MICHIGAN DEPARTMENT OF ENVIRONMENTAL QUALITY

INTEROFFICE COMMUNICATION

January 29, 2001

TO: File for 4-vinylcyclohexene (100-40-3)

FROM: Marco Bianchi, Toxics Unit, Air Quality Division

SUBJECT: Initial Threshold Screening Level

The Initial Threshold Screening Level (ITSL) for 4-vinylcyclohexene (VCH) is 4 $\mu g/m^3$ based on an 8 hour averaging time. The following references or databases were searched to identify data to determine the ITSL/IRSL: IRIS-online, HEAST, NTP Management Status Report-online, RTECS, EPB-CCD, EPB library, CAS-online, NLM-online, IARC-online, NIOSH Pocket Guide, and ACGIH Guide.

The above databases provided a diverse quantity of studies to review the toxicity of VCH. The National Toxicology Program (NTP) conducted an oral 14-day, 13-week and 2-year bioassay on rats and mice for VCH, and a dermal 14-day, 13-week and 2-year bioassay on rats and mice for VCH-diepoxide. VCH-diepoxide is a major metabolite of VCH that is known to cause ovarian toxicity in mice, but not in rats. Reproductive/developmental testing confirmed the ovotoxicity of VCH in different strains of mice orally dosed for 18-weeks or by intraperitoneal injections for 30 days. In addition to these studies, the American Council of Governmental Hygienists (ACGIH) established a Threshold Limit Value (TLV) for 4-vinylcyclohexene of 0.1 ppm (0.44 mg/m^3).

4-Vinylcyclohexene (VCH), a dimer of butadiene, is a colorless liquid used primarily as an intermediate in the production of 4-vinylcyclohexene diepoxide (IARC, 1994). It is produced commercially by the dimerization of butadiene, a process that also occurs during rubber curing. VCH also present in gases discharged during the production of synthetic rubber, especially as a result of the process of curing rubber for tire manufacturing (Rappaport and Fraser 1976).

Acute Studies

In acute studies, data obtained from the ACGIH documentation showed the VCH oral LD_{50} value for the Carworth-Wistar rat is 3.08 (2.49 to 3.81) ml/kg. For short-term inhalation exposures, rats (strain unknown) could tolerate a concentrated VCH vapor for up to 15 minutes without death, but inhalation of 8000 ppm VCH for 4 hours killed four of six rats. Another acute inhalation study resulted in a rat LC_{50} of 6095 ppm. Comparatively, the mouse inhalation LC_{50} has been determined to be 10,610 ppm. The dermal LD_{50} value for the rabbit is 20 ml/kg (exposure site covered), while skin irritation after application of undiluted VCH to shaved rabbit skin (exposure site uncovered) was rated moderate. A small necrotic area on the cornea resulted from instillation of 0.5 ml undiluted VCH into a rabbit eye.

Subchronic Studies

Subchronic studies have shown that administration of VCH (stock solution containing 0.01% butylated hydroxytoluene) by gavage in corn oil at 0, 300, 600, 1250, 2500, or 5000 mg/kg/day for 14 days to groups of five male and five female F344 rats and B6C3F1 mice killed all rats dosed with ≥1250 mg/kg, and killed all mice dosed with 2500 or 5000 mg/kg (NTP 1986). Prior to death, rats showed central nervous system (CNS) depression, tremors, and gastrointestinal

distress. No compound-related gross or histologic changes were observed. Tremors and inactivity were observed in the mice that died (NTP 1986).

Oral intubation of VCH in corn oil 5 days/week for 13 weeks to groups of ten male and ten female F344 rats at 0, 50, 100, 200, 400, or 800 mg/kg/day showed depressed body weight gain and hyaline droplet degeneration of the male rat kidney proximal convoluted tubules (NTP 1986). In B6C3F1 mice given 0, 75, 150, 300, 600, or 1200 mg/kg/day, extensive mortality occurred in the high-dose mice. The major effect in mice was the reduction in the numbers of primary follicles and mature graafian follicles in the ovaries of all high-dose female mice. The ovaries of mice given less than 1200 mg/kg/day were not examined for histopathologic change (NTP 1986).

Male and female Sprague-Dawley rats and B6C3F1 mice were exposed by inhalation to VCH 6 hour/day, 5 days/week for 13 weeks. Rats were exposed to 0, 250, 1000 or 1500 ppm, and mice were exposed to 0, 50, 250, or 1000 ppm. In addition, another group of rats and mice was exposed to 1000 ppm butadiene so that a comparison could be made between two compounds. 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 female 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 to1000 or 1500 ppm VCH or 1000 ppm 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, a no-observed-adverse-effect-level (NOAEL) is 1000 ppm for rats based on lethargy and lowered body weights and 250 ppm for mice base on mortality and ovarian atrophy.

Reproductive/Developmental Studies

In reproductive/developmental studies, Hooser et al., (1991) gave 15 female B6C3F1 mice daily intraperitoneal injections of 6 mmol/kg body weight of VCH in sesame seed oil and an equal number of control mice equivalent injections of the vehicle alone for 30 days. Daily vaginal smears were used to determine estrus. On day 31, all mice were sacrificed and the numbers of primary (22 ± 10) and secondary (26 ± 7) ovarian follicles were found to be reduced in the VCH-treated mice compared to the controls (298 ± 63 and 115 ± 32, respectively). The number of estrous cycles per 30 days was also reduced significantly in the VCH-treated mice (3.2 ± 1.0) compared to the control (4.8 ± 1.5). In a study by Grizzle et al., (1994) F_0 generation CD-1 Swiss mice were given daily oral doses of 0, 100, 250, or 500 mg/kg body weight of VCH for 18 weeks. No alterations in reproductive competence (litters/pair, pups/litter, % live born) or in food or water consumption were observed (Grizzle 1994). In the second generation, (F_1), males

given 500 mg/kg had decreased spermatid head count (with normal sperm number, normal testis, and epididymal weight), and F_1 females given 500 mg/kg had decreased numbers of primordial, growing, and antral follicles. Grizzle et al., (1994) concluded that VCH exposure at up to 500 mg/kg/day decreased body weight and reduced the numbers of gametes, but no alterations of reproductive parameters in the F_0 or F_1 CD-1 mice occurred.

Carcinogenicity Studies

In carcinogenicity studies, VCH (> 98% pure) was administered by oral intubation in corn oil 5 days/week for 2 years at 0, 200, or 400 mg/kg/day to groups of 50 F344/N rats and B6C3F1 mice of each sex (NTP 1986). Mortality among the rats was so great that it compromised the study, but marginally suggestive increases in squamous cell papillomas or carcinomas in male rat skin were observed. Among the mice, acute inflammation and epithelial hyperplasia of the forestomach with assorted histopathologic changes (lung congestion, splenic red pulp atrophy, and adrenal gland congestion) that were not necessarily dose-dependent were observed. Mortality among the high-dose male mice confounded interpretation of the scattered instances of lymphomas and cancers of the lung. There was also some suggestion that the increased numbers of adrenal gland adenomas (found only in the high-dose female mice) may have been related to VCH oral intubation. The numbers of uncommon ovarian neoplasms and ovarian pathologies were increased significantly among VCH-treated mice.

Application of 45 mg VCH in 50% benzene to the shaved dorsal skin of 30 Swiss mice, 3 days/week for 54 weeks resulted in extensive skin damage and produced one squamous cell carcinoma, a result attributed by the authors to trace concentrations of VCH hydroperoxide, a known animal carcinogen. It is noteworthy that similar skin-painting bioassay or intraperitoneal injection with the VCH metabolite VCH-1,2-diepoxide produced squamous cell carcinomas and sarcomas or peritoneal sarcomas, respectively, confirming earlier dermal and parenteral VCH-1,2-diepoxide studies in rodents. IARC found that the skin painting studies were inadequate for evaluation, but concluded that there was sufficient evidence for the carcinogenicity of VCH in animals.

Genotoxicity Studies

In genotoxicity studies, VCH was not mutagenic in Salmonella typhimurium TAI00, TA1535, TAI537, or TA98, either in the presence or absence of an Arochlor®1254-induced male Sprague Dawley rat or male Syrian hamster *in vitro* hepatic (S9) metabolic activating system. Turchi et al. (1981), studied the genotoxic potential of mouse liver microsome-generated VCH metabolites in base substitution assay with Salmonella typhimurium TA100 and in point mutation and cytogenetic analyses with cultured V79 Chinese hamster ovary (CHO) cells. In Salmonella typhimurium, only the VCH-diepoxide showed a mutagenic response, a result confirming previous reports. Other investigators expanded studies with the VCH-diepoxide to include Salmonella typhimurium tester strains TA100, TA1535, TA98, or TA 1537; however, a positive response was observed only in TA100 or TA1535. In point mutation studies with V79 cells, a significant increase in mutation frequency was observed only with VCH-diepoxide; however, addition of 2.0-2.5 mmol of either VCH-1,2-epoxides or VCH-7,8-epoxide-1,2-diol led to anaphase bridges and lagging chromosomes; micronuclei in cultured V79 cells occurred at 25 to 40 hours after treatment with VCH-1,2-epoxide or VCH-7,8-epoxide-1,2-diol, but no such chromosomal aberrations occurred after incubation with VCH-diepoxide. The VCH-diepoxide metabolite was mutagenic for Klebsiella *pneumoniae (26) and* in studies on VCH metabolite alkylation potential towards 4-(p-nitrobenzyl)pyridine, the metabolites showed relatively little reactivity, save for the VCH-diepoxide.

Pharmacokinetic/Metabolism Studies

In pharmacokinetic/metabolism studies male Wistar rat microsomes transformed VCH *in vitro* to VCH-1,2-epoxide and VCH-7,8-epoxide-1,2-diol in the ratio 3.5:1. Trace amounts (0.001 %) of vinylcyclohexene diepoxide were produced.

In similar studies with microsomes from female rat and mouse liver, lung, and ovary, the mouse liver V_{max} for production of the VCH-1,2-epoxide was 56 times that of the rat and that for mouse lung was 2 to 4 times that of the rat. Formation of the VCH-1,2-epoxide could not be detected in studies with rat or mouse ovarian microsomes. The 7,8-epoxide was formed only slowly by rat; mouse liver was 2 to 12 times less efficient in formation of the 7,8-epoxide than in production of the 1,2-congener. Mouse lung microsomes were more efficient than liver microsomes in catalyzing formation of the 7,8-epoxide. Formation of the diepoxide from VCH-1,2-epoxide occurred primarily with liver preparations; no such metabolism occurred with ovarian microsomes. Hydrolysis of these epoxides to the corresponding diols occurred with greater efficiency in rat tissues where mouse displayed greater efficiency in production of the epoxides; these factors combine to suggest that the rat metabolized dose would be reduced compared to mouse. Multiple intraperitoneal injections of VCH at 500 mg/kg induced the activities of several liver function oxidase enzymes, including those enzymes responsible for VCH biotransformation to VCH-diepoxide. This induction was not as great as that seen for classic inducing agents (phenobarbital, polycyclics) but it was comparable to that resulting from styrene exposure. Further, intraperitoneal injection of VCH VCH-diepoxide metabolite rapidly depleted mouse hepatic reduced glutathione, and VCH-diepoxide substrate for mouse glutathione-S-transferase.

A single oral dose of 400 mg/kg 4-[ethylene-^{14}C] (99%, containing tert-butylcatechol as antioxidant) in corn oil was given to fasted female B6C3F1 mice and female F344 rats. The mice excreted 95% of the administered dose within 24 hours, and the rats excreted 95% of the dose within 48 hours via urine (50% to 60%) and expired air (30% to 40%). Negligible amounts of VCH appeared as $^{14}C0_2$; fecal elimination accounted for 3% to 9% of the dose. Total tissue radioactivity in mice hours constituted < 1% of the dose; in rats, 3.4%, 1.1%, and 1.1 % of the dose was retained in adipose tissue, skeletal muscle, and skin, respectively. Neither parent VCH nor its metabolites accumulated in the ovaries of either species [accounting for 0.05% of the administered dose], and there were no species-specific differences in ovarian distribution. After a single intraperitoneal injection of 800 mg/kg VCH in corn oil to female rats or mice, the VCH-1,2-epoxide metabolite appeared in mouse at concentrations 20 times those found in rats. In studies with rat and mouse hepatic microsomes, the rate of VCH epoxidation by mice was 6.5 times greater than rats.

In mice, hepatic biotransformation of VCH results in the production of a circulating reactive epoxide that can reach peripheral tissues; when VCH or its epoxide metabolites were injected into either weanling B6C3F1 female mice or F344 female rats at doses ranging from 0.07 to 7.4 mmol/kg/day (10 to 800 mg/kg/day) for 30 days, VCH-1,2-diepoxide proved the most potent ovotoxic compound. In mice, the epoxides were 5 to 10 more potent ovotoxins than VCH, and in rats, 30 intraperitoneal injections at 800 mg/kg of the parent VCH failed to cause detectable ovotoxicity. Pre-treatment of mice with chloramphenicol inhibited VCH epoxidation by 69% and protected mice from VCH-induced ovotoxicity. Consistent with the above metabolic paradigm VCH epoxidation was increased in phenobarbital induced female B6C3F1 mice. With increasing VCH dose in B6C3F1 mice, corresponding increases in circulating VCH epoxide were observed. Thus, the VCH diepoxide metabolite was the most potent ovotoxin, and it was also the most potent mutagen compared to VCH and its 1,2-epoxide metabolite.

The lack of ovotoxicity after VCH exposure in rats compared to mice can be traced to slower rates of VCH biotransformation to reactive epoxides and a rate of epoxide detoxification twice that in mice. The biochemical basis for these differences in rates of VCH metabolism has been attributed to the facts that female B6C3F1 mice express hepatic microsomal P450IIA and P-450IIB enzymes, whereas female F344 rats completely lack the P-450 isoform that is immunochemically related to mouse P-450IIA, and female rats have only very low amounts of P-450IIB, the enzyme forms responsible for VCH epoxidation. In comparison to B6C3F1 mice, female 129/J mice, as with female F344 rats, possess relatively little P-450118 enzyme and have lower rates of microsomal VCH epoxidase. Based on experiments with antirat P-450IIIA IgG, it was apparent that mouse P-450IIIA forms did not participate in VCH epoxidation.

Human Studies

In human studies, microsomes prepared from 12 cadavers or from liver resected at surgery metabolized VCH to VCH1,2- or 7,8-epoxides *in vitro* even in the absence of glucose-6-phosphate (required for NADPH production). The 7,8-epoxide was formed six times slower than the 1,2-epoxide; there was no substantial difference in the rates measured for males and females. The rates of VCH epoxidation by human female microsomes *in vitro* were comparable to the rates measured for female rat microsomes. It appeared that the reactive epoxide metabolites were subject to detoxification by human epoxide hydrolase.

Summary

From the information presented above, it can be concluded that VCH requires metabolic activation in order to elicit mutagenic, carcinogenic, and ovotoxic properties. The VCH-diepoxide appears to be the most potent metabolite identified, mainly affecting female mice by all routes of administration. This finding is supported by studies which showed that female mice microsomes metabolized VCH to VCH-1,2-epoxide 56 times the rate as rats. Corresponding to this faster rate of biotransformation, female mice metabolized the toxicVCH-1,2-epoxide to the less toxic VCH-diols twice as slowly as rats. Therefore, the toxic effects of VCH seem to favor female mice more than other test species.

The NTP bioassay showed that oral exposure to VCH was carcinogenic in female B6C3F1 mice. Results showed 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; and 11/47; granulosa cell tumor:1/49; 9/48; and 11/47; granulosa cell tumor or carcinoma (combined): 1/49; 10/48; and 13/47. Therefore, the NTP Peer Review Committee concluded that 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. Data on ovarian granulosa cell tumors and carcinomas combined in B6C3F1 mice were used to determine the IRSL because these data produced the highest potency. A slope factor was derived for these data using the Global82 Linearized Multistage Model and equaled 2.26×10^{-3} $(mg/kg/day)^{-1}$. This unit risk value would be equivalent to an IRSL value of 2 $\mu g/m^3$ annual averaging.

The ACGIH did a thorough job in their assessment of this compound by summarizing a number of key studies that have been conducted. However, it is uncertain how they obtained the TLV of 0.1 ppm (0.44 mg/m^3) for VCH, since no dose level was selected from any of the animal or human studies to use as a basis for this number. It appears the ACGIH-TLV committee was uncomfortable with the current collection of toxicity data to base their conclusions on a particular study. Instead, they qualitatively described the basis for the TLV as "the lack of VCH mutagenicity in standard short-term tests, the finding of VCH-induced cancer in mice, and the clear evidence for VCH-1,2-epoxide induced carcinogenesis in rats and mice." Their rationale is

justified considering the number of problems that plagued the subchronic and chronic NTP studies. The major problem was excessive mortality in the mid- and high-dose groups for two test species with no identified cause. Additionally, the 13-week inhalation study that held promise of obtaining a highly confident NOAEL was compromised because histopathology was conducted on the high dose (1000 ppm) females, but not done routinely on low- (50 ppm) or mid-exposed (250 ppm) females. A histological sample was taken only if gross ovarian lesions were noticed during necropsy. It is puzzling why a histopathologic examination was not conducted on ovarian tissues from all dose groups when it was already known that the compound was ovotoxic. This decision by the study investigators raises serious doubts in the confidence of a 250 ppm NOAEL for VCH. Therefore, the 13-week subchronic study will not be used to derive an inhalation Reference Concentration (RfC); instead the ITSL will be derived by using the occupational exposure level (OEL) according to Rule 232(1)(c). Likewise, the NTP carcinogenicity study will not be used to derive an IRSL for this compound for the following reason. It is difficult to extrapolate the oral corn oil bioassay data to a situation where VCH dermal and inhalation exposures may occur. For rigorous interspecies dose extrapolations, it is necessary to utilize physiologically based pharmacokinetic (PBPK) data on retained (or absorbed) dose after VCH inhalation and the comparative mouse-human or rat-human VCH-epoxide metabolized dose. Human microsomes metabolized VCH to genotoxic and carcinogenic metabolites, but no data on rates of detoxification by conjugation or hydrolysis are available to conduct PBPK interspecies dose scaling. A comprehensive PBPK literature search was conducted on 4-vinylcyclohexene, but no information was found. The OEL of 0.44 mg/m^3 (or an ITSL of 4 µg/m^3 8 hour averaging) is set far enough below any published concentration that was shown to cause any animal toxicity and, therefore, is expected to be protective of target organ toxicity and skin sensitivities. When compared to the IRSL of 2 µg/m^3 annual averaging and adjusted for averaging times, the ITSL would be protective from both an acute and chronic exposure.

Screening Level	ITSL/IRSL	Averaging Time Adjusted
ITSL	4 µg/m^3; 8 hour	0.2 µg/m^3; annual
IRSL	2 µg/m^3; annual	43.5 µg/m^3; 8 hour

The ITSL was determined as follows:

ACGIH TLV = 0.44 mg/m^3

0.44 mg/m^3 ÷ 100 = 0.0044 mg/m^3

$$0.0044 \text{ mg/m}^3 \times \frac{1000 \text{ µg/m}^3}{1 \text{ mg/m}^3} = 4.4 \text{ µg/m}^3$$

The ITSL for 4-vinylcyclohexene = 4 µg/m^3 based on an 8 hour averaging time

References:

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MB:DB
cc: Cathy Simon, AQD
Mary Lee Hultin, AQD
Sheila Blais, AQD