 3.1.1 and Table 3.

4.1.2 Key and Supporting Studies

4.1.2.1 Key Study (Bevan et al. 1996)

Bevan et al. (1996) reports on a subchronic inhalation study conducted by Stadler (1994b). The study was conducted using EPA Good Laboratory Practice Standards (40CFR792). Groups of 10 male or female Sprague-Dawley rats or B6C3F$_1$ mice per concentration were exposed for 6 h/day, 5 days/wk for 13 wks using whole-body exposure. VCH and BD concentrations were:

- for rats: 0, 250, 1000, or 1500 ppm VCH (0, 250, 1000, 1500 ppm analytical);
- for mice: 0, 50, 250, or 1000 ppm VCH (0, 53, 250, 1000 ppm analytical).
- another group of rats and mice: 1000 ppm BD (980 ppm analytical) for comparison.

A range of endpoints was evaluated: clinical observations, clinical pathology (i.e., hematology, serum chemistry), body weights, food consumption, urinary analysis, organ weights,

macroscopic and microscopic pathology. According to Bevan et al. (1996), the following effects were observed:

> "Exposure to 1000 ppm VCH resulted in deaths of all male mice and 5/10 female mice on Test Days 11 or 12. Three additional mice exposed to 1000 ppm VCH died prior to study completion. The most notable compound-related clinical sign was lethargy observed in the 1500 ppm VCH-exposed rats and 1000 ppm VCH-exposed mice. Male rats exposed to 1500 ppm VCH had significantly lower body weights compared to controls, and male and female rats in the 1500 ppm group had significantly lower body weight gains. None of the VCH-exposed animals or butadiene-exposed rats showed any compound related hematological effects. However, mice exposed to 1000 ppm butadiene exhibited mild macrocytic anemia. Clinical chemistry evaluation and urinalysis showed no compound-related effects in rats exposed to either VCH or butadiene. Male and female rats exposed to 1000 or 1500 ppm VCH or 1000 butadiene had increased absolute and/or relative liver weights, and male rats in these same exposure groups had increased relative kidney weights. Microscopically, increased accumulation of hyaline droplets was observed in the kidneys of male rats from all VCH exposure groups. Although compound-related, the droplets were not accompanied by cytotoxicity. In mice, the most notable adverse histopathological effect was ovarian atrophy in females exposed to 1000 ppm VCH or 1000 ppm butadiene. The atrophy was slightly more severe in the VCH-exposed females than in the butadiene-exposed females. There were no other compound-related pathological effects in male or female mice exposed to VCH. Additionally, butadiene-exposed male mice had decreased testicular weights, accompanied by slight testicular degeneration and atrophy. For VCH exposure, the no-observed-adverse-effect level is 1000 ppm for rats based on lethargy and lowered body weights and 250 ppm for mice based on mortality and ovarian atrophy."

Stadler (1994b) provided additional clinical observations of mice that died. Some of the mice that were still alive in the 1000 ppm exposure group on test day 10 displayed signs of tremors while in the inhalation chambers. Collins and Manus (1987) also observed tremors and inactivity in mice and CNS depression and tremors in rats that died when animals were exposed to VCH via oral gavage. There were no deaths in mice exposed to 1000 ppm BD.

Minimal to mild ovarian atrophy was present in 5/10 female mice (5/5 mice that survived in study beyond test day 12). This was characterized by a paucity of all developmental stages of ovarian follicles and was slightly more severe in the VCH group as compared to the BD-exposed animals. Collins and Manus (1987) also observed ovarian atrophy in mice exposed to VCH via oral gavage in a 13-wk study. Ovarian atrophy was also noted in 2/10 female rats exposed to 1500 ppm VCH, although the primary change was a decrease in the numbers of corpora lutea

and was morphologically distinct from that seen in mice. Effects on the testes in male rats could not be evaluated at 1000 ppm because all male mice died on test day 11 and 12, but adverse testicular effects were not observed at 250 ppm.

The subchronic LOAEL in mice for minimal to mild ovarian atrophy, lethargy, and tremor/mortality was 1000 ppm and the NOAEL was 250 ppm. In rats, the subchronic LOAEL for lethargy and decreased body weight and weight gain was 1500 ppm and the NOAEL was 1000 ppm. There were significant increases in liver and kidney weights in rats at 1000 and 1500 ppm, but there was no indication of liver or kidney damage relevant to humans (i.e., hyaline droplet formation associated with α_{2u} globulin in the kidney were observed in male rats) based on histological evaluations or clinical chemistry.

4.1.2.2 Reproductive Study (Grizzle et al. 1994)

An oral gavage study that assessed reproductive effects in Swiss (CD-1) mice using a continuous breeding protocol was reported by Grizzle et al. (1994) [conducted by NTP (1989a; NTP 1991)]. Mice were administered VCH in corn oil by oral gavage at doses of 0, 100, 250, and 500 mg/kg/day for 18 wks. Reproductive competence (litters/pair, pups/litter, percent born alive) was not affected and neither was consumption of food or water. The following results were observed:

- decreased spermatid head count (with normal sperm number, normal testis, and epididymal weight) was observed in the second generation (F_1) males given 500 mg/kg;
- decreased numbers of primordial, growing, and antral follicles were observed in F_1 females given 500 mg/kg; and
- The gamete pool in both the ovary (markedly) and testis (slightly) was reduced at the highest dose of 500 mg/kg/day, a dose that produced slight generalized toxicity (i.e., slight decreases in body weight in the F_0 and F_1 generation). However, these gamete pool reductions did not have significant adverse effects on the ability to reproduce in either the F_0 or F_1 generation.

Grizzle and colleagues (1994) concluded that VCH exposure in CD-1 mice did not alter reproductive function in F_0 or F_1 generation up to 500 mg/kg/day, even though decreased body weight and reduction in the numbers of gametes were observed. The NOAEL was 250 mg/kg/day.

4.1.3 Mode-of-Action (MOA) Analysis

4.1.3.1 MOA for Lethargy and Tremors/Mortality

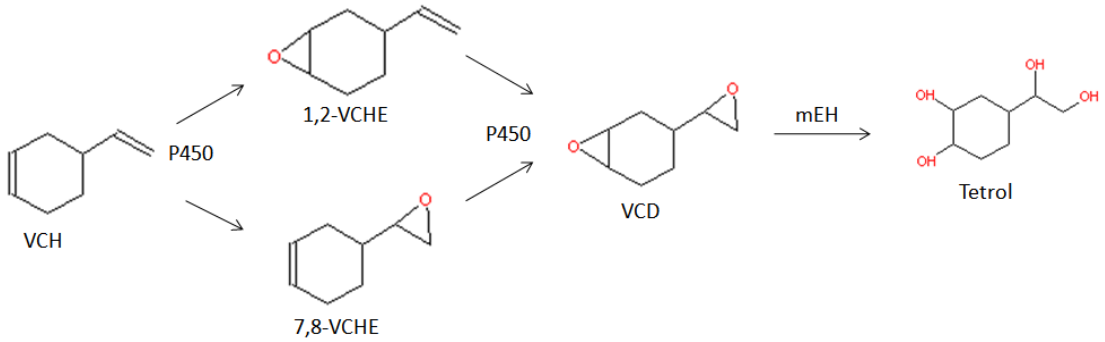
Significant compound-related mortality preceded by tremors and lethargy was observed in mice exposed to 1000 ppm VCH. Bevan et al. (1996) stated that there were no compound-related gross or microscopic lesions observed, so the specific cause of death was not identified. However, Stadler (1994a, 1994b) and Grizzle et al. (1994) reported that lethargy, CNS depression, and tremors occurred in both mice and rats exposed to VCH preceding VCH-induced

mortality. The MOA for lethargy and tremors/mortality is unknown, but appears to be due to CNS effects. Lethargy and tremor/mortality are considered to have a nonlinear MOA and be relevant to humans.

4.1.3.2 Ovarian Atrophy

4.1.3.2.1 Metabolism

In rat and mouse liver microsomes, VCH is metabolized by microsomal cytochrome (CYP) 450 enzymes to either a 1,2 or 7,8-monoepoxide (1,2-VCHE or 7,8-VCHE) and subsequently to vinylcyclohexene diepoxide (VCD) (Gervasi, Abbondandolo et al. 1980; Watabe, Hiratsuka et al. 1981) (Figure 1). The rate of epoxidation of VCH (1 mM) to 1,2-VCHE was 6.5 fold greater in mouse liver microsomes than in rat liver microsomes (Smith et al. 1990a). The rate of 1,2-VCHE formation by female human liver microsomes was 13- and 2-fold lower than that observed in mice and rats, respectively.



4-vinylcyclohexene (VCH); VCH-1,2-epoxide (1,2-VCH); VCH-7,8-epoxide (7,8-VCHE); VCH-diepoxide (VCD); 1,2-dihydroxyethyl-1,2-dihydrixycyclohexane (Tetrol)

Figure 1. Metabolism of VCH

Pathways for bioactivation of VCH to VCD and subsequent detoxification of VCD by microsomal epoxide hydrolase (mEH)

Giannarini et al. (1981) showed that i.p. administration at 500 mg/kg of VCH or VCHE to male Swiss mice induced a number of hepatic xenobiotic biotransforming enzymes involved in the metabolism of these compounds, including CYP 450, cytochrome b_5, NADPH-cytochrome c reductase, aminopyrine N-demethylase, and epoxide hydrolase. It appears that CYP2A and CYP2B play a role in the epoxidation of VCH in the liver, but not CYP2E1 (Fontaine, Hoyer et al. 2001; Fontaine, Hoyer et al. 2001).

Fontaine and colleagues (2001) investigated induction of CYP 450 enzymes involved in VCH and 1,2-VCHE metabolism due to repeated exposure in mice and rats. Repeated exposure resulted in induction of CYP P450 enzymes involved in its bioactivation. Total hepatic CYP levels were elevated only in microsomes from mice pretreated with VCH and 1,2-VCHE. Immunoblotting analysis of microsomes from VCH-treated rodents revealed elevated levels of CYP2A and CYP2B in mice but not rats. 1,2-VCHE pretreatment also increased CYP2B levels in the mouse. Activities toward specific substrates for CYP2A and CYP2B (coumarin and pentoxyresorufin, respectively) confirmed that VCH and 1,2-VCHE pretreatments increased catalytic activities of CYP2A and CYP2B in the mouse but not the rat. These data indicate that induction of CYP enzymes increase the metabolism of VCH to reactive metabolites in mice, but not rats.

The liver is the primary site of activation of VCH, although Keller et al. (1997) showed that both rat and mouse liver and lung microsomes metabolized VCH to 1,2-VCHE and 1,2-VCHE to VCD at detectable rates. Microsomes from ovarian tissue were not able to metabolize VCH. However, Cannady et al. (2003) demonstrated that mRNA and catalytic activity for CYP2A and CYP2B could be induced in the ovary after repeated exposure to VCH and VCD. Therefore, the ovary may participate in the metabolism of VCH or its metabolites after enzyme induction.

Hydrolysis by epoxide hydrolases and/or conjugation with glutathione catalyzed by glutathione S transferases is known to be important in epoxide degradation. This appears to be the case for the epoxides of VCH. Smith, Mattison, and Sipes (1991a) determined the rate of 1,2-VCHE hydrolysis to its corresponding dihydrodiol (Figure 1) by hepatic cytosol or microsomes to assay the activity of epoxide hydrolases toward 1,2-VCHE. Although little enzymatic activity was present in hepatic cytosol from either species, hepatic microsomal hydrolysis of 1,2-VCHE correlated well with protein concentration and incubation time in both species. The rate of hydrolysis was almost completely inhibited by the epoxide hydrolase inhibitor 3,3,3-trichloropropene oxide. Smith, Mattison, and Sipes (1991a) also compared the rate of hydrolysis of 1,2-VCHE using female mouse and rat hepatic microsomes. Under the conditions of the assay, rat microsomes catalyzed the hydrolysis of 1,2-VCHE at a 2-fold greater rate compared to mouse microsomes. In the human microsome studies conducted by Smith et al (1990c), epoxide hydrolase inhibition was required in order to detect the appearance of VCD, suggesting the presence of significant epoxide hydrolase activity toward VCD in humans. This may indicate that the rat is the more appropriate animal model for extrapolation of animal data. Cannady et al. (2002) demonstrated that mRNA and catalytic activity for epoxide hydrolase in ovarian follicles could be induced after repeated exposure to VCH and VCD.

VCD and 1,2-VCHE have been shown to be substrates for glutathione S-transferase. Giannarini et al. (1981) showed that hepatic glutathione levels in mice were reduced after oral doses of 500 mg/kg of VCH, VCH monoepoxides, or VCH diepoxide, which indicate that glutathione is probably involved in the metabolism/detoxification of VCH. Devine, Sipes, and Hoyer (2001) investigated the effects of an i.p. injection of VCD in rats. Animals were euthanized 2, 6, or 26 h

following a single dose, and 2 or 26 h following 15 days of dosing. Reduced hepatic GSH was seen within 2 h of a single dose or 2 h after 15 daily doses of VCD, but ovarian GSH was not depleted. The authors concluded that alterations in ovarian GSH levels were not involved in VCD-induced ovotoxicity.

4.1.3.2.2 Toxicokinetics

The toxicokinetics of VCH have not been investigated after inhalation exposure. However, Smith, Carter, and Sipes (1990a) investigated the disposition after a single oral dose of 400 mg/kg [C^{14}]VCH in female B6C3F_1 mice and Fisher 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 a parent compound or as radioactive equivalents. A dramatic difference was observed between the rat and mouse in the appearance of a monoepoxide 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 by liver microsomes. The rate of epoxidation of VCH (1 mM) to VCH-1,2-epoxide was 6.5 fold greater in mouse liver microsomes than that in rat liver 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."

Toxicokinetic results from an oral study may not be relevant to inhalation exposure.

4.1.3.2.3 MOA for Ovarian Atrophy

Mice exposed to 1000 ppm VCH developed minimal to mild ovarian atrophy including follicle loss. Rats, on the other hand, did not develop ovarian atrophy after exposure to higher VCH concentrations of 1500 ppm (Bevan et al. 1996; Stadler 1994b). This species-specific difference in susceptibility to VCH ovotoxicity is thought to be largely due to differences in the formation of VCH epoxides. Smith et al. (1990b) reported the epoxide metabolites of VCH are more potent ovotoxicants than VCH, the parent compound. The dose which reduced the small oocyte count by 50% in mice (ED_{50}) is as follows:

- 2.7 mmol/kg for VCH,
- 0.5 mmol/kg for 1,2-VCHE,

- 0.7 mmol/kg for 7,8-VCHE, and
- 0.2 mmol/kg for VCD.

The diepoxide appears to be the metabolite responsible for ovarian atrophy and follicle destruction. Doerr *et al.* (1995) and Doerr and Sipes (1996) evaluated the ovarian effects of the metabolites of VCH in mice and rats and also examined BD, a structurally similar compound known to induce ovarian atrophy. Doerr *et al.* (1995, 1996) showed that the VCH diepoxide or the BD diepoxide was required for ovarian toxicity to occur in the rat.

As early as 1991, Smith et al. (1991a) theorized that VCH is metabolized in the liver to a reactive intermediate that is delivered to the ovary by the blood. The subsequent reactions which occur in the ovary cause oocyte destruction. Hoyer (2010) provided the most up-to-date discussion of the MOA for the ovotoxicity caused by VCD at a 2010 Society of Toxicology (SOT) meeting which was based on several well-conducted mechanistic studies (1976; Springer, Flaws et al. 1996; Springer, McAsey et al. 1996; Springer, Tilly et al. 1996; Hoyer, Cannady et al. 2001; Hoyer, Devine et al. 2001; Hu, Christian et al. 2001a; Hu, Christian et al. 2001b; Hu, Flaws et al. 2002; Hoyer and Sipes 2007):

> "The occupational chemical 4-vinylcyclohexene (VCH) has been shown to cause destruction of small pre-antral (primordial and primary) follicles in ovaries of mice. Further, its monoepoxide (VCMEs) and diepoxide (VCD) metabolites have been shown to cause pre-antral follicle loss in rats as well as mice. Chemicals that destroy small pre-antral follicles are of concern to women because exposure can result in premature ovarian failure (early menopause). Animal studies working with this chemical have determined that VCD is the bioactive form, and that it selectively targets primordial and primary ovarian follicles and requires repeated exposure. Mechanistic studies in rats have determined that VCD causes ovotoxicity by accelerating the natural process of atresia (apoptosis), and this can result in early ovarian failure. Insight has been gained into several ovarian intracellular pathways with which VCD interacts. Pro-apoptotic signaling events in the Bcl-2 proto-oncogene and mitogen activated protein kinase (MAPK) families are selectively activated in isolated fractions of small pre-antral follicles collected from rats dosed (15d) with VCD. Furthermore, an in vitro system using cultured whole ovaries from neonatal rats has been developed to expand the potential for more mechanistic investigations into VCD-ovarian interactions. By this approach, activity of the cell survival c-kit (oocyte-associated)/kit ligand (granulosa cell-associated) pathway has been shown to be reduced by VCD exposure. Specifically, the direct initiating event appears to occur within the oocyte involving post-transcriptional decreases in intracellular signaling by c-kit via its downstream mediators PI3kinase, Akt, and Foxo3a. Additionally, studies using this culture

system have uncovered a possible role for ovarian metabolism in modulating ovotoxic effects of VCD."

4.1.3.2.4 Relevance to Humans

A document entitled "4-Vinylcyclohexene: Proposed Alternative Acute and Chronic ReV and ESL Values" was prepared for International Specialty Products and submitted to the Texas Commission on Environmental Quality (The Sapphire Group 2008). Sections 5.0 and 5.1 provide an evaluation of the MOA by which VCH produces ovarian atrophy and ovarian tumors and whether these effects are relevant to humans using the International Programme on Chemical Safety (IPCS) Human Relevance Framework (Meek, Bucher et al. 2001; Boobis, Cohen et al. 2006). The Sapphire Group (2008) examined the weight of evidence; strength, consistency, specificity of association; dose-response concordance; temporal relationship; and biological plausibility and coherence. They also addressed whether key events in the animal MOA are plausible in humans. These findings were reported at the 2009 SOT meeting by Bevan et al. (2009), but have not yet been published. Therefore, Sections 5.0 and 5.1 are provided in Appendix B. Based on an independent critical review of the scientific evidence, the TD considers ovarian atrophy observed in mice that metabolize VCH to a reactive diepoxide to a much greater extent than humans to be relevant to humans. However, humans are expected to be much less susceptible to the ovarian effects than mice, given comparable doses because they produce much less of the reactive diepoxide metabolite.

4.1.4 Dose Metric

4.1.4.1 Lethargy and Tremor/Mortality

The MOA for lethargy and tremor/mortality is not known, although adverse effects on the CNS appear to play a role. Exposure concentration of VCH will be used as the dose metric. Concentration appears to play a greater role in CNS effects which lead to tremor/mortality, but duration plays a role as demonstrated by the following experimental data in mice:

- Clinical signs of toxicity were not noted in mice exposed to 250, 490, and 1000 ppm VCH for 2 days (Bentley 1992; Bevan, Keller et al. 2001), a NOAEL of 1000 ppm.
- The NOAEL for lethargy was 240 ppm and the LOAEL was 720 ppm in the 2-wk study (Stadler 1994b) and for mortality, the NOAEL was 720 ppm with a LOAEL of 1500 ppm.
- The NOAEL for lethargy and mortality was 250 ppm and the LOAEL was 1000 ppm in the 13-wk study (Bevan, Stadler et al. 1996).

4.1.4.2 Ovarian Atrophy

For ovarian atrophy, area under the curve blood levels of VCH monoepoxides or diepoxide would be an appropriate dose metric based on the MOA of VCH, but experimental data or a physiological-based pharmacokinetic (PBPK) model are not available, so exposure concentration

of the parent chemical will be used as the default dose metric. It is assumed that both concentration and duration play a role for the critical effect of ovarian atrophy.

4.1.5 Point of Departure (POD) for Key Study and Dosimetric Adjustments

Based on the Bevan et al. (1996) and Stadler (1994b) 13-wk subchronic mouse study, the LOAEL for minimal to mild ovarian atrophy, lethargy, and tremor/mortality was 1000 ppm and the NOAEL was 250 ppm. Benchmark dose modeling was not conducted because adverse effects were only observed at the highest concentration of 1000 ppm. For mortality, 18/20 mice died after exposure to 1000 ppm, a 90% mortality rate. Only 5 female mice lived past days 11 or 12, and these mice all developed ovarian atrophy (i.e., 100% of the five surviving animals developed ovarian atrophy). These data are not amenable to benchmark dose modeling. The NOAEL of 250 ppm is the POD.

4.1.5.1 Default Exposure Duration Adjustments

The mice used in the key study were exposed to VCH for 6 h/day, 5 days/wk. The study POD was adjusted from a discontinuous animal exposure scenario to a continuous exposure scenario POD_{ADJ} by using the following equation:

$$POD_{ADJ} = POD \times (D/24\ h) \times (F/days)$$

where:

D =Exposure duration, h/day
F = Exposure frequency, days per wk
POD_{ADJ} = 250 ppm x (6/24 h) x (5/7 days)
POD_{ADJ} = 44.64 ppm

4.1.5.2 Default Dosimetry Adjustments from Animal-to-Human Exposure

VCH causes lethargy, tremor/mortality, and minimal to mild ovarian atrophy which are systemic rather than point-of-entry respiratory effects. VCH was therefore considered a Category 3 vapor (USEPA 1994). As discussed previously in Section 3.1.3.2, the default RGDR is 1 and the POD_{HEC} is calculated using the following equation:

$$POD_{HEC} = POD_{ADJ} \times RGDR$$

$$= 44.64\ ppm \times 1 = 44.64\ ppm$$

4.1.6 Adjustments of the POD_{HEC} and Critical Effect

4.1.6.1 Uncertainty Factors (UFs)

4.1.6.1.1 Lethargy/Tremor/Mortality

Exposure to VCH results in lethargy and tremor/mortality, which are effects assumed to have a threshold. Therefore, UFs were applied to the POD_{HEC} to derive a ReV (i.e., assume a nonlinear MOA). The following UFs were applied to the POD_{HEC} of 44.64 ppm to calculate the chronic ReV: 10 for UF_H, 3 for UF_A, 3 for a subchronic to chronic UF (UF_{Sub}), and 6 for UF_D, for a total UF = 600:

- A UF_H of 10 was used to account for variation in sensitivity among members of the human population.
- A UF_A of 3 was used for extrapolation from animals to humans because default dosimetric adjustments from animal-to-human exposure were conducted, which account for toxicokinetic differences but not toxicodynamic differences.
- A UF_{Sub} of 3 instead of 10 was used to account for the uncertainty of using a subchronic study to calculate a chronic value since the possible MOA and experimental data indicate that concentration plays a greater role in toxicity than duration (i.e., the animal PODs for neurological effects for the acute ReV of 240 ppm and the chronic ReV of 250 ppm are similar);
- A UF_D of 6 was used. A 13-wk inhalation study in rats and mice was available but the highest concentration tested, which was the LOAEL, was well above the maximum tolerated concentration. Although a two-generation oral gavage reproductive study in mice did not indicate reproductive effects (Grizzle et al. 1994), inhalation studies investigating two-generation reproductive effects as well as inhalation developmental studies are not available. Confidence in the database is medium.

$$\text{chronic ReV} = POD_{HEC} / (UF_H \times UF_A \times UF_{Sub} \times UF_D)$$
$$= 44.64 \text{ ppm} / (10 \times 3 \times 3 \times 6)$$
$$= 0.0744 \text{ ppm}$$
$$= 74 \text{ ppb (rounded to two significant figures)}$$

4.1.6.1.2 Ovarian Atrophy

Exposure to VCH results in ovarian atrophy, which is an effect that is assumed to have a threshold. Therefore, UFs were applied to the POD_{HEC} to derive a ReV (i.e., assume a nonlinear MOA). The following UFs were applied to the POD_{HEC} of 44.64 ppm for ovarian atrophy: 10 for UF_H, 1 for UF_A, 10 for UF_{Sub}, and 6 for UF_D, for a total UF = 600:

- A UF_H of 10 was used to account for variation in sensitivity among members of the human population.

- A UF_A of 1 was used for extrapolation from animals to humans because of the following data:
- Experimental data indicate mice produce more VCD than humans as discussed in Section 4.1.3.2.1 Metabolism and in Appendix B (The Sapphire Group 2008). VCD is the reactive metabolite responsible for ovarian atrophy.
- VCH is structurally similar to BD and is metabolized similarly. The TD has determined that a UF_A of 1 is defensible for BD for ovarian atrophy (TCEQ 2008; Grant, Haney et al. 2010), and experimental data on the species differences in metabolism of VCH support the same UF_A of 1.
- A UF_{Sub} of 10 was used because minimal to mild ovarian atrophy was observed and ovarian atrophy may have been observed at lower concentrations if mice were chronically exposed;
- A UF_D of 6 was used. A 13-wk inhalation study in rats and mice was available but the highest concentration tested, which was the LOAEL, was well above the maximum tolerated concentration. Although a two-generation oral gavage reproductive study in mice did not indicate reproductive effects (Grizzle et al. 1994), inhalation studies investigating two-generation reproductive effects as well as inhalation developmental studies are not available. Confidence in the database is medium.

$$\text{chronic ReV} = POD_{HEC} / (UF_H \times UF_A \times UF_S \times UF_D)$$
$$= 44.64 \text{ ppm} / (10 \times 1 \times 10 \times 6)$$
$$= 0.0744 \text{ ppm}$$
$$= 74 \text{ ppb (rounded to two significant figures)}$$

4.1.6.2 Critical Effect

The noncarcinogenic critical effect is lethargy/tremor/mortality/ovarian atrophy produced in mice after exposure to VCH (Bevan et al. 1996; Stadler 1994b). The chronic ReV based on ovarian atrophy will be used to develop a carcinogenic chronic ReV, as discussed in Section 4.2 Carcinogenic Potential.

4.1.7 Health-Based Chronic ReV and $^{chronic}ESL_{nonlinear(nc)}$

The noncarcinogenic chronic ReV was rounded to two significant figures at the end of all calculations resulting in a chronic ReV of 74 ppb (330 µg/m^3). The rounded chronic ReV was then multiplied by 0.3 to calculate the $^{chronic}ESL_{nonlinear(nc)}$. At the target hazard quotient (HQ) of 0.3, the $^{chronic}ESL_{nonlinear(nc)}$ is 22 ppb (97 µg/m^3) (Table 5).

Table 5. Derivation of the Noncarcinogenic Chronic ReV and $^{chronic}ESL_{nonlinear(nc)}$

Parameter	**Summary**
Study	Bevan et al. (1996); Stadler 1994b
Study population	10 $B6C3F_1$ mice per sex per concentration
Study quality	Medium
Exposure methods	Exposures via inhalation at 0, 53, 250, 1000 ppm (analytical)
Critical effects	Lethargy/tremor/mortality/ovarian atrophy
LOAEL	1000
POD (NOAEL)	250 ppm
Exposure duration	6 h/day, 5 days/wk for 13 wks
POD_{ADJ}	44.64 ppm
POD_{HEC}	44.64 ppm (Category 3 vapor)
Total uncertainty factors (UFs)	600 (for both lethargy/tremor/mortality and ovarian atrophy, although individual UFs vary)
Interspecies UF	10
Intraspecies UF	3 (lethargy/tremor/mortality) 1 (ovarian atrophy)
Subchronic to chronic UF	3 (for lethargy/tremor/mortality 10 (ovarian atrophy)
LOAEL UF	Not applicable
Incomplete Database UF *Database Quality*	6 Medium
Chronic ReV [1 h] (HQ = 1)	**330 µg/m^3 (74 ppb)**
$^{chronic}ESL_{nonlinear(nc)}$ [1 h] (HQ = 0.3)	**97 µg/m^3 (22 ppb)**

4.2 Carcinogenic Potential

Epidemiology studies in humans and inhalation studies in animals investigating the potential carcinogenicity of VCH are not available. Chronic oral and dermal studies in rodents as well as i*n vivo* and *in vitro* mutagenicity assays on VCH and VCH metabolites are available. These studies are discussed in the following sections.

The TD developed a nonlinear carcinogenic ReV for VCH based on preventing ovarian atrophy in female mice because of strong MOA evidence indicating that ovarian atrophy is a precursor to the development of ovarian tumors observed in chronic oral and dermal studies after exposure of female mice to VCH or VCD (NTP 1986, 1989b). Female rats did not develop ovarian tumors. Inhalation studies investigating ovarian atrophy were available whereas inhalation studies investigating carcinogenic potential were not. By preventing ovarian atrophy, a known precursor to development of ovarian tumors, the risk for developing ovarian tumors is reduced.

4.2.1 Oral and Dermal Studies in Rodents

NTP (1986) performed an oral gavage chronic study in mice and rats which indicated there was clear evidence of carcinogenicity of VCH in female mice, as shown by markedly increased incidences of uncommon ovarian neoplasms at 200 or 400 mg/kg body weight. In addition, a slight increase in the incidence of adrenal gland adenomas in high dose female mice was observed. The studies were considered to be inadequate for assessment of carcinogenicity for male and female rats and male mice because of extensive and early mortality at the high dose or at both doses and the lack of conclusive evidence of a carcinogenic effect, as discussed in Appendix C.

NTP (1989b) also conducted a carcinogenicity study for VCD via the dermal route. Groups of 60 male and female F344/N rats and $B6C3F_1$ mice received VCD by topical application at doses of 0 (vehicle), 15, or 30 mg/animal (rats), and 0 (vehicle), 2.5, 5, or 10 mg/animal (mice), 5 days/wk for 105 wks. Under the conditions of these 2-year dermal studies, there was clear evidence of carcinogenic activity of VCD for male and female F344/N rats, as shown by squamous cell and basal cell neoplasms of the skin. There was clear evidence of carcinogenic activity of VCD for male and female $B6C3F_1$ mice, as shown by squamous cell carcinomas of the skin in males and squamous cell carcinomas of the skin and ovarian neoplasms in females; increased incidences of lung neoplasms in females may also have been related to chemical application. The survival rate in rats was very low for all groups, including the vehicle controls.

4.2.2 Carcinogenic Weight of Evidence

4.2.2.1 Carcinogenic Weight of Evidence

Based on experimental data and guidance in USEPA's Guidelines for Carcinogenic Risk Assessment (2005), there is inadequate information to assess carcinogenic potential via inhalation since only oral and dermal carcinogenic studies are available. Evidence that indicates

an association between exposure to VCH in humans and development of tumors is not available. *In vivo* and *in vitro* evidence that VCH is mutagenic is not strong. However, VCH can be metabolized to VCD, and there is clear evidence of carcinogenic activity of VCD for mice and rats, as shown by squamous cell carcinomas of the skin in males and squamous cell carcinomas of the skin and ovarian neoplasms in female mice after dermal exposure to VCD. *In vivo* and *in vitro* evidence indicates that VCD may be mutagenic. Humans are capable of metabolizing VCH to VCD, so the findings in rats and mice exposed to VCD are relevant to humans. There is some evidence that VCH works through a mutagenic mode of action, as discussed by Collins, Montali, and Manus (1987).

An inhalation unit risk factor (URF) was not developed for VCH because an inhalation chronic study investigating carcinogenic potential is not available. However, a 13-wk subchronic inhalation study investigating ovarian atrophy is available (Stadler 1994b). Scientific data support the fact that ovarian tumors may be formed due to ovarian atrophy (The Sapphire Group 2008) so a carcinogenic chronic ReV was derived based on the potential for VCH to produce ovarian atrophy, as discussed below. The nongenotoxic, nonlinear MOA for ovarian tumors is discussed below.

4.2.2.2 Carcinogenic Weight of Evidence from Other Organizations

Table 6 provides information on the carcinogenic weight of evidence provided by other organizations.

Table 6. Carcinogenic Weight of Evidence

Organization	Weight of Evidence
International Agency for Research on Cancer (IARC 1994)	Group 2B, possibly carcinogenic to humans
American Conference of Governmental Industrial Hygienists (ACGIH 2001)	A3, Confirmed animal carcinogen with unknown relevance to humans
Health Council of the Netherlands (2008)	Should be considered to be carcinogenic to humans and should be considered a genotoxic agent

4.2.3 MOA

4.2.3.1 Mutagenicity Studies

Collins, Montali, and Manus (1987) provide a discussion on mutagenicity studies conducted *in vivo* and *in vitro* that support a genotoxic MOA for the carcinogenic effects of VCH (refer to Collins, Montali, and Manus 1987 for references):

> "With respect to the mechanism mediating the carcinogenic effects of VCH in female $B6C3F_1$ mice, several in vivo and in vitro studies support the possibility that VCH may be metabolized in vivo to yield mutagenic/carcinogenic derivatives. While VCH was found not to be mutagenic in several strains of Salmonella, with or without activation (National Toxicology Program, 1986), a number of its metabolites, and especially VCH diepoxide, have been shown to be highly mutagenic in both prokaryotic and eukaryotic cells (Murray and Cummins, 1979; El-Tantawy and Hammock, 1980; Simmon and Baden, 1980; Frantz and Sinsheimer, 1981; Turchi et al., 1981; Watabe et al., 1981; Voogd et al., 1981; Mortelmans et al., 1986). In addition, VCH diepoxide has been shown to induce chromosomal abnormalities (anaphase bridges) in Chinese hamster lung cells (Turchi et al., 1981) and sister-chromatid exchanges and chromosomal aberrations in Chinese hamsters ovary cells (E. Zeiger, unpublished data). The carcinogenicity of VCH diepoxide has been demonstrated in long-term dermal studies in Swiss-Millerton mice (Van Duuren et al., 1963, 1967), and a threshold limit value of 10 ppm has been established (American Conference of Governmental Industrial Hygienists, 1983). These results have been confirmed by the current NTP 2-yr dermal studies of VCH diepoxide, in which papillomas and squamous-cell carcinomas of the skin were induced in dosed $B6C3F_1$ mice, even by the interim kill at wk 65 (R. Chhabra, unpublished data). Of particular interest is the observation that a variety of neoplastic and nonneoplastic ovarian lesions are apparent in female $B6C3F_1$ mice receiving VCH diepoxide in these chronic dermal studies."

Table 7 provides mutagenicity results from recent studies on VCH (reproduced from The Sapphire Group (2008) – for references, refer to Appendix D). The evidence for VCH working through a mutagenic MOA is not strong, although *in vitro* evidence for VCD indicates it may work through a mutagenic MOA as shown in Table 8 (reproduced from The Sapphire Group (2008) - for references, refer to Appendix D) . Evidence for VCH not working through a mutagenic MOA is provided by The Sapphire Group (2008):

> "A mutagenic MOA for VCH is also not consistent with the toxicity data set for this compound. Although VCH-diepoxide is a DNA-reactive chemical, at least in in vitro systems, tumors were not observed at multiple sites, or in multiple species subsequent to BCH *(i.e., VCH)* exposure. Furthermore, there was not an increase in tumor-bearing animals, increase in tumor multiplicity, or early tumor response in the long-term studies. Contrast the findings of VCH with that of 1,3-butadiene (BD), which also produces both monoepoxide and diepoxide metabolites and produces at least some tumors through a possible mutagenic MOA (Preston et al., 2007). Like VCH, BD produces toxicity when it is metabolized to its reactive epoxide metabolites. Mice are more sensitive than rats due to differences in metabolism: there is greater production of mono-

and di-epoxide metabolites in mice compared to rats, with mice having lower capacity for detoxification of these reactive intermediates. BD is genotoxic in vitro and in vivo; the in vivo studies are positive for mice, but negative in rats due to the species difference in metabolism of the DNA-reactive epoxide metabolites. Ovarian atrophy (follicular loss) and ovarian tumors are also seen in BD-exposed mice, but not rats, due to the presence of the diepoxide of BD. But, unlike VCH, BD produces toxicity and tumors at multiple sites. Thus, when compared to the structurally-related genotoxic analogue BD, VCH does not appear to have the same profile. VCH selectively targets the ovarian follicles in mice, without showing effects at any other sites, a pattern of toxicity that is inconsistent with a mutagenic MOA."

Table 7. *In vitro* and *in vivo* Genotoxicity Studies on VCH (The Sapphire Group 2008)

Endpoint	Test System	Results	Reference
Mutagenicity	Ames/Salmonella TA98, TA100, TA1535, TA1537	Negative (+ S9)	Zeiger et al. (1997)
Chromosomal aberrations	Chinese Hamster Ovary (CHO) cells	Negative	NTP (1989), USEPA (1994)
Sister chromatid exchange	Chinese Hamster Ovary (CHO) cells	Negative	NTP (1989), USEPA (1994)
Mutagenicity	L5178Y mouse lymphoma cells	Equivocal (at most)	USEPA (1994)
Micronuclei	Mouse bone marrow (2-day inhalation)	Negative	Bevan et al. (2001)
Micronuclei	Mouse bone marrow (90-day inhalation)	Negative	Bevan et al. (2001)

Table 8. *In vitro* Genotoxicity Studies on VCH-Diepoxide (The Sapphire Group 2008)

Endpoint	Test System	Results	Reference
Mutagenicity	Ames/S. typhimurium TA100 (standard plate test)	Negative	Turchi et al. (1981)
Mutagenicity	Ames/S. typhimurium TA100 (liquid test)	Positive	Turchi et al. (1981)
Mutagenicity	Ames/S. typhimurium TA100, TA98, TA1535, TA1537	Positive (TA 100 only; -S9)	Watabe et al. (1981)
Mutagenicity	Ames/S. typhimurium TA100, TA98 (spot test)	Positive (TA 100 only; +/-S9)	Wade et al. (1979)
Mutagenicity	Ames/ S. typhimurium TA100, TA98, TA1535, TA1537	Positive (TA 100, TA 1535 only; -S9)	El-Tantawy et al. (1980)
Mutagenicity	S. cerevisiae D7	Positive (-S9)	Bronzetti et al. (1980)
Mutagenicity	V79 Chinese hamster cells	Positive	Gervasi et al. (1980)
Mutagenicity	V79 Chinese hamster cells	Positive	Turchi et al. (1981)
Chromosomal aberrations	V79 Chinese hamster cells	Positive	Turchi et al. (1981)
Micronuclei	V79 Chinese hamster cells	Negative	Turchi et al. (1981)

Based on scientific evidence presented by The Sapphire Group (2008) (Appendix D) and an independent review of the scientific data, the TD concludes that VCH acts through a non-genotoxic, nonlinear (threshold) MOA in producing ovarian tumors in female mice, as discussed in the following sections.

4.2.3.2 Nongenotoxic, Nonlinear MOA for Ovarian Tumors

The Sapphire Group (2008) presented scientific data that indicates the MOA for ovarian tumors is nongenotoxic, nonlinear and provides a discussion of the key events involved in ovarian tumors. Refer to Appendix B for a detailed discussion. The following is a summary of the key findings:

> Following exposure and uptake, VCH is metabolized, primarily in the liver, to VCH-1,2-epoxide or VCH-7,8-epoxide, which are further metabolized to VCH-diepoxide. VCH-diepoxide enters the blood and circulates through the body. Upon reaching the ovary, VCH-diepoxide selectively destroys the primordial

and primary follicles through a mechanism involving programmed cell death or apoptosis. Repeated exposures to VCH ultimately result in premature ovarian failure, due to complete follicular loss. Since 17β-estradiol and inhibin are no longer produced from the primordial and primary follicles in the ovary, loss of the negative feedback inhibition of (follicle stimulating hormone) FSH release from the hypothalamus and pituitary occurs, leading to high plasma levels of FSH. Increased plasma levels of FSH results in the initiation and/or promotion of ovarian tumors.

Key Events:

Following exposure and uptake of VCH, the key events leading to ovarian toxicity and tumors are as follows:

- Systemic levels of VCH diepoxide [bioactivation of VCH to VCH diepoxide (via VCH-1,2-epoxide) and hydrolysis of VCH epoxide metabolites by epoxide hydrolase]
- Increased follicular loss in ovaries from VCH diepoxide
- Selective destruction of primordial and primary follicles through apoptosis
- Ovarian failure (no estrous cyclicity) from complete oocyte loss
- Increased plasma FSH levels from release of negative feedback of 17β – extradiol and inhibin on hypothalamus and pituitary
- Initiation and/or promotion of ovarian tumors from increased plasma FSH levels

4.2.4 Key Studies

As mentioned previously, the NTP (1986, 1989b) studies were considered to be inadequate for assessment of carcinogenicity. However, The Sapphire Group (2008) proposed a nongenotoxic, nonlinear MOA for VCH. By preventing ovarian atrophy, and the increase in plasma FSH levels, the promotion of ovarian tumors may be prevented. Therefore, the TD developed a nonlinear carcinogenic chronic ReV. The chronic ReV developed in Section 4.1.6.1.2 for minimal to mild ovarian atrophy based on the Bevan et al. (1996) and Stadler (1994b) 13-wk subchronic study is the carcinogenic chronic ReV. Refer to Section 4.1.2.1 for study details.

4.2.5 Chronic Carcinogenic ReV and $^{chronic}ESL_{nonlinear(c)}$

Please refer to Section 4.1 for the derivation of the chronic ReV for the critical effect of minimal to mild ovarian atrophy, which was determined to be 74 ppb, rounded to two significant figures. The carcinogenic chronic ReV of 74 ppb (330 µg/m^3) was then multiplied by 0.3 to calculate the $^{chronic}ESL_{nonlinear(c)}$. At the target HQ of 0.3, the $^{chronic}ESL_{nonlinear(c)}$ is 22 ppb (97 µg/m^3) (Table 7). The $^{chronic}ESL_{nonlinear(nc)}$ of 22 ppb (97 µg/m^3) (Table 5) is equal to the $^{chronic}ESL_{nonlinear(c)}$.

Table 9. Derivation of the Carcinogenic Chronic ReV and $^{chronic}ESL_{nonlinear(c)}$

Parameter	**Summary**
Study	Bevan et al. (1996); Stadler (1994b)
Study population	10 $B6C3F_1$ mice per sex per concentration
Study quality	Medium
Exposure methods	Exposures via inhalation at 0, 53, 250, 1000 ppm (analytical)
Critical effects	Minimal to mild ovarian atrophy
LOAEL	1000
POD (NOAEL)	250 ppm
Exposure duration	6 h/day, 5 days/wk for 13 wks
POD_{ADJ}	44.64 ppm
POD_{HEC}	44.64 ppm (Category 3 vapor)
Total uncertainty factors (UFs)	600
Interspecies UF	10
Intraspecies UF	1
Subchronic to chronic UF	10
LOAEL UF	Not applicable
Incomplete Database UF *Database Quality*	6 Medium
Chronic ReV [1 h] (HQ = 1)	**330 µg/m^3 (74 ppb)**
$^{chronic}ESL_{nonlinear(nc)}$ [1 h] (HQ = 0.3)	**97 µg/m^3 (22 ppb)**

4.3 Welfare-Based Chronic ESL

No data were found regarding long-term vegetative effects.

4.4 Long-Term ESL

The chronic evaluation resulted in the derivation of the following values:

- noncarcinogenic chronic ReV = 330 µg/m^3 (74 ppb)
- $^{chronic}ESL_{nonlinear(nc)}$ = 97 µg/m^3 (22 ppb)
- carcinogenic chronic ReV = 330 µg/m^3 (74 ppb)
- $^{chronic}ESL_{nonlinear(c)}$ = 97 µg/m^3 (22 ppb)

The long-term ESL for air permit reviews is the $^{chronic}ESL_{nonlinear(c)}$ of 97 µg/m^3 (22 ppb) (Table 2).

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Appendix A Summary of VCH Reproductive Studies from USEPA (2002)

Refer to Table 5-8 (USEPA 2002)

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Appendix B Sections 5.0 and 5.1 from the Sapphire Group (2008)

5.0 Mode of Action(s) of Mouse Ovarian Tumors

An evaluation of the mode of action (MOA) by which VCH produces ovarian tumors in rodents was conducted using the IPCS Human Relevance Framework (Meek *et al.,* 2003; Boobis *et al.,* 2006). In this framework, three fundamental questions are considered for the MOA:

1. Is the weight of evidence sufficient to establish an MOA in animals?
2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?
3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

Following a consideration of these three questions, a confidence statement is given, along with a discussion of the implications of the MOA to the risk assessment.

5.1. Proposed Mode of Action for Mouse Ovarian Tumors

Following exposure and uptake, VCH is metabolized, primarily in the liver, to VCH-1 ,2-epoxide or VCH-7,8-epoxide, which are further metabolized to VCH-diepoxide. VCH-diepoxide enters the blood and circulates through the body. Upon reaching the ovary, VCH-diepoxide selectively destroys the primordial and primary follicles through a mechanism involving programmed cell death or apoptosis. Repeated exposures to VCH ultimately result in premature ovarian failure, due to complete follicular loss. Since 17β-estradiol and inhibin are no longer produced from the primordial and primary follicles in the ovary, loss of the negative feedback inhibition of FSH release from the hypothalamus and pituitary occurs, leading to high plasma levels of FSH. Increased plasma levels of FSH results in the initiation and/or promotion of ovarian tumors.

5.1.1. Key Events

Following exposure and uptake of VCH, the key events leading to ovarian toxicity and tumors are presented in Table 4 and outlined below.

1. Systemic levels of VCHD
   1a. Bioactivation of VCH to VCHD (via VCH-l ,2-epoxide)
   1 b. Hydrolysis of VCH epoxide metabolites by epoxide hydrolase
2. Decreased follicular loss in ovaries from VCHD
3. Selective destruction of primordial and primary follicles through apoptosis
4. Ovarian failure (no estrous cyclicity) from complete oocyte loss
5. Ovarian failure (no estrous cyclicity) from complete oocyte loss

6. Increased plasma FSH levels from release of negative feedback of 17β-estradiol and inhibin on hypothalamus and pituitary.
7. Initiation and/or promotion of ovarian tumors from increased plasma FSH levels.

Table 4 Key Events in the Proposed MOA for VCH-Induced Ovarian Tumors

Key Events	Evidence in Animals	Confidence	Key References
1. Systemic levels of VCH diepoxide	Blood levels inferred from studies showing blood levels of VCH-1,2-epoxide in VCH-dose mice, and from toxicity studies with VCHD	High	Smith et al. 1990 a,b,c; Keller et al. 1997
1a. bioactivation of VCH to VCH diepoxide (via VCH-1,2-epoxide)	In vitro liver, lung, and ovary microsome studies, with formation greater in mice than rats. Inhibition of cytochrome P450 reduces VDH-1,2-epoxide formation in vivo and in vitro	High	Smith et al. 1990 a,b,c; Keller et al. 1997
1b hydrolysis of VCH epoxide metabolites by epoxide hydrolase]	In vitro liver, lung and ovary microsome studies, with rats having higher epoxide hydrolase rates than mice	High	Smith et al. 1990 a,b,c; Keller et al. 1997
3. Selective destruction of primordial and primary follicles through apoptosis	Secondary follicles not directly affected by VCHD treatment; morphological and biochemical pathway studies.	High	Hooser et al. 1994; Flaws et al. 1994; Springer et al. 1996; Kao et al. 1999; Mayer et al. 2002 ; Hu et al. 2001a,b ; 2002
4. Ovarian failure (no estrous cyclicity) from complete oocyte loss	Long-term studies (up to one year) from 30 day treatment with either VCH (mice) or VCHD (rats);	High	Hooser et al. 1994; Mayer et al. 2002
5. Increased plasma FSH levels from release of negative feedback of 17β – extradiol and inhibin on hypothalamus and pituitary	Long-term studies (up to one year) from 30 day treatment with either VCH (mice) or VCHD (rats)	High	Hooser et al. 1994; Mayer et al. 2002 ; Lohff et al. 2006
6. Initiation and/or promotion of ovarian tumors from increased plasma FSH levels	Cystic structures in VCH-treated mice similar to preneoplastic lesions in genetically altered mice predisposed to granulose cell tumors. Prolonged increased FSH plasma levels associated with initiation/development of ovarian tumors	Moderate	Hooser et al. 1994; Tennent et al. 1990 ; Murphy and Beamer 1973 ; Murphy 1980 ; Fuller et al. 2002

5.1.2. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

The Weight of Evidence for this MOA is assessed using an approach based on the Hill criteria for causality, originally developed for application in epidemiologic investigations (Hill, 1965).

5.1.2.1. Strength, Consistency, Specificity of Association

Chronic oral exposure of female mice to VCH resulted in ovarian granulosa cell tumors (Collins *et al.,* 1987). Preceding the tumors, a reduction in the number of follicles, particularly the primary follicles, were noted in the ovaries of female mice exposed orally or by inhalation for 13 weeks to VCH (Collins and Manus, 1987; Bevan *et al.,* 1996*).* Although it is difficult to reach any strong conclusions about the ovarian tumor incidence in rats due to poor survival in the oral chronic study, rats exposed to VCH did not show any ovarian toxicity or increased incidence of ovarian tumors (Collins and Manus, 1987; Collins *et al.,* 1987; Bevan *et al.,* 1996*).*

The species difference in ovarian toxicity appears largely due to differences in the rate of bioactivation of VCH to VCH epoxides (VCH-1,2-epoxide, VCH-7,8-epoxide and VCH-diepoxide) by cytochrome P-450 enzymes. Following treatment of female mice and rats with a single intraperitoneal dose of VCH, only mice had detectable blood levels of VCH-I,2-epoxide (Smith *et al.,* 1990a). Pretreatment of VCH-dosed mice with the cytochrome P450 inhibitor chloramphenicol resulted in reduced VCH-1,2-epoxide blood concentrations compared to non-pretreated VCH-dosed mice (Smith *et al.,* 1990a).

The evidence is compelling that VCH-diepoxide is the metabolite responsible for follicular destruction. Analogues of VCH that have the potential to form only a monoepoxide metabolite failed to deplete small follicles (Hooser *et al.,* 1993; Doerr *et al.,* 1994), whereas compounds which can form diepoxides, such as BD and isoprene, significantly depleted follicles (Doerr *et al.,* 1994). Indeed, the study by Smith *et al.* (1990b) showed that the potency of VCH-diepoxide to induce oocyte loss was considerably greater than the monoepoxide metabolites and the parent compound VCH (Table 5). Furthermore, when male Fischer 344 rats and B6C3Fl mice were given intraperitoneal injections of VCH, VCH-1,2-epoxide, VCH-7,8-epoxide (mice only), or VCH-diepoxide for 30 days, VCH reduced the number of small (pre-antral) oocytes in mice, whereas no detectable oocyte loss occurred in rats at the highest dose tested (Table 4). However, this difference in susceptibility between mice and rats to oocyte destruction disappeared when animals were administered VCH-diepoxide. A 13-week dermal mouse study of VCH-diepoxide has also been conducted and showed atrophy of the ovaries, characterized by a decreased number of follicles (Chhabra *et al.,* 1990a).

Table 5
ED_{50}[a] Values for the Reduction in Small Oocyte Counts in Mice and Rats[1]

Species	VCH	VCH-1,2-epoxide	VCH-7,8-epoxide	VCH-diepoxide
Mouse	2.7	0.5	0.7	0.2
Rat	>7.4[b]	1.4	ND[c]	0.4

[1]Results from Smith *et al.* (1990b)
[a]Dose in mmnol/kg-day which reduces the small oocyte count to 50% of that observed in control animals.
[b]Highest dose given.
[c]Not determined.

The follicles that are selectively targeted by VCH-diepoxide are the primordial and primary follicles, but not the secondary follicles (Springer *et al.,* 1996). Results from time-course studies indicate that following 12 days of dosing with VCH-diepoxide, there is a significant loss of primordial and primary follicles in both rats and mice, with no effect on secondary follicle numbers (Kao *et al.,* 1999). Longer periods of dosing (30 days) with either VCH in mice or VCD in rats result in additional reduction of secondary follicles, but this is likely a result of a reduced population of primordial and primary follicles from which to recruit (Hooser *et al.,* 1994; Flaws *et al.,* 1994). Mechanistic studies in rats have determined that VCH-diepoxide causes ovotoxicity by accelerating the natural process of atresia - which occurs through apoptosis - and this requires repeated exposures (Hoyer and Sipes, 2007).

In mice dosed with VCH/VCH-diepoxide or rats dosed with VCH-diepoxide, the pathological changes in the ovary are identical (Flaws *et al.,* 1994; Springer *et al., 1996;* Mayer *et al.,* 2002). VCH-diepoxide selectively destroys the primordial and primary follicles, accelerating the normal process of atresia via apoptosis (Springer *et al., 1996).* Accelerated oocyte depletion leads eventually to premature ovarian failure and cessation of the estrous cycle. Highly elevated FSH plasma levels occur in both in rats, mice and nonhuman primates treated with either VCH (mice only) or VCH-diepoxide. At the time of ovarian failure, VCH-treated mice showed lesions in the ovary that appear similar to preneoplastic lesions reported in a genetically susceptible strain of mice for granulosa cell tumors (Hooser *et al.,* 1994; Tennant *et al.,* 1990). Elevated FSH levels have been consistently seen in various animal models of ovarian cancer and are thought to be the underlying mechanism to ovarian cancer, perhaps through alteration in signaling pathways affecting cell growth (Murphy, 1980; Fuller *et al.,* 2002*).*

Mice, but not rats, are susceptible to ovarian toxicity by VCH. However, a consistent association has been observed across species (mice, rats, and nonhuman primate) between VCH-diepoxide administration and primordial and primary follicle loss in the ovary (Springer *et al.,* 1996; Kao *et al.,* 1999; Mayer *et al.,* 2002; Appt *et al.,* 2006*).* Unfortunately, no data are available for the tumorigenic response of VCH or VCH-diepoxide across species. The consistent association of VCH-diepoxide exposure with follicular loss across species, in contrast to VCH exposure where only the mouse is susceptible, can be explained by species differences in the kinetics of the metabolism of VCH and its metabolites. The balance of activation versus detoxification reactions

in rats and mice suggest that the mouse may be more susceptible to VCH toxicity because of generation of high levels of epoxide metabolites. In general, the mouse is more efficient at metabolism of VCH to epoxides than is the rat. In contrast, the rat may be more efficient at hydrolysis of epoxides. Thus, the rat would tend to have a lower circulating concentration of epoxide metabolites than the mouse at equal doses of VCH. If, however, the ultimate metabolite VCH-diepoxide is administered to either rats or mice, metabolism does not play a limiting role in the ability of the diepoxide to be formed in sufficient quantity so that it can reach the ovary and target primordial and primary follicles. No VCH metabolism data exist for nonhuman primates. Data on olefinic compounds, such as BD, indicate that nonhuman primates are similar to humans with respect to cytochrome P-450 bioactivation of olefins to its epoxide metabolites (Dahl and Henderson, 2000). Limited *in vitro* data with human liver microsomes suggest that VCH metabolism in nonhuman primates is likely to be more like the rat than the mouse (Smith and Sipes, 1991).

In summary, there is strong evidence for an association of follicular loss in the mouse ovary via VCH-diepoxide by a non-genotoxic pathway and the formaton of ovarian tumors. The key events show strength, consistency and specificity of association.

5.1.2.2. Dose-Response Concordance

The dose-response concordance between the ovarian toxicity and tumors in VCH-exposed mice cannot be evaluated. For inhalation exposure, a 13-week, but not a 2-year chronic bioassay, was conducted. The NTP conducted both 13-week and 2-year oral studies in mice; however, only the high-dose (1200 mg/kg) female mice were evaluated for ovarian effects (follicle loss), and it is not known whether the ovarian effects were also present at the lower doses (75 to 600 mg/kg). The continuous breeding protocol study showed ovarian effects in mice dosed with 500 mg/kg VCH for 17 to 18 weeks; but, here again, the lower doses were not evaluated. Increased incidence of ovarian tumors were seen in the 200 and 400 mg/kg dose female mice in the NTP chronic bioassay.

Smith *et al.* (1990b) compared the dose-response relationship of the reduction in small oocyte counts in the ovaries of mice and rats following 30 days of intraperitoneal treatment with VCH, VCH-1,2-epoxide, VCH-7,8-epoxide, and VCH-diepoxide. The doses of VCH and its epoxide metabolites that reduced the small oocyte count to 50% that of control are shown in the Table 5. In mice, the destruction of the small oocytes was dependent on the administered dose of VCH. In contrast, VCH treatment produced no detectable change in oocyte number in the ovaries of rats. However, in both species, VCH mono- and di-epoxide metabolites were much more potent than the parent compound in destroying small oocytes. The ED_{50} of VCH-1,2-epoxide, VCH-7,8-epoxide and VCH-diepoxide was 5.4-, 3.9-, and 14-fold lower than that of VCH. The potency of VCH-diepoxide >> YCH-1,2-epoxide~VCH-7,8-epoxide > VCH in oocyte destruction would be expected if VCH-diepoxide was the ultimate ovotoxicant. VCH-diepoxide is metabolically produced from VCH-1,2-epoxide, which in turn is metabolically formed from VCH. A comparison of the ED_{50} of VCH between mice and rats is consistent with the susceptibility of

mice to VCH-induced ovarian toxicity observed in the 13-week inhalation and oral toxicity studies, compared to rats. This susceptibility difference disappears when the animals are treated with VCH-diepoxide, and to a lesser extent with VCH-1,2-epoxide.

Nonhuman primates given a single daily intramuscular injection of VCH-diepoxide for 15 days had nearly complete elimination of primordial, intermediate, primary and secondary follicles in the ovaries 27 days after treatment with a 250 mg/kg dose, a 50% reduction in primordial and primary follicles with 160 mg/kg, and no effect with 80 mg/kg (Appt *et al.,* 2006). No studies, however, have been conducted to determine the dose-response relationship of follicle loss and ovarian tumors in nonhuman primates.

In summary, a dose-response relationship is observed in the potency of VCH and its epoxide metabolites in inducing follicular loss in the ovary, providing strong evidence for VCH-diepoxide as the compound responsible for the ovarian toxicity, as well as the reason for the species differences in susceptibility.

5.1.2.3. Temporal Relationship

A single intraperitoneal dose of 320 mg/kg VCH-diepoxide resulted in a time-dependent decrease of both primordial and small primary follicles beginning 6 days later (Devine *et al.,* 2004). Larger follicle stages were not affected over the time period studied (12 days following dosing).

The follicles that are selectively targeted by VCH-diepoxide are the primordial and primary follicles, but not the secondary follicles (Springer *et al.,* 1996). Results from time-course studies indicate that following 12 days of dosing with VCH-diepoxide, there is a significant loss of primordial and primary follicles in both rats and mice, with no effect on secondary follicle numbers (Kao *et al.,* 1999). Longer periods of dosing (30 days) with either VCH in mice or VCD in rats result in additional reduction of secondary follicles, but this is likely a result of a reduced population of primordial and primary follicles from which to recruit (Hooser *et al.,* 1994; Flaws *et al.,* 1994). Mechanistic studies in rats have determined that VCH-diepoxide causes ovotoxicity by accelerating the natural process of atresia - which occurs through apoptosis - and this requires repeated exposures (Hoyer and Sipes, 2007).

Ovarian failure (premature menopause) is a consequence of VCH-induced primordial and primary follicle loss (Hooser *et al.,* 1994; Mayer *et a!.,* 2002). In mice given daily intraperitoneal injections of 800 mg/kg VCH for 30 days, there was >90% loss of the small pre-antral follicles at the end of the dosing period (Hooser *et al.,* 1994; Table 6). At 240 days of the study (210 days following VCH treatment), there were few widely scattered oocytes in small and growing follicles; however, at 360 days, no oocytes at any stage were observed in the VCH-treated mice. The complete loss of oocytes at 360 days coincided with the loss of estrous cyclicity, indicating ovarian failure. Follicular loss also resulted in increased follicle stimulating hormone (FSH) plasma levels, presumably due to the lack of 17β-estradiol and inhibin production from the

follicles. 17β -Estradiol and inhibin exert negative feedback inhibition of FSH production in the hypothalamus and/or pituitary. Plasma FSH levels were not elevated above control levels until 240 days following the initiation of dosing, suggesting that virtually complete loss of follicles is needed before the release of the negative feedback inhibition at the hypothalamus/pituitary.

Table 6
Long-Term Effects of 30 Days Dosing of Female$B6C3F_1$ Mice With 800 mg/kg VCH by Intraperitoneal Injection[1]

Day	Small follicles (% control)	Serum FSH (% above control)	Estrous cyclicity
30	11%*	30	Yes
120	3%*	50	Yes
240	<1%*	130*	Yes
360	0%*	160*	No

[1]Results from Hooser *et al.*, 1994. Day = day after onset of dosing.
*Different from controls, $p<0.05$.

A similar pattern was reported for rats dosed intraperitoneally for 30 days with 80 mg/kg VCH-diepoxide (Mayer *et al.*, 2002). Rats dosed with VCH-diepoxide had reduced number of preantral follicles by day 30. Following cessation of dosing, relative to controls, primordial, primary, and secondary follicles were progressively lost with time. Circulating FSH levels in VCH-treated rats were greater (days 120,240 and 360) than in controls. Cyclicity was disrupted in the VCH-diepoxide treated animals by day 360.

In the two-year NTP mouse bioassay on VCH, there were increased incidences of uncommon ovarian tumors, including mixed benign tumors, granulosa-cell tumors, and granulosa-cell tumors or carcinomas (combined), in female B6C3F1 mice given oral doses of VCH (in corn oil) for 103 weeks (Collins *et al.*, 1987). The incidence of tubular cell or granulosa cell hyperplasia was also increased in the VCH-treated groups. These tumors were preceded by ovarian toxicity, characterized by a reduction in the number of primary follicles and mature graafian follicles, which was observed in female mice given oral doses of VCH for 13 weeks (Collins and Manus, 1987).

Likewise, in the two-year NTP dermal study on VCH-diepoxide, benign or malignant granulosa cell tumors and or benign mixed tumors of the ovary was preceded by atrophy of the ovaries (decreased number of follicles), which was seen in the 13-week dermal study (Chhabra *et al.*, 1990a,b).

In summary, there is strong evidence for the temporal progression of the key events in the proposed MOA, leading to the formation of ovarian tumors. Metabolism precedes follicular loss.

Complete follicular loss is required before the elevation in plasma FSH levels and the subsequent appearance of pre-neoplastic lesions.

5.1.2.4. Biological Plausibility and Coherence

Embryonic development of the ovary involves extensive proliferation of germ cells and somatic cells. In the later stages of this development, germ cells differentiate into oocytes when they cease to divide mitotically and begin to undergo meiosis (Hirshfield, 1991). However, the meiotic process is not completed and oocytes are arrested in an early stage of prophase known as the diplotene stage of meiosis (Buccione *et al., 1990;* Hirshfield, 1991). Somatic follicular (granulosa) cells in the embryonic ovary continue to proliferate and envelop small oocytes within a single layer to form primordial follicles (Gondos, 1970; Bacharova, 1985). Therefore, at birth, the ovary contains a finite number of primordial follicles containing oocytes arrested in prophase of the first meiotic division (Hirshfield, 1991). A primordial follicle contains an oocyte surrounded by a single layer of fusiform-shaped granulosa cells. During follicular development, the oocyte enlarges and the granulosa cells become cubiodal in appearance to form a primary follicle. A growing follicle results from proliferation of the granulosa cells into multiple layers. All of these stages of development occur in the pre-antral stage (25-250 μm in diameter). Larger, more mature follicles have developed a fluid-filled antrum, and thus classified as antral follicles (>250 μm in diameter). The process by which follicles are selected for ovulation is not known, but the pool of available follicles is considerably greater than those selected for ovulation. So, there is also a gradual loss of follicles at various stages of development by an apoptotic process called atresia. Agents that damage primordial and primary follicles to the extent of complete depletion of the available follicle pool produce permanent infertility and premature menopause since, once destroyed, those follicles cannot be replaced.

Several animal models initially drew attention to the possible involvement of gonadotropins in ovarian tumorigenesis. Biskind and Biskind (1944) reported a high incidence of ovarian tumors in rats whose ovaries were autotransplanted to the spleen. However, the formation of the ovarian tumors did not occur when one ovary was left intact or when the ovary was autotransplanted in previously hypophysectomized animals (Biskind and Biskind, 1948). This tumorigenesis has been attributed to elevated pituitary gonadotropins due to the deactivation of estrogen in the liver and the consequent depletion of negative feedback of estrogen on the pituitary. Since then, the development of ovarian tumors has been reported in several transgenic or knockout animal models that exhibit hypergonadotropism with high levels of circulating FSH and LH similar to the postmenopausal state in women (Kumar *et al.,* 1999; Risma *et al.,* 1995). Granulosa cell tumors can also be induced by genetic deletion of germ cells (Murphy, 1972; Murphy and Beamer, 1973), neonatal thymectomy (Nishizuka *et al.,* 1972), or X-irradiaton (Marchant, 1987).

The hormonal tumorigenesis hypothesis for ovarian granulosa cell cancers is that endocrine factors that control the normal growth of target organs can also provide suitable conditions for neoplastic transformation. The gonadotropin hypothesis has been proposed as an underlying mechanism to ovarian cancer, in that excessive levels of gonadotropins, related to the surge

occurring during ovulation and the loss of gonadal negative feedback in menopause and premature ovarian failure (oocyte depletion), may play a role in the development and progression of ovarian (granulosa cell) cancer. The incidence of ovarian cancer in women climbs dramatically around the age at which most women reach menopause. The onset of menopause, which happens at approximately 51 years of age, involves changes in gonadotropin levels as a result of cessation of ovarian function and menstrual cycle. The complete cessation of ovarian function results in the loss of negative feedback of ovarian steroids (i.e., 17β-estradiol) on gonadotropins. In 2 to 3 years after menopause, gonadotropin levels are particularly high, such that the concentrations of FSH and LH reach a peak of 10-20 times and 3-4 times the values recorded during the proliferative phase of the menstrual cycle, respectively (Chakravarti *et al.,* 1976; Speroff *et al.,* 1999). The increase in plasma gonadotropin levels is a result of the loss of feedback inhibition from 17 β-estradiol and inhibin, both of which are produced from follicles. In the case of ovarian failure where there is complete loss of oocytes in the ovary, the loss of 17 β-estradiol and inhibin from the follicles leads to increased plasma gonadotropin levels.

In summary, there is strong evidence of biological plausibility and coherence in the proposed MOA for mouse ovarian tumors by a non-genotoxic, threshold mechanism.

5.1.3. Are Key Events in the Animal MOA Plausible in Humans?

The key events in the animal MOA are plausible in humans. VCH-diepoxide has been shown to selectively deplete primordial and primary follicles in the ovaries of nonhuman primates *(Macaca fascicularis)* (Appt *et al.,* 2006). The physiology and anatomy of nonhuman primates are more similar to humans than rodents. The finding that VCH-diepoxide depletes primordial and primary follicles in nonhuman primates is strong evidence that the MOA for VCH-induced ovarian cancer is plausible in humans. Humans and nonhuman primates possess the same ability to metabolize VCH as rodents, specifically cytochrome P-450 CYP 2A, 2B and 2E1 and epoxide hydrolase, as well as glutathione transferase in organs, such as the liver, lung and ovaries. Female human liver microsomes have been shown to metabolize VCH to VCH-1,2-epoxide, but at lower rates than rat (2-fold) and mouse (13-fold) liver microsomes (Smith *et al.,* 1991).

1-3-Butadiene (BD), a structural analogue of VCH, also produces ovarian atrophy (follicular loss) and ovarian tumors in mice, but not rats. The diepoxide of BD (DEB) is believed to be the metabolite responsible for the ovarian effects, and the species susceptibility is likely due to the decreased ability of the rat to produce BD diepoxide. Filser *et al.* (2007) was unable to detect DEB in venous blood of male Sprague-Dawley rats (detection limit 0.01 µmol/L) exposed to 1,200 ppm for 6-8 hours, whereas DEB was detected in mice 3.2 µmol/L at 1,280 ppm BD. Humans appear to be similar to rats in their inability to produce the diepoxide metabolite. Albertini *et al.,* (2007) reported findings of a molecular epidemiology study in the Czech Republic of occupationally- exposed workers with cumulative exposures up to 6.3 ppm-weeks. Any N,N-(2,3- dihydroxy-1,4-butadiyl) valine (pry-Val) hemoglobin adduct of DEB that may have been present in these workers were below the limit of detection of the assay used. Swenberg *et al.* (2007) compared results in the Czech Republic occupationally-exposed workers

to results in mice and rats for a pry-Val adduct at similar BD concentrations. It was concluded that production of DEB in humans is below levels produced in both mice and rats exposed to as little as 1 ppm BD by inhalation. Subsequently, Georgieva *et al.* (2007) reported in an abstract that these adducts were detected at a low concentrations in Czech Republic workers when a more sensitive analytical method to measure pry-Val adducts was used. There was, however, no clear dose-response relationship between pry-Val adducts and BD concentrations, indicating that pry-Val adducts may be formed from other unknown sources besides the BD in the workplace environment.

Thus, using BD as an analogy, it is possible that VCH may be metabolized in human to VCH diepoxide in humans; but as is the case with BD, at extremely low levels when compared to the mouse.

5.1.4. Taking into Account Kinetic and Dynamic Factors, is the Animal MOA Plausible in Humans?

The diepoxide appears to be the metabolite of VCH responsible for the specific targeted destruction of promordial and primary follicles. There are species differences in the rates of formation or activation of the epoxide metabolites of VCH, as well as in the rate of detoxification. Mice have significantly greater capacity to metabolize VCH to the mono- and di-epoxides than do rats, and in many cases performs the reactions more efficiently than rats. In particular, mouse liver and lung tissue are very active in their ability to metabolize VCH to the epoxide metabolites. In contrast, the mouse does not hydrolyze epoxides well, while the rat hydrolyzes the epoxides to a greater extent than the mouse. This balance of activation reactions with detoxification reactions leads to the conclusion that the mouse may be more susceptible to the toxic effects of VCH, since the VCH diepoxide is considered the metabolite responsible for follicular destruction in the ovary. The prediction that VCH epoxidation rate in the liver and lung is the major factor which determines the ovotoxicity and carcinogenesis of VCH is supported by the toxicity data. Ovarian effects are only observed in mice exposed to VCH either orally or by inhalation, with tumors seen in mice dosed orally with VCH. Further support of the role of metabolism in the susceptiblity of animal species to the ovotoxicity of VCH comes from studies which show that the rat develops follicular loss and, ultimately ovarian failure, if dosed with VCH-diepoxide. Thus, if the epoxidation rate of VCH is the critical factor which determines the ovotoxicity and carcinogenicity of VCH, then the rat would be the more appropriate animal model for extrapolation of the VCH animal data to humans. Based on the available *in vitro* human liver microsomes data, human metabolism of VCH is expected to be more similar to the rat than the mouse. Given that the rat did not develop ovarian effects (follicular loss) either from oral or inhalation exposure, humans would also not be expected to develop ovotoxicity, and thus would not be expected to develop ovarian tumors at or below the exposures used in these animal studies.

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Appendix C National Toxicology Program (1986)

The National Toxicology Program (1986) performed chronic animal studies to assess toxicity and carcinogenicity of VCH in rats and mice in oral gavage studies. Groups of 50 F344JN rats and B6C3F1 mice of each sex were administered VCH in corn oil by gavage at a dose of 0, 200, or 400 mg/kg body weight, 5 days/week for 103 weeks. The NTP concluded:

> "4-Vinylcyclohexene was administered by gavage in corn oil to F344JN rats and B6C3F1 mice of each sex at doses of 200 or 400 mg/kg for 103 weeks. Under these conditions, the 2-year gavage studies of 4-vinylcyclohexene in male and female rats and male mice were considered inadequate studies of carcinogenicity because of extensive and early mortality at the high dose or at both doses and the lack of conclusive evidence of a carcinogenic effect. There was clear evidence of carcinogenicity of 4-vinylcyclohexene for female mice, as shown by markedly increased incidences of uncommon ovarian neoplasms at both doses. In addition, the increased incidence of adrenal gland adenomas in high dose female mice may have been related to the administration of 4-vinylcyclohexene."

The following summary of results was obtained from NTP (1986):

> "Many dosed rats died early in the 2-year studies (male: vehicle control, 17/50; low dose, 37/50; high dose, 45/50; female: vehicle control, 10/50; low dose, 22/50; high dose, 36/50; $P > 0.001$ for all groups except low dose female rats, for which $P = 0.022$). The poor survival of dosed male and female rats reduced the sensitivity of the studies for detecting the possible carcinogenic effects of 4-vinylcyclohexene. Mean body weights of dosed rats were comparable to those of their respective vehicle controls, except for high dose males late in the study. Survival of high dose mice of each sex was lower ($P < 0.001$) than that of the vehicle controls, whereas survival of low dose mice of each sex was comparable to that of the vehicle controls. Mean body weights of high dose mice of each sex were generally lower than those of the vehicle controls throughout most of the 2-year studies.
>
> Administration of 4-vinylcyclohexene to F344/N rats by gavage for 2 years was associated with a slightly increased incidence of epithelial hyperplasia of the forestomach (1/50; 3/50; 5/47) and squamous cell papillomas or carcinomas (combined) of the skin in high dose males (0/50; 1/50; 4/50). Low dose female rats, whose survival was more similar to that of the vehicle controls, had a marginally increased incidence of adenomas or squamous cell carcinomas (combined) of the clitoral gland (1/50; 5/50; 0/49).

In B6C3F1 mice, administration of 4-vinylcyclohexene for 2 years by gavage was associated with mild, acute inflammatory lesions and epithelial hyperplasia of the forestomach, especially in males (0/47; 7/50; 7/46), and with an increased incidence of a number of other nonneoplastic lesions, including lung congestion in high dose males and females, splenic red pulp atrophy in high dose males, congestion of the adrenal gland in high dose females, and cytologic alteration of the adrenal cortex in low dose and high dose females.

The incidences of uncommon ovarian neoplasms were markedly increased ($P < 0.01$) in both groups of dosed female mice (mixed tumor, benign: 0/49; 25/48, 52%; 11/47, 23%; granulosa cell tumor: 1/49, 2%; 9/48, 19%; 11/47, 23%; granulosa cell tumor or carcinoma [combined]: 1/49, 2%; 10/48, 21%; 13/47, 28%). In addition, a slight increase in the incidence of adrenal gland adenomas in high dose females was observed (0/50; 3/49, 6%; 4/48, 8%). The extensive mortality seen in the high dose male mice confounded interpretation of the increased incidences of malignant lymphomas and alveolar/bronchiolar adenomas or carcinomas (combined) of the lung seen in these animals surviving to the end of the study (malignant lymphomas: 3/37,8%; 5/39, 13%; 4/7, 57%; alveolar/bronchiolar adenomas or carcinomas [combined]: 3/37, 8%; 9/39,23%; 3/7,43%).”

Appendix D Sections 5.2 and 5.3 from the Sapphire Group (2008)

5.2 Mutagenic MOA

Mutation as a MOA for the VCH-induced mouse ovarian tumors is an alternative MOA to that proposed in section 5.1. In the mutation MOA, mutation is the only key event in the pathway to tumor induction. The text of the U.S. EPA 2005 document *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposures to Carcinogen* describes effects that are indicators for determining a mutagenic MOA for cancer in the following words.

"Key data for a mutagenic mode of action may be evidence that the carcinogen or a metabolite is DNA reactive and/or has the ability to bind DNA. Also, such carcinogens usually produce positive effects in multiple test systems for different genetic endpoints, particularly gene mutations and structural chromosomal aberrations, and in tests performed *in vivo* which generally are supported by positive tests *in vitro."*

The genotoxicity studies conducted on VCH, VCH-1,2-epoxide and VCH-diepoxide are listed in Table 7 through 9. VCH does not appear to be genotoxic when tested both *in vitro* and *in vivo.* The epoxides of VCH have also been tested for genotoxicity *in vitro,* but not *in vivo* (Tables 8 and 9). VCH-1,2-epoxide is not mutagenic either in bacteria or in V79 Chinese hamster cells, but one study reported increased chromosomal aberrations and micronuclei V79 Chinese hamster cells. For VCH-diepoxide, the results generally suggest a genotoxic response in bacteria and V79 Chinese hamster cells *in vitro.* Without *in vivo* studies on the VCH epoxide metabolites, the genotoxic potential of these compounds cannot be determined. Given the greater weight on the genotoxicity data set for VCH versus the epoxides of VCH, since VCH has been tested in multiple test systems for different genetic endpoints, the weight of evidence does not support a mutagenic MOA for VCH.

A mutagenic MOA for VCH is also not consistent with the toxicity data set for this compound. Although VCH-diepoxide is a DNA-reactive chemical, at least in *in vitro* systems, tumors were not observed at multiple sites, or in multiple species subsequent to BCH *(i.e., VCH)* exposure. Furthermore, there was not an increase in tumor-bearing animals, increase in tumor muliplicity, or early tumor response in the long-term studies. Contrast the findings of VCH with that of 1,3-butadiene (BD), which also produces both monoepoxide and diepoxide metabolites and produces at least some tumors through a possible mutagenic MOA (Preston *et al.,* 2007). Like VCH, BD produces toxicity when it is metabolized to its reactive epoxide metabolites. Mice are more sensitive than rats due to differences in metabolism: there is greater production of mono- and di-epoxide metabolites in mice compared to rats, with mice having lower capacity for detoxification of these reactive intermediates. BD is genotoxic *in vitro* and *in vivo;* the *in vivo* studies are positive for mice, but negative in rats due to the species difference in metabolism of the DNA-reactive epoxide metabolites. Ovarian atrophy (follicular loss) and ovarian tumors are also seen in BD-exposed mice, but not rats, due to the presence of the diepoxide of BD. But, unlike VCH, BD produces toxicity and tumors at multiple sites. Thus, when compared to the structurally-

related genotoxic analogue BD, VCH does not appear to have the same profile. VCH selectively targets the ovarian follicles in mice, without showing effects at any other sites, a pattern of toxicity that is inconsistent with a mutagenic MOA.

Table 7
***In vitro* and *In vivo* Genotoxicity Studies on VCH**

Endpoint	Test System	Results	Reference
Mutagenicity	Ames/Salmonella TA98, TA100, TA1535, TA1537	Negative (± S9)	Zeiger *et al.* (1997)
Chromosomal aberrations	Chinese Hamster Ovary (CHO) cells	Negative	NTP (1989), U.S. EPA (1994)
Sister chromatid exchange	Chinese Hamster Ovary (CHO) cells	Negative	NTP (1989), U.S. EPA (1994)
Mutagenicity	L5178Y mouse lymphoma cells	Equivocal (at most)	U.S. EPA (1994)
Micronuclei	Mouse bone marrow (2-day inhalation)	Negative	Bevan *et al.* (2001)
Micronuclei	Mouse bone marrow (90-day inhalation)	Negative	Bevan *et al.* (2001)

Table 8
***In vitro* Genotoxicity Studies on VCH-1,2-epoxide**

Endpoint	Test System	Results	Reference
Mutagenicity	Ames/S. typhimurium TA100 (standard plate test)	Negative	Turchi *et al.* (1981)
Mutagenicity	Ames/S. typhimurium TA100 (liquid test)	Negative	Turchi *et al.* (1981)
Mutagenicity	Ames/ S. typhimurium TA100, TA98, TA1535, TA1537	Negative (+/- S9)	Watabe *et al.* (1981)
Mutagenicity	V79 Chinese hamster cells	Negative	Gervasi *et al.* (1980)
Mutagenicity	V79 Chinese hamster cells	Negative	Turchi *et al.* (1981)
Chromosomal aberrations	V79 Chinese hamster cells	Positive	Turchi *et al.* (1981)
Micronuclei	V79 Chinese hamster cells	Positive	Turchi *et al.* (1981)

Table 9
***In vitro* Genotoxicity Studies on VCH-diepoxide**

Endpoint	Test system	Results	Reference
Mutagenicity	Ames/S. typhimurium TA100 (standard plate test)	Negative	Turchi *et al.* (1981)
Mutagenicity	Ames/S. typhimurium TA100 (liquid test)	Positive	Turchi *et al.* (1981)
Mutagenicity	Ames/S. typhimurium TA100, TA98, TA1535, TA1537	Positive (TA100 only; -S9)	Watabe *et al.* (1981)
Mutagenicity	Ames/S. typhimurium TA100, TA98 (spot test)	Positive (TA100 only; +/- S9)	Wade *et al.* (1979)
Mutagenicity	Ames/S. typhimuirum TA100, TA98, TA1535, TA1537	Positive (TA100, TA1535 only; -S9)	El-Tantawy *et al.*, 1980
Mutagenicity	S. cerevisiae D7	Positive (-S9)	Bronzetti *et al.* (1980)
Mutagenicity	V79 Chinese hamster cells	Positive	Gervasi *et al.* (1980)
Mutagenicity	V79 Chinese hamster cells	Positive	Turchi *et al.* (1981)
Chromosomal aberrations	V79 Chinese hamster cells	Positive	Turchi *et al.* (1981)
Micronuclei	V79 Chinese hamster cells	Negative	Turchi *et al.* (1981)

5.3 Conclusion

There is a high degree of confidence that VCH acts through a non-genotoxic, non-linear (threshold) MOA in producing ovarian tumors in mice. The critical event is the destruction of primordial and primary follicles in the ovary by the diepoxide metabolite. The MOA is assumed to be relevant to humans; but, due to metabolic differences between the mouse and humans, humans are expected to be less susceptible to the ovarian effects than mice, given comparable doses. Although there is limited *in vitro* data indicating potential genotoxic potential from the epoxide metabolites of VCH, particularly VCH-diepoxide, the weight of evidence for a genotoxic MOA is not compelling.

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