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**Toxicity, Mutagenicity, and Mutational Spectra of  
Vinyl Chloride, 2-Chloroethylene Oxide, and Chloroacetaldehyde  
in a Human Lymphoblastoid Line Expressing Cytochrome P450IIE1**

by

Wade Henderson Weisman

A Thesis submitted to the faculty of the School of Public Health of The University of North Carolina at Chapel Hill in Partial fulfillment of the requirements for the degree of Master of Public Health in the Department of Environmental Sciences and Engineering.

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**WADE HENDERSON WEISMAN. Toxicity, Mutagenicity and Mutational Spectra of Vinyl Chloride, 2-Chloroethylene Oxide, and Chloroacetaldehyde in a Human Lymphoblastoid Line Expressing Cytochrome P450IIE1.**

(Under the direction of Thomas R. Skopek)

### **ABSTRACT**

Vinyl Chloride Monomer (VCM, CAS # 75-01-4) is one of the few chemicals identified as a human carcinogen (IARC, 1979). In this report, the toxicity and mutagenicity of VCM and its two metabolic products, 2-chloroethylene oxide (CEO) and 2-chloroacetaldehyde (CAA) were studied in a human B-lymphoblastoid line containing cloned cytochrome P450IIE1. Cytochrome P450IIE1 is capable of metabolizing VCM to CEO which non-enzymatically rearranges to CAA. Toxicity was determined by plating cells immediately after exposure and comparing their clone-forming ability to that of untreated cells. Mutagenicity at the hprt locus was determined by plating cells in the presence of the selective agent 6-thioguanine. Delivered doses of 25  $\mu$ M to 400  $\mu$ M VCM x 24 hours produced no measureable toxicity but resulted in induced mutation frequencies that ranged from  $0.5 \times 10^{-6}$  to  $5.6 \times 10^{-6}$ . Although increases in mutation frequency were consistently seen, a clear dose-response was not apparent. Dose dependent increases in toxicity and mutagenicity were observed with both CEO and CAA. Treatments of 25  $\mu$ M CEO x 24 hours resulted in survival of 0.82 and induced mutation frequency of  $8 \times 10^{-6}$ , while similar treatments with CAA produced a

survival of 0.06 and induced mutation frequency of  $9 \times 10^{-6}$ . Comparison of mutation frequency/ survival ratios for the three compounds at the same induced mutation frequency suggest the majority of mutations induced by VCM must be produced by CEO.

Denaturing gradient gel electrophoresis (DGGE) was used to identify unique VCM, CEO, and CAA mutations in exon 3 of the hprt gene. DGGE banding patterns from CAA and CEO isolated mutants were compared to the banding patterns from VCM. 118 VCM mutants analyzed in the low temperature melting domain of exon 3, 8.5% (10 isolates) produced identical DGGE banding patterns; sequencing of the isolated mutant bands revealed a G-T transversion at base pair 292. In the high temperature melting domain, 3.4% (4 of 118 isolates) exhibited identical DGGE bands; sequencing revealed a G→A transition at base pair 197. These sequence changes represent potential VCM mutational "hotspots". The low-temperature "hotspot" banding pattern was evident in less than 2% (1 isolate) of the spontaneous mutants in the VCM experiment. However, it was present in 5.6% (11 isolates) of the CEO mutants analyzed.

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As for the direction and incredible planning abilities to get me done "on-time", I know I could not have worked for a better investigator, thank-you Tom.

Whatever significance may have been attached to this document will quickly pass. For this reason I dedicate it to someone whose work has infinitely more significance than mine ever will, my patient and understanding wife Debbie, whose job in training up our two boys has eternal significance.

## FOREWORD

The identification of biomarkers that can serve as indicators of human exposure to specific chemicals is an important goal of the study of carcinogenesis. The use of biomarkers takes into account a variety of factors affecting a compound's toxicity, including differences in route of uptake, the metabolism to activate or detoxify the compound, the ability to excrete the compound or its metabolites and the ability to repair damage (Wogan, et al., 1987).

Biomarkers include a broad range of analytical targets that can be grouped in two general categories: 1) measurements of concentrations of chemicals or their derivatives in body fluids or excreta, and 2) measurement of biological reaction products such as DNA adducts, mutation, chromosomal aberrations or sister chromatid exchanges produced in the tissues of exposed individuals (Wogan, et al., 1987). Two important elements of a biomarker are 1) its relevance to the exposure of concern (correlation of the measured biomarker to an adverse human health effect); and 2) the sensitivity of the biomarker, (whether it can detect differences between exposed and non-exposed individuals).

DNA adduct formation is an endpoint relevant to exposure to genotoxic agents. Furthermore, it can be measured with sufficient sensitivity to differentiate between exposed and non-exposed individuals and can function over a wide range of doses (Swenberg et al., 1990). The molecular dose of a compound is the actual amount of chemical that reacts with macromolecular targets, such as DNA. This dose is very small in comparison to the environmental concentration of the chemical (Swenberg et al., 1990). The determination of DNA adducts can clarify the relationship between the external exposure and the molecular

dose, at exposure concentrations which are much less than those used in bioassays (Swenberg et al., 1990).

DNA adducts do not always lead to mutations. Replication of the DNA is required to convert the adducts formed (promutagenic lesions) to actual mutations (Swenberg et al., 1990). DNA repair systems can repair some of the adducts before replication of DNA thus avoiding mutation. Also, many adducts are intrinsically nonmutagenic while others are very promutagenic. Some sites on DNA called, "hot spots", are more susceptible than other locations to the effects of chemical agents (Benzer, 1961 and Briscoe et al., 1984). Many mutagens exhibit characteristic patterns of mutation in the DNA that can be distinguished by comparison to patterns of mutation induced by spontaneous processes or by other mutagens (Fuchs, 1981; Skopek, 1982; Duane, 1986; Schaaper, 1986; Drobetsky, 1987; Richardson, 1987; Giroux, 1988; Vrieling, 1988; Liber, 1989). The patterns of these mutations are termed mutational spectra. Within these spectra, the active locations or "hot spots" can readily be discriminated by the location, type and frequency of the mutation in relation to other mutational events. Because of this mutational specificity, the base pair changes produced by the mutagen can imply additional information about the DNA adducts and the molecular events that produce the mutations (Fuchs, 1981; Skopek, 1982; Duane, 1986; Schaaper, 1986; Drobetsky, 1987; Richardson, 1987; Giroux, 1988; Vrieling, 1988; Liber, 1989). The study of DNA adducts and mutational spectra can help to determine causal relationships and may improve extrapolation of risk for cancer from experimental animals to humans. Measurement of DNA adducts and mutational spectra may also be used in biomonitoring programs to increase the power of these studies to detect early health hazards.

VCM is a proven human carcinogen and exposures are still a concern. The goal of the present project was to identify characteristic sequence changes induced by VCM and its metabolites, CEO and CAA. These specific mutation patterns could ultimately serve as biomarkers relevant to VCM exposure. Furthermore, these changes can be used to aid in determining which DNA adduct(s) is (are) involved in VCM mutagenesis; these adducts would serve as additional relevant indicators of health threats due to VCM exposure.

## **SPECIFIC AIMS**

The primary goal of the planned work was to study the frequency and nature of mutations induced by VCM and its metabolites, CEO and CAA, in a human cell line. This information would then be used to conclude which metabolite is mainly responsible for the mutagenic activity of VCM. Specifically, the following two aims were proposed:

1. To determine the toxicity and mutation frequency at the *hprt* gene induced by VCM, CEO, and CAA in a human lymphoblastoid cell line expressing cytochrome P450IIE1.
2. To characterize the mutational spectra of VCM, CEO, and CAA using denaturing gradient gel electrophoresis technology and DNA sequencing.

## **CHAPTER I**

### **TOXICOLOGY OF VCM**

Vinyl Chloride is a synthetic chlorinated monomer manufactured from petrochemical feedstock and chlorine. The compound is a colorless, explosive gas with a boiling point of  $-14^{\circ}\text{C}$  at atmospheric pressure and is slightly water soluble (EPA, 1975). About 96% of the VCM manufactured worldwide is used in the production of vinyl chloride homopolymer and copolymer resins. One of the major commercial products made with VCM is polyvinyl chloride (PVC) which is then used in the manufacture of a myraid of products (Sax, 1988). The estimate for vinyl chloride production in the U.S. for 1993 is approximately 11.0 billion pounds (ATSDR, 1992). The Occupational Safety and Health Administration (OSHA) has set a regulatory standard for VCM exposure in the workplace of 1 part per million (ppm) weighted over an 8-hour time period. Other OSHA regulations prohibit operations which result in VCM coming in contact with the skin. The National Institute of Occupational Safety and Health (NIOSH) has recommended the lowest limit of detectability as the limit for a ten-hour averaged exposure (Sax, 1988). A NIOSH survey conducted from 1981-1983 estimated that 81,000 U.S. workers are potentially exposed to VCM in the workplace (NOES, 1990). The major route of VCM exposure in an industrial setting is through inhalation; however, skin absorption is possible in the cleaning of polymerization ovens. In the 1970's the acute effects seen in VCM polymerization workers were believed to be the only hazardous properties of vinyl chloride. Monitoring of vinyl chloride at this time was directed primarily toward maintaining levels below the lower explosive limit (LEL) of 4% (Sax, 1988). Because of the low level of concern and the high levels permitted, VCM workers (especially those who cleaned the polymerization ovens), could have been exposed to levels in the thousands of parts per million (Purchase et al., 1987). This speculation agrees

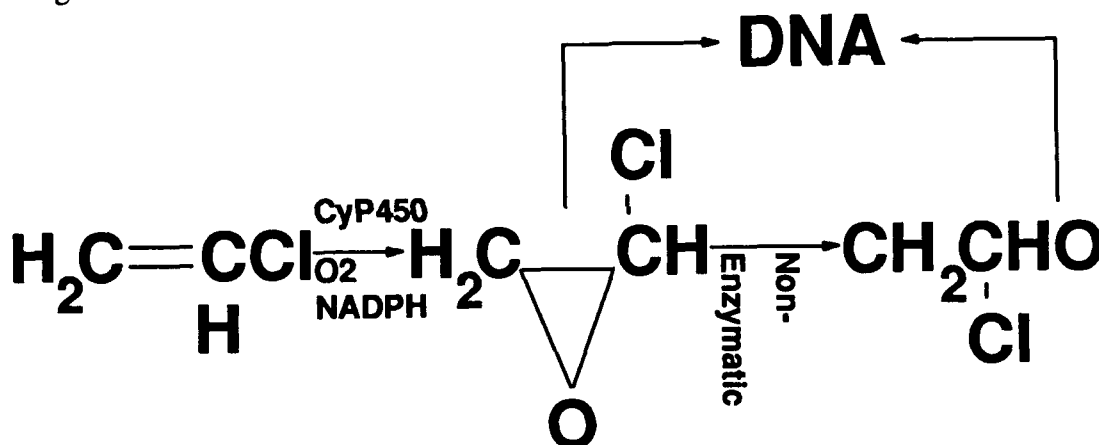
with reports that VCM oven workers would be removed from the work area in an unconscious or semi-conscious state due to the high levels of VCM exposure. Acute exposure to VCM in levels over 10,000 ppm can cause central nervous system depression, dizziness, lightheadedness, nausea, dulling of the senses and headache (Lester et al., 1963). Additionally, two fatalities had been reported in 1960 due to extremely high acute exposures (Danziger, 1960). Chronic adverse health effects are also seen from VCM exposure. These effects include: degenerative bone changes, circulatory disturbances, thrombocytopenia, splenomegaly, hepatomegaly and hepatic fibrosis (La Dou, 1990). More central to the focus of this research is the confirmation that angiosarcoma of the liver, a rare form of liver cancer, is caused almost exclusively by VCM exposure and is an often studied endpoint for chronic exposures (Purchase et al., 1987). Since 1974 there have been 118 cases worldwide of angiosarcoma of the liver reported to a central registry. Most of these individuals were workers involved in the cleaning of ovens used for VCM polymerization. This registry, along with numerous epidemiological studies estimate an additional 200-350 deaths over the next 30 years associated with past VCM exposure (Forman et al., 1985). The level of exposure to VCM capable of causing angiosarcoma has not been definitively shown to cause any other carcinogenic effects in man, as indicated by epidemiological studies with endpoints other than angiosarcoma (Purchase et al., 1987). This is another reason why most cohort studies of VCM workers use angiosarcoma of the liver as a definitive endpoint. Many epidemiological studies have centered on VCM workers, and a summary of these studies can be found in separate reviews by I.F.H. Purchase et al. (1987), and Sax (1988). These studies looked at a variety of cancer sites including liver, lung and brain. The excess liver cancers

found in most of these studies was due to an excess of angiosarcoma. One retrospective cohort study of 1151 workers at four vinyl chloride production facilities with over 12,000 person years at risk demonstrated that of the fourteen cases of biliary and liver cancer observed, eleven were cases of angiosarcoma (Sax, 1988). While the preponderance of epidemiological data strongly indicate that exposure to vinyl chloride increases a persons risk to a variety of cancers, angiosarcoma of the liver is the only cancer type that can be attributed unambiguously to VCM exposure.

Environmental sources of VCM contamination include product manufacturing and hazardous waste sites (EPA, 1985, Stephens et al., 1986). VCM was identified in at least 10% of the hazardous waste sites listed on the Environmental Protection Agency's National Priorities' List (NPL). Vinyl chloride emanating from VCM and PVC processing facilities accounts for the majority of VCM entering the environment. VCM has a half-life in the environment of only 1-2 days as sunlight will cause the monomer to polymerize. People who live near production facilities are the only segment of the non-occupationally exposed public to receive any measureable exposure. Their estimated daily intake of VCM is from 0-2,100  $\mu\text{g}/\text{person}/\text{day}$  (ATSDR, 1992). Other potential routes of non-occuapional VCM exposure is from ingestion of food and water contaminated with VCM through leaching of packaging material and pipes. A study on bottled water estimated the average daily intake of VCM at 120ng VCM/person/day if bottled water were the only source of drinking water. Many pipes that supply homes drinking water are made from PVC and the potential exists that the migration of uncured monomer from the pipes is contributing to the total amount of VCM ingested (Benfanati et al., 1991). VCM is also produced via the natural anerobic microbial

degradation pathways of two of the most common pollutants at hazardous waste sites, trichloroethylene (PCE) and perchloroethylene (Vogel et al., 1985).

Vinyl chloride is metabolized through an NADPH-dependent cytochrome P450IIE1 mixed function oxidase system to the reactive species 2-chloroethylene oxide (CEO), which non-enzymatically rearranges to form 2-chloroacetyldehyde (CAA). Both CEO and CAA are highly reactive and are capable of modifying DNA. Consequently, the carcinogenic effects of VCM have been attributed to these electrophilic metabolites rather than the parent compound (Miller, 1976). The general reaction and rearrangement for this process is given in Fig. 1-1:



(From Purchase et al., 1987 and Zajedela et al., 1980)

VCM is similar to many other industrial chemicals, in that its adverse effects are attributable to one or more of the metabolites. The concentration of metabolite produced from a parent compound does not continue to increase proportionately with increasing levels of exposure. Extremely high doses of a chemical can overwhelm the metabolic system responsible for activating or detoxifying the compound. Gehring et al., (1978) discovered that the toxicity of vinyl chloride exposed male Sprague-Dawley rats was a saturable process

following Michaelis-Menten kinetics. Using  $^{14}\text{C}$  labeled VCM, they measured total radioactivity in animals sacrificed after equal exposure time to various concentrations of VCM. They discovered that the amount of VCM metabolized reached a plateau between 9 and 25 parts per million (ppm). They also concluded from this study that VCM-induced angiosarcoma is a function of the metabolic activation rather than the exposure concentration (Gehring et al., 1978).

A large number of *in vivo* animal studies as well as *in vitro* studies have been carried out to examine the mechanism of action of VCM. An extensive listing which summarizes these studies is provided in Sax (1988).

Work by C. Malaveille et al. (1975), using histidine reversion in *S. typhimurium* as an endpoint, demonstrated the need for metabolism in VCM mutagenesis. They exposed bacteria to gaseous VCM, both with and without S-9 liver fractions from humans, rats and mice. They demonstrated a mutagenic effect from CAA and an even greater effect from CEO in bacterial strains which revert by base pair substitution. No mutagenic activity was demonstrated in strains reverted by a frameshift (Maleville, et al., 1975). In the same study, using the 4-(p-nitrobenzyl)pyridine assay with CEO or CAA, they determined that CEO possess a greater alkylating activity than CAA. In a review of *in vitro* studies involving VCM exposure to microorganisms and VCM exposures to Chinese hamster ovary cells, Bartsch et al.,(1976) concluded that both CAA and CEO are mutagenic in these test systems, but that CEO was far more effective; in bacterial studies the difference was as great as 1000 fold (Bartsch et al., 1976). They concluded that CEO was the metabolite most responsible for the mutagenic effects of VCM.

Guengerich et al., (1979) using reconstituted and microsomal cytochrome P450 from rats concluded that CEO was the alkylating agent involved in conversion of adenosine to 1,N<sup>6</sup>-ethenoadenosine. 1,N<sup>6</sup>- Ethenoadenosine was not produced by CEO when epoxide hydrolase was present, but formation did occur in the presence of alcohol dehydrogenase. They concluded through this and earlier studies that CEO is sufficiently reactive to alkylate DNA efficiently, but is not so reactive as to bind solely to P450 proteins (Guengerich, et al., 1979).

L.M. Gwinner et al., ( 1983) compared alkylation produced by VCM and 2,2'-dichlorodiethylether (BCME) in hepatocytes of male Wistar rats. The latter compound is metabolized completely to CAA (partly through an intermediate). They concluded that CAA was responsible for protein alkylation while the VCM ( and therefore CEO) was mainly responsible for binding to DNA. This study was unique in its use of BCME which is oxidized to CAA and 2-chloroethanol (CE), which in turn is completely metabolized to CAA. Unlike VCM, 2,2'-dichlorodiethyl ether does not pass through a reactive epoxide; consequently, effects from the epoxide and the aldehyde could be differentiated *in vivo*, something heretofore only possible *in vitro* (Gwinner, et al., 1983).

Zajdela et al. administered CEO subcutaneously to eight-to ten-week- old male and female XVllnc/Z mice. The same was done with BCME, a compound that is a direct acting human and animal carcinogen, similar in structure to CEO. Tumor formation from CEO was comparable to BCME. CAA was also included in this study, but was used in an initiation/promotion skin painting experiment (CAA could not be tested subcutaneously because of its necrotizing effects). VCM and CEO were also used in the painting study. 12-

O-n-tetradecanoylphorbol-13-acetate was used as a promoter with all three chemicals. Both CEO and BCME produced skin tumors, while CAA did not. These results led them to conclude that CEO was the reactive metabolite of VCM and therefore, responsible for its carcinogenic activity. (Zajdela et al., 1980).

In conclusion, CAA and CEO were demonstrated to react with DNA *in vitro* causing mutations, however CEO was more strongly mutagenic. Additionally, CEO was carcinogenic *in vivo* while CAA was not. This brief summary of the growing body of evidence in numerous *in vivo* and *in vitro* test systems strengthens the following conclusions about VCM: 1) VCM requires metabolic activation to exert its mutagenic and carcinogenic effects, 2) CEO and CAA, two reactive products of metabolism manifest their effects in different ways, 3) CAA, although the more cytotoxic compound, shows less mutagenic potential *in vitro* and less carcinogenic potential *in vivo* compared to CEO.

#### DNA Adduct Formation and Relevance

DNA is a highly nucleophilic molecule and contains thirteen potentially reactive sites (Singer, 1985). Chemicals which are strongly electrophilic are capable of reacting with these sites and producing modified DNA bases. Upon DNA replication these modified bases can result in base mispairings at the adduct site. The electrophilic attack of chemicals can result in a wide variety of DNA adducts (Swenberg, 1990). Both CAA and CEO can react with nucleophilic cellular macromolecules, including DNA and can form stable DNA adducts.

Several factors control the probability of an adduct undergoing a mispairing event in DNA. First, modified bases differ in their ability to base pair in DNA, possibly due to differences in hydrogen bonding between the two Watson-Crick paired bases. The length of

time that an adduct remains on the DNA is also an important factor in the ultimate biological outcome of DNA modification. This is dependent on both the chemical stability of the adduct and the function and availability of various DNA repair enzymes (Singer, 1985).

The production of DNA adducts shows specificity for certain sites on the DNA (Richardson 1989, Warpehoske 1988). The propensity of sites to react is affected by the base adducted and usually the neighboring base or bases (5' or 3'). The differences in reactivity no doubt contribute to "hot spots" and cold spots of base substitutions (Cooper, 1990). The frequency of mutations occurring at different modified bases may be influenced by several factors including differences in repair efficiencies and differences in the rate of base misinsertion, both of which are expected to be influenced by local DNA sequence context. Briscoe and Cotter conducted a study in 1984 to determine whether there was any effect of cytosines or guanines as nearest neighbors upon the alkylation of a guanine residue in DNA. N-methyl-N-nitrosourea (MNU) was reacted with a synthetic polynucleotide. They concluded that the alkylation pattern of guanine was dependent on the neighboring bases and this finding would be relevant in defining hot spots of mutational activity within a genome (Briscoe et al., 1984). Richardson et al., (1989) investigated the formation of O<sup>6</sup>-methyldeoxyguanosine (O<sup>6</sup>MedGuo) from N-methyl-N-nitrosourea at four specific sites in a synthetic oligonucleotide resembling a mutational hotspot in the *Escherichia coli* xanthine-guanine phosphoribosyltransferase gene. The sites studied on the oligonucleotide were tritiated and the formation of O<sup>6</sup>MedGuo quantitated. It was determined that the site where O<sup>6</sup>MedGuo most frequently formed corresponded to the site frequently mutated in *E. coli* by the same compound. The O<sup>6</sup>MedGuo formation was not random along the oligonucleotide

and the distribution was only evident in the dsDNA oligonucleotide tested, not the ssDNA. These results suggest a sequence dependent mutation site that is also dependent on the secondary DNA structure (Richardson et al., 1989).

*In vivo* and *in vitro* studies by Laib et al., (1981) concluded that the DNA adduct N<sup>7</sup>-(2-oxoethyl) guanine (7-OEG) was the major product of alkylation from the reactive VCM metabolites. This was true both for rats exposed to [1,2-<sup>14</sup>C] vinyl chloride and *in vitro* studies with rat liver microsomes, an NADPH-regenerating system, DNA and [<sup>14</sup>C] vinyl chloride (Laib, et al., 1981). It was proposed that this adduct in the cyclic hemiacetal form could be read by the polymerase as adenine and therefore cause mispairing. However numerous studies have shown that modification at the N7 position of guanine does not prompt mispairing. Alkylation at the N7 position does promote depurination which can also occur spontaneously or through the action of glycosylases. Apurinic sites have been shown to be promutagenic but their production and conversion to mutations is expected to be inefficient relative to other adducts formed by VCM (see below). Therefore, even though 7-OEG accounts for up to 98% of the modified bases in DNA, it was predicted that it would contribute little to induction of mutagenicity by vinyl chloride or its metabolites (Barbin et al., 1985; Laib et al., 1981; Swenberg et al., 1990). Furthermore, it has a half-life of approximately 62 hours in DNA and therefore is not extremely persistent (Fedtke et al., 1990, Swenberg et al., 1990).

Different methods of adduct detection and quantification revealed an additional three adducts formed by the interaction of DNA with the metabolites of VCM. These are: 1,N<sup>6</sup>-etheno-2-deoxyadenosine ( $\epsilon$ dAdo), 3,N<sup>4</sup>-etheno-2-deoxycytidine ( $\epsilon$ dCyd); and the most

recently identified VCM adduct  $N^2,3\epsilon$ G (Singer, 1983). These adducts and the point mutations which have been inferred to result upon DNA replication are pictured in figure 1.2.

The biological importance of these adducts in mutagenesis and carcinogenesis remains to be determined. As stated above, 7-OEG is found *in vivo* and *in vitro* in the greatest concentrations relative to other VCM adducts, but no studies have demonstrated the mutagenic properties of this adduct. Laib et al., (1985) identified the presence of  $N^2,3\epsilon$ G in VCM-exposed fourteen-day-old rats. Singer et al., (1987) went on to determine that this adduct can act like a guanine or adenine (in reference to recognition by DNA polymerase) causing GC→AT transitions approximately twenty percent of the time. Their study involved the synthesis of  $N^2,3\epsilon$ G, and its copolymerization with cytosine diphosphate (CDP) into an oligonucleotide. They then measured the rate of incorporation of incorrect nucleosides using the oligonucleotide as a template. Based on these results, they proposed that  $N^2,3\epsilon$ G is an important promutagenic adduct of vinyl chloride (Singer et al., 1987). Fedtke, et al., (1990) studied the formation and persistence of both 7-OEG and  $N^2,3\epsilon$ G in target and non-target tissues of preweanling Sprague-Dawley rats exposed 5 days to gaseous VCM. The ratio of  $N^2,3\epsilon$ G:7-OEG was 1:100 in animals analyzed immediately after exposure. This value changed dramatically in animals analyzed at two weeks (1:14.1) indicating the greater persistence of  $N^2,3\epsilon$ G. From their study they calculated a half-life of 62 hours for 7-OEG and greater than 30 days for  $N^2,3\epsilon$ G (Fedtke, et al., 1990). These results suggest the  $N^2,3\epsilon$ G is likely to accumulate to high levels *in vivo* (Swenberg et al., 1990).

Eberle et al., (1989) raised monoclonal antibodies to  $\epsilon$ dAdo and  $\epsilon$ dCyd to permit their quantification *in vivo*. Young Sprague-Dawley rats were exposed by inhalation to 2000 ppm of VCM for ten days and the levels of  $\epsilon$ dAdo and  $\epsilon$ dCyd were quantified in the lung and liver tissues through the use of the Mabs. Both adducts were found in lung and liver tissues, with greater concentrations of both in the lung. Additionally, the levels of  $\epsilon$ dCyd were 2.5 times and 3.2 times higher in the lung and liver, (respectively), than the levels of  $\epsilon$ dAdo. These results demonstrated that both of these adducts were formed efficiently *in vivo* during VCM exposure (Eberle, et al., 1989).

To determine whether CEO or CAA is primarily responsible for the formation of these minor, yet persistent and promutagenic adducts Guengerich et al., (1992) reacted these compounds directly with calf thymus DNA (Guengerich et al. 1992). They found that the etheno adenine and guanine adducts were formed much more effectively by CEO than CAA (by at least an order of magnitude). The key to their study was the conditions of metabolism of vinyl chloride. 1,N<sup>6</sup>-ethenoadenosine ( $\epsilon$ dAdo) formation was almost completely blocked (from either DNA or adenosine) when purified epoxide hydrolase was included in the reaction, but when the CAA destroying enzyme (alcohol dehydrogenase) was added, there was little effect on  $\epsilon$ dAdo formation. The *in vitro* study indicated formation of adducts from CEO exposure at the following relative levels : 7-OEG >>  $\epsilon$ dAdo > N<sup>2</sup>,3 $\epsilon$ G. The lack of formation of 7-OEG by CAA in this *in vitro* study was consistent with the reported lack of formation *in vivo*. The very low levels of  $\epsilon$ dAdo and N<sup>2</sup>,3 $\epsilon$ G formation from CAA led the authors to conclude the CEO is the major metabolite responsible for DNA alkylation (Guengerich, 1992). These results have led the authors to conclude that both DNA adducts

$N^2,3\epsilon G$  and  $\epsilon dAdo$  are formed in DNA primarily through the reactive epoxide versus the aldehyde.

As mentioned previously, 7-OEG is not expected to demonstrate any miscoding properties in DNA. This is not the case for the other three adducts, all of which have demonstrated miscoding properties.  $\epsilon dAdo$  was found to be a miscoding lesion during RNA transcription, resulting in AT→GC transitions and AT→CG and AT→TA transversions (Spengler and Singer, 1981, de los Santos et al., 1991).  $\epsilon dCyd$  was found to cause errors during both DNA replication and RNA transcription, resulting in CG→AT transversions, CG→TA transitions, and, at a much lower frequency, CG→GC transversions (Spengler and Singer, 1981; Singer and Spengler, 1986). As mentioned previously, the adduct,  $N^2,3\epsilon G$  causes GC→AT transitions during replication.

Because of the efficiency of formation of these etheno DNA adducts of VCM, their promutagenic properties and persistence in DNA, exposures to even very low concentrations of VCM could pose a significant health risk (Swenberg, 1991). The work with VCM and its metabolites in this research will provide additional information on the role of CEO and CAA in VCM mutagenesis in human cells and contribute to the knowledge of the molecular nature of VCM-induced mutations.

### Mutagenicity

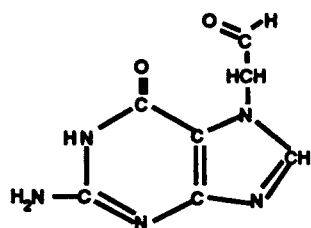
A mutation in DNA can be defined as a stably inherited change in the ordering of nucleotide bases in the genome. An organism with a genomic change is called a mutant (for that change) and the same organism without the change is referred to as wild type. The frequency of occurrence of mutants in a population is referred to as the mutation frequency

(MF) and represents the fraction of mutants in the population. Mutations can be classified in several categories. A point mutation affects only a single base in the DNA. The most common form of point mutations are substitutions, of which there are two types. Transitions substitute one pyrimidine base for another or one purine base for another purine (AT→GC, CG→TA) and transversions exchange a purine for a pyrimidine or a pyrimidine for a purine (AT→CG,TA; GC→TA,CG). Point mutations can lead to three outcomes in the transcription of the changed sequence. There may be no change at all in the amino acid sequence from the wild type to the mutant sequence; this is due to the degenerate nature of the code. The change can result in a "missense" mutation, in which a different amino acid is inserted. This can affect a unique enzymatic function of the cell resulting in reduced or negligible enzyme activity, or possibly a new function. If the change in sequence results in the production of a stop codon, then premature termination of protein synthesis will occur. This type of change is called a "nonsense" mutation. Rearrangements usually effect large portions of the gene and are manifested through the loss (deletions) or gains (insertions) of nucleotides into the DNA. Mutations of this kind can result in shifts of the reading frame of the DNA sequence due to the fact that information is stored in codons consisting of three nucleotides (Lewin, 1990).

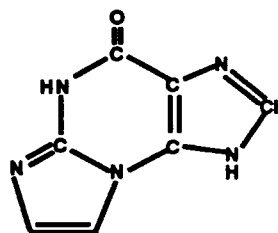
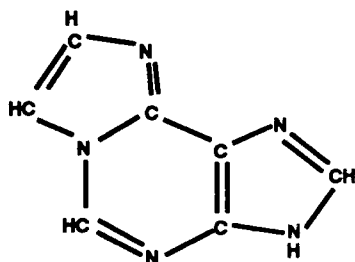
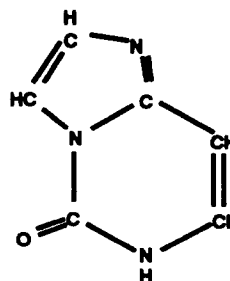
Mutations can also be classified as to their general causative source. "Naturally-arising" mutations are usually called spontaneous mutations and the level at which they occur for an organism (or cell line) is termed the background mutant frequency (MF). This level of mutation can be determined from the zero treatment controls used for the mutation studies. The cell line used in the current study has an average background MF at the hprt locus (see

chapter II) of  $3.0 \times 10^{-6}$  (or three mutants out of one million wild type cells with HPRT activity) (Gentest, 1990). These spontaneous events can arise due to a variety of causes including the processes of DNA replication and DNA repair. Giroux et al., (1988) in a study to determine the DNA sequence of 196 spontaneous mutants in the SUP4-o gene of *S. cerevisiae* identified all possible types of base pair substitutions, deletions, and complex alterations involving multiple changes, as well as insertions and transpositions. Their work led them to support the hypothesis that many spontaneous mutations are actually the result of DNA sequence-directed events (Giroux et al., 1988). Similar results were obtained by Schaaper et al., (1986) in their analysis of the spectrum of spontaneous mutation in the *Escherichia coli* lac I gene. They analyzed 174 spontaneous mutations and determined that the spectrum consisted of base pair substitutions, frameshifts, deletions, duplications, and transpositions. These results led them to conclude that the spontaneous mutations occurring in the organism studied were the result of a variety of endogenous mutational mechanisms (Schaaper, et al., 1986). Mutations induced by chemical or physical damage can be ascertained in a test system when the level of mutations induced is significantly greater than the level of spontaneous mutations. The level of MF increase over background determines the strength of mutagenic compounds and allows for their comparison. Many mutagenic compounds exert their effect by covalently binding to (adducting) a particular base or intercalating (Lewin, 1990). If this bound chemical/DNA complex is stable it can be processed through the repair, replication, and recombination systems of the cell, and may be converted to a mutation (Fuchs, et al., 1981).

Vinyl chloride was shown to be mutagenic in many test systems including bacteria (*Escherichia coli* K12, *Salmonella typhimurium* TA1530, TA1535, 646) yeast (*Schizosaccharomyces pombe*) and *Drosophila melanogaster*. All of the *in vitro* studies indicated the necessity for some metabolic activation system for the compound to show definitive mutagenicity (Sax, 1988).



7-(2-OXOETHYL)GUANINE

N<sup>2</sup>,3-ETHENOGUANINE1,N<sup>6</sup>-ETHENOADENINE1,N<sup>4</sup>-ETHENOCYTOSINE

| REACTIONS WITH DNA IN VITRO |            | MISCODING PROPERTIES             |                       |
|-----------------------------|------------|----------------------------------|-----------------------|
| <u>CEO</u>                  | <u>CAA</u> |                                  |                       |
| X                           |            | 7-(2-OXOETHYL)GUANINE            | NONE                  |
| X                           | X          | N <sup>2</sup> ,3-ETHENOGUANINE  | G → A                 |
| X                           | X          | 1,N <sup>6</sup> -ETHENOADENINE  | A → T   A → G   A → C |
| X                           | X          | 1,N <sup>4</sup> -ETHENOCYTOSINE | C → A   C → T   C → G |

Fig 1.2. Identified DNA adducts from *in vitro* and *in vivo* exposures to VCM and its metabolites along with inferred base pair changes. Compiled from: Barbin et al., 1985; Laib et al., 1981; Swenberg et al., 1990; Spengler and Singer, 1981; de los Santos et al., 1991; Singer and Spengler, 1986.

## **CHAPTER II**

### **HPRT MUTATION ASSAY: THEORY AND DESIGN**

The hypoxanthine phosphoribosyl transferase (hprt) gene was the genetic target used in all the mutation assays reported here. HPRT (protein) is a purine salvage enzyme that catalyzes the condensation of free hypoxanthine or guanine with 5-phosphoribosyl-1-pyrophosphate (pp-ribose-p). Although non-essential for cells growing in culture, the lack of HPRT can have serious effects in man. Complete lack of HPRT activity results in the genetic disease known as Lesch-Nyhan syndrome (Holden et al., 1978). Less severe deficiencies can result in gouty arthritis (Kelly et al., 1967). HPRT enzyme activity has an influence directly or indirectly on cellular processes related to purine synthesis. This is known primarily through examination of people with gout who have been shown to exhibit elevated rates of purine synthesis (Caskey, 1979).

Hprt is located on the long arm of the X chromosome (Holden et al., 1978) and consists of nine exons and eight introns which total 57,000 base pairs in length. The molecular weight of HPRT is 24,300, and it exists *in vivo* as a tetramer (Holden, 1978). Since hprt is located on the X-chromosome, males possess only one copy and females possess only one active copy due to X-chromosome inactivation. Consequently, hprt is effectively hemizygous in both males and females and only a single mutational event is required to render a cell phenotypically hprt<sup>-</sup>. HPRT substrates include: hypoxanthine, guanine, 6-mercaptopurine, and 6-thioguanine (6TG) (Krenitsky et al., 1969).

The key advantage for the use of hprt in gene mutation studies is the existence of stringent selective conditions for both hprt<sup>+</sup> and hprt<sup>-</sup> phenotypes. Hprt<sup>+</sup> cells can be selected in media containing hypoxanthine, aminopterin and thymidine (HAT). Aminopterin inhibits purine (and thymidine) *de novo* synthesis and its presence forces a cell to rely on its

salvage pathways (hprt, tk) for survival. Hprt<sup>+</sup> cells can phosphoribosylate and utilize hypoxanthine in HAT media and grow, while hprt<sup>-</sup> cells cannot (Thomas, 1979; see figure 2.1.) The process of treating a cell culture in HAT media prior to a mutation study reduces the level of background mutants (those caused by means other than the test substances) and increases the sensitivity of the assay.

When grown in the presence of 6TG a cell with active HPRT will metabolize 6TG to the ribonucleoside monophosphate 6-thioGMP (6TGMP). 6TGMP is further metabolized, incorporated into nucleic acid and kills the cell. Consequently, hprt<sup>+</sup> cells are killed in the presence of 6TG (Nelson, 1975). However, cells without HPRT activity (hprt<sup>-</sup>) cannot phosphoribosylate 6TG and can survive and grow in its presence. The ability to select hprt<sup>-</sup> mutants provides a straight-forward means to detect mutation induction resulting from a chemical exposure.

Cells mutated at the hprt locus no longer produce functional hprt mRNA; however, for a period of time they still possess HPRT enzymatic activity due to the presence of preexisting hprt mRNA and HPRT proteins. Prior to 6TG-mediated selection, the cells must pass through several cell cycles (without selection) to ensure that newly formed hprt<sup>-</sup> cells lose their residual HPRT activity. This length of time is known as the phenotypic expression period and for most cells is greater than five days (Crespi, 1984).

The hprt locus has been widely studied and the types of mutations from different chemicals reported. A review of the hprt locus by Caskey in 1979 indicated studies at that time had demonstrated missense mutants (point mutations), frameshifts, deletions, and suspected premature chain termination mutations (nonsense mutations) (Caskey et al., 1979).

Subsequent studies verified and added to the location, type and frequency of mutations at *hprt* in different systems. Cariello et al., (1988) identified a point mutation in *hprt* in a patient with gout, utilizing denaturing gradient gel electrophoresis and direct sequencing as analytical tools (Cariello et al., 1988). Deletions primarily of exon 2 and 3 were identified by McGinniss et al (1989) in their analysis of *in vivo* *hprt* mutations in human new born T-lymphocytes (McGinniss et al., 1989). A study by Recio et al., (1990) of spontaneous *in vivo* *hprt* mutations in human T-lymphocytes identified single base substitutions, insertions, small deletions and large deletions of entire exons. The large deletions suggested mutations in *hprt* sequences that regulate splicing of *hprt* mRNA. The growing amount of scientific data on *hprt* is significant. Currently a data base is being compiled of all mutations at *hprt* produced by chemicals and physical agents in a wide variety of model systems (Cariello et al., 1992).

The truly unique capability of the cell line used in this study is their ability to express cytochrome P450IIE1 activity. Most mamalian cell lines used in mutation studies lack the ability to metabolize xenobiotic compounds and convert them to their mutagenic form. To circumvent this deficiency, some form of exogeneous activating enzymes must also be provided to metabolize promutagenic chemicals to their active form. This limitation also demands that the metabolites are produced outside of the plasma membrane and must be transported across to interact with the genetic target (Gonzalez et al., 1991).

Since cell lines can be stably transfected with extrachromosomal vectors, the opportunity exists to transform the cells with cloned P450 activity needed to metabolize the compounds of interest (Gonzalez et al., 1991). The AHH-1 cell line have within their genome, an integrated Epstein Barr Virus (EBV) genome. Epstein-Barr Virus (EBV) is

responsible for the immortalization of the cell line and is necessary for the cells' continued propagation in culture. The cytochrome introduced extrachromosomally into AHH-1 cells, is IIE1 (Gonzalez, 1991). This P450 is capable of oxidizing many compounds including VCM (Guengerrich et al., 1991). The vector used to transfect the cDNA with P450IIE1 in this cell line was pEBVHistk. This vector contains the origin of replication (P element) of EBV and allows replication of the extrachromosomal plasmid carrying the P450IIE1 cDNAs (Sugden et al., 1985 and Yates et al., 1984). The selectable marker in the vector is the *Escherichia coli* HisD (histidinol dehydrogenase) gene which confers resistance to 1-histidinol.

Histidinol is a potent protein synthesis inhibitor. Histidinol dehydrogenase can convert the histidinol present in the cell to the essential (and non-toxic) amino acid L - histidine. Cells that have lost all copies of the extrachromosomal vector (or have lost enough copies so there is not sufficient histidinol dehydrogenase activity present to convert the histidinol) will not survive. Therefore, maintaining cells in media containing 1- histidinol provides adequate selective pressure to ensure cells in culture contain the transfected plasmid and thus the desired cytochrome P450IIE1 enzymatic capability (Crespi,1990). Constant selection maintains approximately 40 extrachromosomal copies per cell. This methodology of selection in mammalian cell culture was originally reported by Hartman (Hartman et al., 1988).

Since induction of the cytochrome P450IIE1 is not possible in these cells, the level of metabolic activity is determined by the plasmid copy number per cell. This vector is stable (in the presence of the selective agent) for at least 70 days of cell culture (Gonzalez, 1991).

A final important aspect of the cell line is its lack of microsomal epoxide hydrolase activity (Crespi, 1990). One would expect that epoxide hydrolase activity near the cytochrome P450 system would catalyze the detoxification of CEO and essentially abolish the alkylating ability of CEO (Guengerich, et al., 1979 and Amdur et al., 1986).

### Toxicity

Defining toxicity at the cellular level is less complicated than for an entire organism, but is still not trivial. Toxic effects in a cell can be observed at many levels, with cell death being the most easy to identify. Toxicity at the cellular level can also include growth delays due to destruction of intracellular macromolecules, alterations in metabolic processes, changes in cellular or nuclear membranes, disruption of the cells replicative cycle, or interactions with proteins, DNA, and RNA and the processes responsible for synthesis and repair of these cellular molecules (Grisham, 1984).

Observations of toxic effects in human cellular systems provide little information on the ultimate toxic effect in humans. However, it is an important and useful parameter to measure in cell systems. First, within a given cell system, it can be used to measure the relative reactivity of closely related test compounds (such as in the present study). More importantly, toxicity determinations are a necessary element in demonstrating that mutation experiments are correctly carried out and contain statistically significant number of surviving mutants. For many chemicals analyzed in human cellular test systems, the toxicity of the compound can limit the dose concentrations that can be tested. In their statistical analysis of human lymphoblast mutation assays, Penman and Crespi determined that the relative survival after exposure should be between 10 and 30%. Maintaining a large number of surviving

mutants after exposure serves to decrease the variability between repeat experiments and increases the sensitivity of the protocol (Penman and Crespi, 1987, and Gentest, 1990). There are two factors which can be altered to ensure that enough mutant cells are present in culture after treatment; one is to treat a higher concentration (or total number) of cells, and the other is to ensure the level of toxicity is within the range mentioned previously. An example will more clearly represent the importance of maintaining the specified range of toxicity in the experimental design: if  $3 \times 10^7$  cells total are treated in culture and the mutation frequency is  $3 \times 10^{-6}$  and there is no measureable toxicity, then there will be 90 total mutants in the cell population after treatment ( $3 \times 10^7 \times 3 \times 10^{-6}$ ), with a standard deviation of the mutants of about 10%. However, if in the same treatment, the relative survival was only 10%, then there would be only 9 mutants total with a 33% standard deviation (from Gentest, 1990). Additionally, careful consideration of the number of surviving mutants is also critical in obtaining mutational spectra (as in the present study). In the latter example, the mutant population analyzed would have been the progeny of only nine different cells, and the analysis of the resultant spectra would have limited significance.

Quantification of cellular toxicity from exposure to VCM, CAA, and CEO is an important aspect of this study. Trypan blue exclusion observed through microscopic examination has been determined to be an un-reliable measure of toxicity in general, and most definitely not a good measure of toxicity for the AHH-1 cell line used in this study (Grisham, 1984 and Gentest, 1990). Trypan blue only measures plasma membrane breaching, and as discussed above, there are more subtle ways in which a cell can be killed. The primary technique used in this study to measure toxicity of VCM, CAA, and CEO

exposures is through the determination of relative macroscopic colony forming ability or plating efficiency (PE) in 96 well microtiter plates. The plating assay is based on the ability of the plated cells to survive treatment and progress through a sufficient number of cycles in a given time period to be visible for counting (scoring). The length of time required for colonies to become visible varies between different cell lines. Fifteen days incubation time has proven to be sufficient for the cell line used in this study.

Another measure of cellular toxicity is growth delay. Cells are counted each day after treatment and the length of time prior to the resumption of logarithmic growth with normal doubling time is determined. This growth delay time may be due to inhibition of cell growth due to the exposure, or to actual cell killing. In the latter case the growth lag time results from the time required by the small number of living cells to repopulate the culture. Comparison of growth delay and PE can distinguish between the two possibilities.

Control populations of untreated cells are a crucial part of the study. Even untreated cells do not form colonies at 100% efficiency. For this reason, untreated, control cells which were handled in a manner identical to the treated cells (less the chemical exposure) are used as comparison when the well count data is processed to ascertain the level of toxicity. The distribution of colony forming units (CFU's) is across a large number of possible locations (96 wells on a single microtiter plate), and is expected to follow the Poission distribution (Furth et. al., 1981). Because of this distribution, it is possible to calculate the average number of CFU's that were distributed to the 96 wells by observing the number of positive and negative wells. According to Poission statistics, the fraction of negative wells  $P(0)$  is equal to  $e^{-m}$ , where  $m$  is the average CFU's per well. Consequently,  $m = -\ln[P(0)]$ . Cells are

plated for toxicity at limiting cell density such that both positive and negative wells are seen.

The plating efficiency (PE) of a culture can be calculated as:

$$PE = \text{CFU per well} / \text{number of cells per well}$$

The number of cells per well is based on physical cell counts and the preparation of known dilutions. The PE calculated for the zero treatment controls can be compared to the PE obtained for different levels of exposure and a percent relative survival can be calculated :

$$\text{Relative Survival} = \text{PE treated cultures} / \text{PE controls}$$

Consequently a culture displaying the same PE as the control has a relative survival (RS) of 1.0; cultures with a lower PE have a RS <1.0. For the cells used in this protocol, average values for PE ranged from 30%-60%. This methodology provides a quantitative measure of toxicity in the cells at different levels of exposure to VCM and its metabolites.

#### Mutagenicity

The plating efficiency of a culture in the presence of 6TG can also be determined in the same fashion described in the previous section. Of course, the resultant colonies are 6TG<sup>r</sup>, hprt<sup>-</sup> mutants. To convert the PE of the culture in the presence of 6TG to actual mutation frequency, the 6TG PE is divided by the PE of the culture in the absence of 6TG:

$$MF = \text{PE in 6TG} / \text{PE without 6TG}$$

# HPRT Metabolic Pathway

(non-essential)

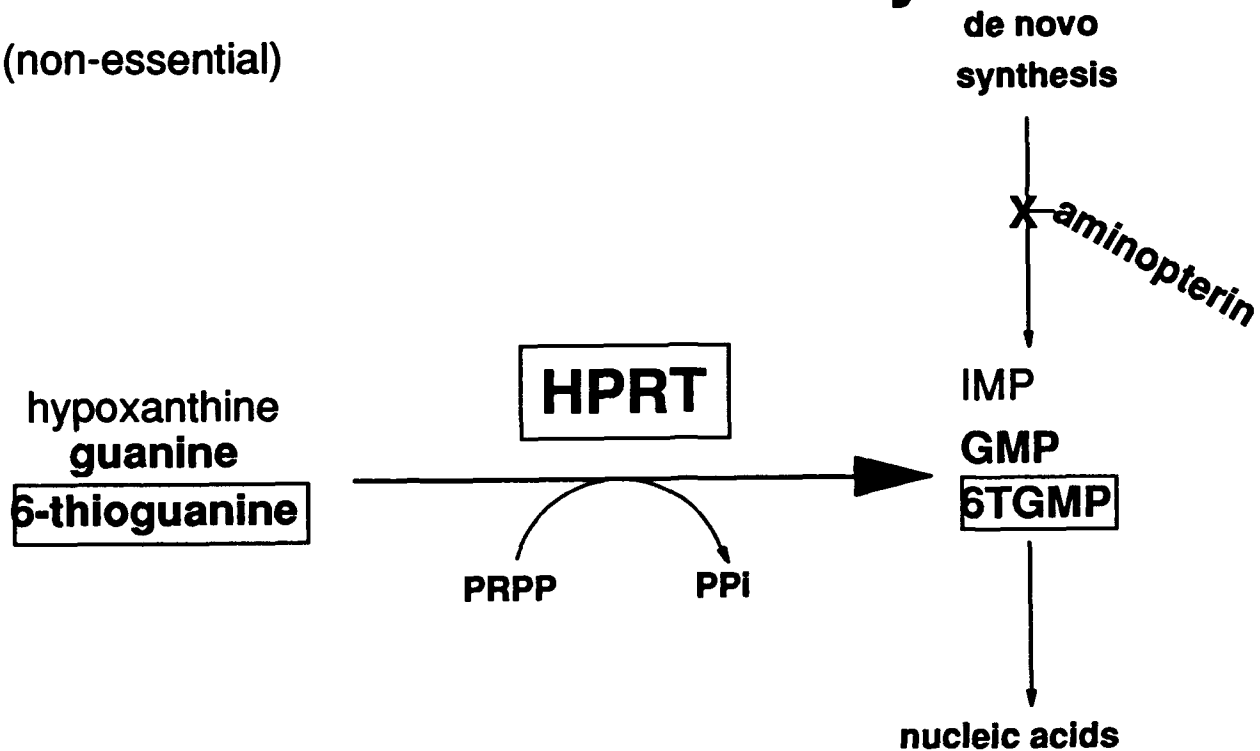


Fig 2.1. The HPRT metabolic pathway. This diagram illustrates the two selection mechanisms for both  $hprt^+$  and  $hprt^-$  phenotypes used in this study.

## **CHAPTER III**

### **MOLECULAR ANALYSIS OF MUTATIONS: THE POLYMERASE CHAIN REACTION, DENATURING GRADIENT GEL ELECTROPHORESIS, AND DNA SEQUENCING**

### Polymerase Chain Reaction

The practical analysis of mutations in defined regions of DNA has been made possible through the advent of the polymerase chain reaction (PCR). PCR is an *in vitro* method for the amplification of defined DNA sequences. Two oligonucleotide primers are used that hybridize to the regions on the DNA flanking the section to be amplified (Erich, 1989). The flanking sequences of the target DNA must be known in detail so that primers can be designed to anneal specifically at those regions. In general, the reaction mixture consists of DNA (which does not need to be purified but only include the desired sequence to be copied), an excess of the two primers, the four different deoxyribonucleoside triphosphates, and DNA polymerase (Mullis, 1987). This reaction mixture is subjected to a specified number of thermal cycles, each of which consists of a high temperature melting step to denature the DNA (94-96 °C), a lower temperature, primer-annealing step (42-47 °C) followed by an intermediate extending step (72 °C) where the deoxyribonucleosides are incorporated into a "new" strand of DNA by polymerase. The reaction produces an exponentially increasing number of dsDNA molecules of the length defined by the two primers (Mullis, 1987). A significant improvement in the PCR process was the discovery of a thermostable DNA polymerase isolated from *Thermus aquaticus* bacteria (Taq) by Gefland and Stoffel. The use of Taq as the DNA polymerase has resulted in an automated PCR process, since Taq polymerase can withstand the high temperatures needed to denature the DNA and therefore does not have to be added at each cycle. Additionally, since Taq also has a higher optimized temperature, higher annealing and extending temperatures can be used. This increases the stringency of the reaction and minimizes production of strands

resulting from primer annealing at incorrect locations (Saki, 1988, and Rychilik, 1990). PCR also allows the incorporation of new base pairs at the ends of amplified molecules through the construction of primers containing the new sequences. This is possible because mismatches between the 5' end of the oligonucleotide primer and the initial flanking regions of DNA are tolerated in the PCR reaction and the production of new strands of specified DNA become incorporated into all subsequent copies produced. After several cycles, the vast majority of product contains the entire primer sequence, including the added base pairs (Erich, 1989). This aspect of PCR is exploited for the analysis of mutant DNA by allowing the incorporation of sections rich in G-C pairs (G-C clamps) and the incorporation of bases that act as recognition sequences for the fluorescent sequencing primers; both of which will be discussed in the following sections.

#### Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) allows the resolution of DNA fragments that differ in sequence by as little as a single base pair (Fisher and Lerman, 1983). This procedure can translate a minor difference in sequence into significant differences in position on a polyacrylamide denaturing gel (Lerman, 1986). These separations are brought about by differences in melting temperatures ( $T_m$ ) of DNA, which is critically dependent on the DNA sequence. Melting of the DNA is defined as an "equilibrium between two well-defined states for each base pair - that of the double helix and that of a more nearly random chain in which bases are neither paired nor stacked on adjacent bases in an orderly way" (Myers et al., 1987). Describing melting domains based on their temperature is analogous to describing the same domain based on the effects of the denaturant concentration used in the

DGGE system (urea and formamide) (Myers et al., 1987). The DNA melts in discrete segments or domain of 25 to several hundred base pairs in length, and for each domain the melting is cooperative across the domain, at the  $T_m$  (Erlich, 1989). These domains are clearly demarcated from one another and a base pair change in one domain, affecting its melting temperature, will usually have little or no effect on the surrounding domain(s) (Myers et al., 1987). In exon three of human hprt, there are two well defined domains. The low temperature melting domain is located from base pair 214-319, of exon 3, while the high-temperature domain is bp 135-213.

The central principle of DGGE is that DNA molecules that are partly helical and partly melted (caused by the melting of a single domain) will migrate at a much slower rate in a polyacrylamide gel than the fully helical molecule and the rate will decrease more as the melted portion increases (Lerman, 1986). In practice, a DNA fragment migrates through a polyacrylamide gel that contains a linear gradient concentration, increasing from top to bottom. As the DNA reaches the denaturant concentration where the lowest melting domain denatures ( $T_m$ ) the DNA is in a partly melted, partly helical state and its mobility is greatly decreased (Erlich, 1989). Staining of the DNA with ethidium bromide or labeling with radioactivity permits the location of the DNA band to be determined.

In its unmodified form, DGGE is only partly successful in separation of all mutants in a DNA fragment of interest. This is due to the fact that DGGE cannot separate fragments where the base pair changes are in the highest melting domain since the mobility of the DNA has already been greatly reduced when the low temperature domain separated, and melting of the higher domain has essentially no significant effect on the reduction of mobility (Myers et

al., 1985). To overcome the inherent inability to resolve mutants in the high temperature melting domain, the melting characteristics of the DNA fragment can be altered through the attachment of a length of nucleotides rich in guanine and cytosine. This attached sequence is termed a GC clamp (Myers et al, 1985a). With a GC-clamp, the entire fragment of interest can be melted as it passes through the denaturant, but the GC clamp remains duplexed. This significant alteration in the melting temperature of the molecule was hypothesized to enable detections of base changes in all melting domains of a DNA fragment (Myers et al., 1985b). In the first studies with this procedure (in the  $\beta$ -globin promoter fragment) the fraction of all substitution that could be detected by DGGE increased from 40 to 95% with the addition of a GC clamp (Myers et al., 1985b). The first clamps utilized in these studies were long (300bp) and were not 100% G+C. It was later determined that a 100% G+C clamp of only 40 base pairs was needed to resolve base substitutions in all of the melting domains of DNA fragment (Sheffield et al., 1989).

The process of altering the melting temperature of a DNA fragment through the clamp, was combined with PCR technology as the means to attach the clamped fragment. This technique increased the ability to detect most base substitutions in all of the melting domains from a very small quantity of initial, unclamped fragment as the starting template (Sheffield et al., 1989). This meant that DGGE technology could be used to detect mutations in human genomic DNA from a starting point of less than 5 ng of DNA (Sheffield et al., 1989).

Heteroduplex DNA, in which there is a mismatch at a single base, always migrates at a much slower rate than either the mutant:mutant or wild-type homoduplexes. This phenomenon is attributed to the destabilizing effects of the mismatch (Myers et al., 1985).

The use of heteroduplex DNA in DGGE analysis is advantageous since their physical separation on the gel from the wild type homoduplex is always greater than that of mutant homoduplexes. This allows for the identification of mutants in cases where the mutant homoduplex does not resolve from the wild-type homoduplex (Erich, 1989). To form heteroduplexes, mutant and wild-type homoduplexes are denatured and allowed to reanneal. Heteroduplex formation insures the resolution of all mutants in the denaturing gel.

The ability of DGGE to distinguish mutant DNA from wild-type DNA is limited by fidelity of the DNA polymerase used in the PCR reactions. Cariello et al., (1990) analysed complex human cell populations containing mutants induced by the alkylating agent MNNG or the intercalating agent ICR-191. In the study they determined that mutants comprising at least one percent of the total population of cells can be distinguished from wild type DNA as a distinct band or set of bands on a denaturing gradient gel. If the mutant population was less than one percent, the background noise on the gel (visualized by a smear) caused by DNA polymerase infidelity would prevent the identification of unique bands (Cariello et al., 1990).

In this present study, all mutants analyzed are *hprt*<sup>-</sup>, but not all mutations reside in exon 3, the target of study. We are concentrating on exon 3 since this is the only exon which can be examined in the *in vivo* mouse model in our laboratory. Also, exon 3 contains 28% of the coding frame in a continuous length and is the exon believed to code for both catalytic sites of the enzyme (Wilson et al., 1983). DGGE analysis of DNA is used to 1) identify mutants with mutations in exon 3 and 2) purify mutant from wild type DNA (Cariello, et al., 1990).

### Automated DNA Sequencing

Sequencing of the amplified mutant DNA is the analytical endpoint for mutants that resolved as unique bands on the denaturing gradient gel. Direct visual comparison of the mutant sequence with the wild type sequence will indicate the exact nature and location of the mutation. The frequency of occurrence of each of the mutations is calculated and a mutational spectrum with mutational "hot spots" constructed.

The automated sequencing is based on the dideoxy termination method of DNA sequencing. In this enzymatic approach, ssDNA template is copied by a polymerase using a specific primer. The reactions are carried out in four separate tubes, each containing ssDNA template, primer, dNTP's and a small amount of a specific dideoxy nucleotide (lacking the 3'-OH). A different dideoxy is added to each of the four reaction mixtures. Incorporation of the dideoxy nucleotide into the elongating dsDNA causes termination at the base specified by the dideoxy ribose. Each of the four reaction tubes will contain varying lengths of DNA and in a given tube all DNA molecules will end in the same dideoxy nucleotide (Lewin, 1990). When the samples are run on a denaturing polyacrylamide gel, the DNA size-fractionates and the sequence pattern can be read horizontally across the gel with the next base in the sequence always indicated by the next higher band in the gel. In the automated sequencing procedure, four different fluorescent primers, each with a unique signal, are used in the four dideoxy reactions. The Applied Biosystems Incorporated model 370a DNA sequencer was used for electrophoresis and detection of sequence data after sequencing reactions were prepared stringently following ABI's recommended protocol. The four different dyes are detected by laser activated fluorescence. Photomultipliers with four color filters collect data

in real time near the bottom of the gel as the data is stored in computer memory for further evaluation.

## **CHAPTER IV**

**TOXICITY AND MUTAGENICITY**

**OF**

**VCM, CEO, AND CAA**

## MATERIALS AND METHODS

An overview in the form of a flow chart of the mutation assays done for this study is provided in figure 4.1.

### Chemicals, Enzymes and Media Compounds:

Materials for the toxicity and mutation assays were obtained from the following sources: RPMI 1640 (1x with L-glutamine), Dulbecco's phosphate buffered saline (PBS) pH 7.5, 8.0 mg/ml NaCl, 0.2 mg/ml KCl, 1.15 mg/ml Na<sub>2</sub>HPO<sub>4</sub>, and 0.2 mg/ml KH<sub>2</sub>PO<sub>4</sub> and penicillin and streptomycin (1000x) were obtained from Lineberger Cancer Research Center, Tissue Culture Facility. L-glutamine; 200mM as 29.2 mg/ml in 0.85M NaCl were from Gibco, Life Technologies Grand Island, NY. Bovine Calf Serum, Lots 21512008 and 21511064, from HyClone Laboratories, Logan UT. Aminopterin, hypoxanthine (6-hydroxypurine), thymidine (1-[2-deoxy  $\alpha$ -D-ribofuranosyl]-5-methyl uracil) 99-100%, deoxycytidine, 6-thioguanine - (2 amino-6-mercaptopurine) 98%, and L-histidinol were purchased from Sigma Chemical Company, St. Louis, MO. Vinyl chloride and ethylene oxide (>99.5%) were obtained from Fluka Chemica, Switzerland. CAA was obtained as a 50% w/w solution in H<sub>2</sub>O from Aldrich Chemical Company, Milwaukee WI. CEO was synthesized by Dr. Avram Gold, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC. through photochemical chlorination of ethylene oxide in the prescence of tertiary butyl hypochlorite (Walling and Fredricks, 1962). The t-butyl hypochlorite was a generous gift of Dr. Amarnath, Department of Pathology, Duke University, Durham, NC. The AHH-1 IIE1 cell line were the generous gift of Gentest Corporation, 6 Henshaw St., Woburn Ma.

### Safety Considerations

Safety in handling chemical carcinogens, selective agents, and the cells in culture was always paramount in experimental design.

The cell line used in this study contains Epstein Barr Virus DNA which necessitates the handling in accordance with NIH guidelines for research with recombinant DNA molecules (Gentest 90-1). EBV transfected cells do have the potential to shed the active virus, although this is highly unlikely. All regular cell culture work was accomplished in a Health and Safety certified biological safety hood with vertical laminar air flow and external exhaust. In addition, latex gloves and a laboratory coat were worn when handling cells in culture. The selective agents aminopterin and 6TG are both toxic compounds and were handled in the biological safety hood only after they were placed in solution. Solutions of these compounds were prepared in an approved chemical laboratory hood.

The exposures to VCM, CAA, and CEO were all accomplished in a chemical lab hood. In addition, a NIOSH approved, fit-tested, half-face respirator with organic vapor cartridges (TC-23c-435) was worn. The cartridges were replaced frequently (every 2-3 weeks) to preclude cartridge saturation and breakthrough. The use of vinyl chloride in the workplace is federally regulated under OSHA Standard 1910.1017. These regulations were implemented where applicable. During treatment with any of the three compounds, the door to the room was closed and a sign posted that warned others that vinyl chloride, a cancer suspect agent, was in use. An incubator was dedicated solely for use during the cell exposures and was appropriately labeled during the exposure period. In addition to its carcinogenic properties, vinyl chloride is an extremely flammable gas and is maintained

under pressure. Consequently, the compound was handled very cautiously when it was in the gaseous state and all sources of ignition were removed from the hood. When the stock gas cylinder was not in use, it was stored inside the chemical lab hood. All contaminated media and disposable plasticware were kept in the chemical laboratory hood until it was packaged for disposal through the Health and Safety office. Material safety data sheets for the compounds used were available in the work area to provide additional safety guidelines.

### Cell Culture

The stocks were maintained at  $-70^{\circ}\text{C}$  in DMSO until ready for use. Cells were quickly thawed at  $37^{\circ}\text{C}$ , diluted in 50 ml of prewarmed RPMI 1640, 10% bovine calf serum without antibiotics (stock media), pelleted with centrifugation (1000g 3 min) and resuspended in 50 ml of stock media. The cytoprotective agent, DMSO inhibits cytochrome P450IIE1 activity and must be removed from the media (Yoo et al., 1987). Selective pressure for the IIE1 plasmid is begun immediately after resuspending the cell pellet in fresh media and is accomplished by the addition of 3mM L-histidinol. L-histidinol was stored as 300x stock in 0.500 ml aliquots in cryogenic vials at  $-20^{\circ}\text{C}$  and thawed individually. (Repeated freezing and thawing of L-histidinol in solution (sterile PBS) is not appropriate according to the technical department of Sigma Chemical Company.) 7.5%NaHCO<sub>3</sub> was added 0.250 ml/ 50 ml whenever L-histidinol was added to buffer the alkalinity of histidinol. Histidinol was added to the cell culture after each dilution (three days per week) up through the treatment with HAT. Cells were maintained in a  $37^{\circ}\text{C}$  incubator with 6% CO<sub>2</sub>, 100% humidity in 75 cm<sup>2</sup> sterile tissue culture flasks. Cells were enumerated approximately every other day using a Coulter Counter (Coulter Electronics, Hialeah FL) and diluted using

prewarmed (37°C stock media) to a concentration of  $0.5 - 4.0 \times 10^5$  cells/ml. Cells were never grown at cell concentrations exceeding  $1.2 \times 10^6$  cells/ml. Cell cultures were tested periodically and found negative for mycoplasma contamination. Cell doubling time was calculated periodically using the formula:

$$\tau = [\ln 2(T)] / [\ln(N/N_0)]$$

Where  $\tau$  is the doubling time in hours, T the difference in hours between the time the cells were last diluted and the current cell count, N is the current cell concentration (cells/ml) and  $N_0$  is the cell concentration when the cells were last counted. Average doubling time for this cell line is 22 hours; longer doubling times (in excess of 30 hours) were viewed as suspect for possible culturing problems. Cell culture was maintained during and after treatment as described, but the cultures were supplemented with penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml).

Pretreatment of cell stocks in HAT media to reduce the level of background mutants was carried out prior to each exposure to the test substance. The HAT treatment consisted of  $2 \times 10^{-4}$  M hypoxanthine,  $8 \times 10^{-7}$  M aminopterin and  $3.5 \times 10^{-5}$  M thymidine. Stock media was used through HAT treatment. HAT was prepared as 333x HT in 0.3N HCl, 0.2  $\mu$ m filter sterilized and 250x A in 0.1N NaOH, 0.2  $\mu$ m filter sterilized and stored at -20°C in a frost-free freezer. HAT treatment was carried out for 72 hours while maintaining exponential growth. Histidinol selection was maintained through the HAT treatment. All cells which were used in any one experiment (cells for controls and treated cultures) were passaged

through the HAT treatment in a single culture. Cells were counted and diluted after 48 hours and the HAT concentrations re-established for an additional 24 hours. Cells were then centrifuged in an IEC Centra 8 table top centrifuge for three minutes at 1000 rpm, 25 °C. The supernatant was carefully removed and the cell pellet resuspended in pre-warmed stock media, and HT (no aminopterin) added in the same concentrations as in the HAT treatment for recovery. Cells were allowed to grow for two to three days after the 24 hour recovery in HT to achieve adequate cell numbers for treatments. Treatments were accomplished in 50 ml cell suspension, ( $4 \times 10^5$ /ml) in 75 cm<sup>2</sup> tissue culture flasks for 24 hours. The media used was RPMI 1640, 10% BCS with penicillin and streptomycin (referred to as treatment media). During treatment, flasks were sealed to prevent carcinogen contamination of the atmosphere in the incubator. While sealed, the CO<sub>2</sub> in the incubator could not reach the media to maintain pH; therefore the treatment media was supplemented with 29 mM HEPES buffer during the 24 hour treatment period. After treatment, the entire volume of cells or a fraction thereof (depending on whether toxicity was anticipated or not) was pelleted, resuspended in fifteen ml prewarmed treatment media in a 50 ml centrifuge tube, pelleted again and resuspended in 50 ml of treatment media in 75 cm<sup>2</sup> flasks. The cell density was determined and two plates were seeded per treatment concentration for toxicity determination. The time at which this initial density was determined was the starting point for evaluating growth delay. The counts from the two plates were pooled to calculate PE. Cells were carried for six days to allow phenotypic expression at the hprt phenotype; during this time the cell cultures were diluted daily to  $4 \times 10^5$  cells/ml. Cell density was ascertained on day seven and the cells were plated for mutant fraction as described previously. Replicate plates (2 - 10

depending on the treatment and anticipated MF) were used for each treatment and the counts of all the plates were pooled to calculate the MF. Three independent cultures were exposed and carried through MF determination for each concentration of compound tested. Actual number of flasks (and consequently number of cells) initially treated varied according to toxicity. Four control flasks were maintained in every experiment.

### Plating

Cells at a determined density were plated in 96-well, flat bottom microtiter plates (well capacity 0.35 ml, surface area of  $0.28 \text{ cm}^2$ , Costar Corp). The volume plated per well was 0.2 ml in all cases. The media used for plating was RPMI 1640 plus 15% bovine calf serum, 1X penicillin and streptomycin, and 1.0 mM L-glutamine. Plates were maintained undisturbed in a water jacket incubator at 6%  $\text{CO}_2$  for 15 days before colonies were scored. Because of the ability of this cell line to form rather large colonies (2 mm in diameter and larger, both mutant colonies and wild type) the plates were scored by counting colonies from the back side of the plate without magnification. Contaminated or empty wells were excluded from the calculation to determine plating efficiency or mutant fraction. Plating efficiencies ranged from 20-60%. Cells for the purpose of determining toxicity or plating efficiency were plated at a density of two cells per well. The only exception was in plating cells after exposure to higher doses of CAA where toxicity required that four cells/well ( $16 \mu\text{M}$  exposure) and 30 cells/well ( $24 \mu\text{M}$  exposure) be plated. To determine the frequency of  $\text{hprt}^-$  mutants generated by exposure to the test substances, cells were plated at an optimized concentration of 30,000 cells per well in the presence of  $1 \mu\text{g/ml}$  of the selective

agent 6-thioguanine (6TG). Plating efficiencies (PE) and mutant fraction (MF) were calculated as described previously.

#### Determination of Treatment Concentrations

Initial cytotoxicity studies were accomplished with the three compounds over a broad range of test substance concentrations. Ten milliliter cultures at  $4 \times 10^5$  cells/ml in 25 cm<sup>2</sup> sterile tissue culture flasks were used. Preliminary results from the VCM toxicity and mutagenicity experiments indicated that 24 hour treatments would be preferable versus shorter periods due to the limited response seen. Therefore, for consistency, all cell exposures were conducted for 24 hours. The preliminary results from the VCM studies indicated that VCM solubility would determine the upper treatment concentration. This level was determined as 500  $\mu$ M. Dose ranges of 8-24  $\mu$ M for CAA and 25-75  $\mu$ M for CEO were selected; the upper level of these doses approached 10% survival.

When the first mutation determinations were made, a very high background MF was observed, ( $9 - 75 \times 10^{-6}$ ). High background levels of mutants can be due to: artifacts from low plating efficiencies, improper aminopterin concentrations, exposure to some other mutagen, or an inadequate selective agent (6TG) (Gentest, 1990). These possibilities were tested and eliminated. We then demonstrated that the high background was dominated by an unusual 6TG<sup>r</sup>, HAT<sup>r</sup> population. Although never tested, it was assumed that this was an hprt<sup>-</sup> mutant lacking activity with 6TG but retaining sufficient activity with hypoxanthine to permit growth in HAT. To obtain cultures free of this mutant, ten milliliter cultures with  $10^4$  cells total were established. After cultures were grown to a concentration of  $10^6$  cells/ml, the cells were plated for MF. The rest of the cell stock was frozen. Plates were scored after

fifteen days incubation. Cultures with very low background MF ( $3 \times 10^{-6}$  or less) were considered to be mutant free and aliquots cryo-preserved. These "mutant free" cell stocks were then used for all mutation assays.

### VCM Treatment

Liquid VCM was prepared by carefully releasing gaseous VCM through tygon tubing into a small-necked glass scintillation vial submerged in a dry ice/acetone bath ( $-70^{\circ}\text{C}$ ) in a chemical laboratory hood. The liquid VCM was quickly transferred to a dry ice/acetone cooled 20 ml amber serum vial containing 10 ml MeOH. The bottle was quickly capped with a septum and the septum locked into place with an aluminum collar. The weight of the bottle and contents was determined and the amount of VCM collected was calculated by subtracting the weight of the bottle, septum, collar, and MeOH. From this, the VCM/MeOH ratio could be established. The VCM/MeOH stock was maintained on dry ice/acetone until needed for preparing the dosing solutions. The final concentration of the VCM stock used to treat the cultures was a compromise between two critical factors. The final MeOH concentration in the culture had to be kept below 0.03% to avoid inhibition of the cytochrome P450IIE1 activity (Gentest, 1990). This demanded a high VCM/MeOH ratio in the stock. However, the MeOH concentration had to be high enough to ensure VCM solubility. Treatment solutions (10mM, 25mM, and 50mM) were prepared from the VCM/MeOH immediately prior to treatment by diluting the VCM/MeOH into cold unsupplemented RPMI 1640, with an ice-cold gas-tight Hamilton syringe. Concentrations of treatment solutions were selected to permit volumes delivered to treated cultures to remain between 0.1 ml and 0.5 ml, a

volume accurately delivered with a 1cc tuberculin syringe. Treatment flasks contained 50 ml of  $4 \times 10^5$  cells/ml. Flasks were sealed with sterile rubber septum stoppers which were sealed with vinyl tape. VCM/MeOH stocks and treatment solutions were maintained as cold as possible during transfers. Vials containing these solutions were kept in dry ice/acetone (stock solutions) or near freezing on dry ice (treatment solutions). Appropriate volumes of treatment solution were measured in a syringe by holding the syringe against dry ice as the solution was drawn into it. Doses delivered into the treatment flasks were always injected through the cell suspension with the opening of the treatment flask pointing down and the mixture gently agitated before setting the flask down. This arrangement ensured: 1) efficient delivery of the treatment solutions with minimal losses, 2) the delivered dose remained in the tissue culture flask (in solution or in the head space) 3) no contamination of either the incubator or the laboratory with VCM. After treatment, the rubber septums were cut off and held for appropriate disposal. Three cultures were treated for each exposure concentration and three MF plates were prepared from each culture (nine plates for each dose). Two MF plates were prepared for each control culture and two plates for each treatment concentration for both PE and toxicity plates. Two consecutive treatments were accomplished at 300 and 500  $\mu$ M to induce greater mutant fractions and allow for the collection of mutants for analysis. Since VCM toxicity was never observed, the second treatment was administered the same day the cells were removed from the first treatment.

#### CAA Treatment

CAA treatment stock was prepared as 100 mM in PBS. This was diluted to 1 mM in unsupplemented RPMI. The 1 mM stock was used for all cell treatments. New CAA stock

was prepared from the purchased CAA stock for each treatment. The 1 mM stock was added to 50 ml cultures at  $4.5 \times 10^5$  cells/ml at a final concentration of 8, 16, or 24  $\mu\text{M}$ . Cultures were tightly capped, gently mixed and placed in the 37°C incubator for 24 hours. Controls were also capped tightly and contained HEPES buffer at the same concentration. Four replicate control cultures, three replicate cultures at 8  $\mu\text{M}$ , 6 cultures at 16  $\mu\text{M}$ , and 15 cultures at 24  $\mu\text{M}$  were treated. After growth resumed in the flasks, the 16 and 24  $\mu\text{M}$  treatments were pooled into three cultures. When cells were plated for MF, there was a total of three cultures for each treatment concentration and four controls.

The following number of plates were used for each treatment concentration to determine MF: two plates per each of the three treatment cultures for 8  $\mu\text{M}$ , six plates per each of the three pooled treatment cultures at 16  $\mu\text{M}$  and ten plates for each of the three pooled treatment cultures at 24  $\mu\text{M}$ . Two plates for each control were plated. To obtain cells for DGGE analysis, two consecutive treatments were carried out at 16  $\mu\text{M}$ ; the second treatment started after the cells had recovered from the first treatment (i.e. log growth with approximately 22 hour doubling times). Attempts were made for bulk selection of mutants in large spinner cultures (900 ml,  $4 \times 10^5$  cells/ml -  $1.0 \times 10^6$  cells/ml in 1 liter jars on a magnetic stir plate) (Carriello et al., 1991). However, the combined effects of toxicity from the CAA exposure and the propensity to grow poorly in bulk spinner cultures (Gentest, 1990) resulted in forgoing this method of isolating mutants for analysis.

#### CEO Exposure

CEO density was determined by weight using a Mettler AE240 analytical balance to be 1.102 gm/ml. CEO purity was determined as 97% for the first three treatments, and 85% for

the fourth treatment. Purity was determined based on nuclear magnetic resonance (NMR) recorded at the time of synthesis. CEO treatment stocks were prepared in acetone from the pure CEO immediately before treatment. The appropriate volume of acetone for treatment solutions was aliquoted into 20ml amber serum vials and kept covered in dry ice/acetone. The CEO was quickly thawed, the volume of CEO needed removed with a pipette, and delivered to the serum bottle which was then sealed with a rubber septum and collar. The CEO was quickly returned to the dry ice acetone and cell cultures were treated as described for the VCM. Treatments of 100 $\mu$ M, 75 $\mu$ M, 50 $\mu$ M and 25 $\mu$ M were conducted. CEO was delivered in acetone at a final level of 0.04% in each culture including controls, (acetone produces signs of toxicity in these cells at 0.5% (Gentest, 1990). Triplicate cultures were treated at each dosing concentration and ultimately three MF plates were prepared from each treatment flask (nine plates per treatment dose).

## RESULTS

### Toxicity

The plating efficiency of treated cultures immediately after treatment was compared to that of controls and expressed as relative survival. CAA and CEO showed a dose-dependent decrease in survival following treatment (Fig 4.2) and (Fig 4.3) respectively. On a molar basis, CAA was much more toxic than CEO. No toxicity was observed at any of the VCM concentrations tested (Fig 4.4). Therefore, the toxicity induced by the three compounds can be compared on a molar basis and represented as: CAA>CEO>>VCM.

### Growth Delay

After treatment the cells were monitored by daily counts. As an example of culture growth following treatment, results obtained with one CAA experiment (the compound that showed the greatest level of toxicity) are provided in Fig 4.5. There was a dose-dependent increase in growth delay from treatment with CAA. One can extrapolate the linear portion of the curves (in Fig 4.5) back to  $t=0$  to obtain the theoretical number of surviving cells required to produce the exponential growth eventually observed in the culture. For example, estimates with  $24\mu\text{M}$  indicate that at least 1% of treated cells had to survive to produce the observed growth. This value is very close to actual cell survival determined by plating. Therefore, the delay in culture growth was due mainly to cell killing and not to delay in cell cycling.

### Mutant Fraction

The average induced  $\text{hprt}^-$  mutant fraction (observed MF minus concurrent control MF) for the three different treatments was determined (Fig 4.6-4.8). For the VCM exposures, each point represents the average of from 3-5 independent experiments. For CEO treatments, each value is the average of four independent experiments and for CAA every value is the average of three experiments. In turn, the determination in each experiment is the average of 3 independent test cultures. Error bars are standard error of the mean based on the number of studies accomplished at each treatment concentration. The background mutant fraction varied for each study with each compound, and the ranges of background M.F. ( $\times 10^{-6}$ ) were 2.1 - 5.6 for VCM, 3.8 - 5.6 for CAA, and 2.1 - 10.8 for CEO. The use of induced mutant fraction allows for comparison between independent experiments with different background MF. On a molar basis, CEO is very similar to CAA in terms of mutagenic potential. The

induced mutant fraction with each compound increased in a linear fashion with increasing concentration. However, as discussed above the two compounds differ greatly in terms of toxicity. Consequently, at equi-mutagenic concentrations, CAA is much more toxic than CEO. VCM consistently produced a MF above background level, but a monotonically-increasing response was not observed.

## DISCUSSION

We have used the human cell line AHH-1 which expressed cytochrome P450IIE1 activity to test the mutagenic potency of vinyl chloride and its metabolic products, chloroethylene oxide, and chloroacetaldehyde. VCM requires metabolic activation through cytochrome P450IIE1 to exhibit its mutagenic and toxic effects. Both CEO and CAA can react with nucleophilic cellular macromolecules, including DNA and can form stable DNA adducts. These adducts, if unrepaired or repaired incorrectly can result in heritable DNA damage.

The concentration-dependence of the VCM results suggest a plateau of mutagenic response, which one would anticipate from the saturation of metabolic activation of VCM. No toxicity was seen at any of the concentrations tested. Substantial fluctuation in measured MF was seen and can be attributed to the difficulty in delivering VCM reproducibly to the treatment flasks and the fact that induced levels were not large compared to background levels. Also, a simple monotonically-increasing response is not apparent. This may be the result of varying two competing parameters in the study: 1) VCM concentrations which will affect the final mutation frequency and 2) methanol concentrations which can affect the

ability of P450 to activate VCM. Methanol concentrations of only 0.03% will have an impact on the metabolizing capabilities of the cell line (Gentest, 1990). This is plausible, since replicate experiments with different MeOH concentrations showed a consistent modulation of VCM mutagenesis as a fraction of MeOH concentration. In comparing mutant fraction induced at the same delivered dose of VCM, the higher mutant fraction usually occurred at a lower MeOH concentration. The data would indicate that in an attempt to ensure delivery of the measured dose, by avoiding VCM loss from VCM coming out of solution, the MeOH concentration contributed to the ineffectiveness of the metabolic system to oxidize VCM to CEO.

The values in Fig 4.8 represent the theoretical delivered dose to the flasks. Due to the volatility of VCM, one expects a certain fraction to escape from the medium and reside in the flask head space during treatment. Therefore the values represent an upper limit of VCM in the medium. Even with exact concentration of VCM unknown, certain conclusions can be drawn. At the lowest concentration tested ( $25\mu\text{M}$ ) a mutagenic response was seen equivalent to  $9\mu\text{M}$  CEO and  $10\mu\text{M}$  CAA delivered externally to the cell in the medium. This suggests that at most, one-half of the VCM was metabolized. The lower limit cannot be determined since intra-cellular generation of CEO and CAA may be much more efficient in terms of mutagenic potency.

It is interesting to note that at the concentrations of CEO and CAA needed to achieve the induced MF of 5 observed with VCM, the CAA dose should have produced detectable toxicity (50%) while the CEO should have produced negligible toxicity (10%). In fact, no toxicity was observed with VCM. These results suggest that the majority of induced MF

produced by VCM must have resulted from CEO production. One must also consider that CEO is rapidly rearranging to CAA (half-life = 1.6 min at 37 °C). It is likely that a substantial portion of the toxicity seen with CEO is due to the appearance of CAA in the media. An increasing body of experimental evidence from other test systems indicates that CEO is the VCM metabolite responsible for much of its genetic activity. The data obtained from toxicity and mutagenicity studies supports this data. It is interesting to note, however, that in this study CEO demonstrated a mutagenic potency equal to CAA, however, CAA was much more cytotoxic than CEO.

Impurities on the CEO included ethylene oxide and t-butyl alcohol. The contribution of EO to the observed mutagenicity and toxicity were of concern. However, Kolman et al., (1992) exposed human diploid fibroblasts (VH-10) ( $5 \times 10^5$  cells in 5ml) to EO in solution for 1 hour and then determined toxicity and hprt<sup>-</sup> mutant fraction. The lowest concentration tested in their study was 11.02 µg/ml which produced a MF of  $18 \times 10^{-6}$ . The highest dose of CEO used in the present study would have contained at most 1.0 µg/ml EO. Therefore, EO could account for no more than  $1.5 \times 10^{-6}$  of the induced MF of  $20 \times 10^{-6}$ , and its contribution to the MF is small (Kolman, et al., 1992). It is interesting to note that in comparison between the two induced mutant fractions on a per molar basis, CEO is 25 times more mutagenic than EO.

# Mutation Assay Exposure Summary

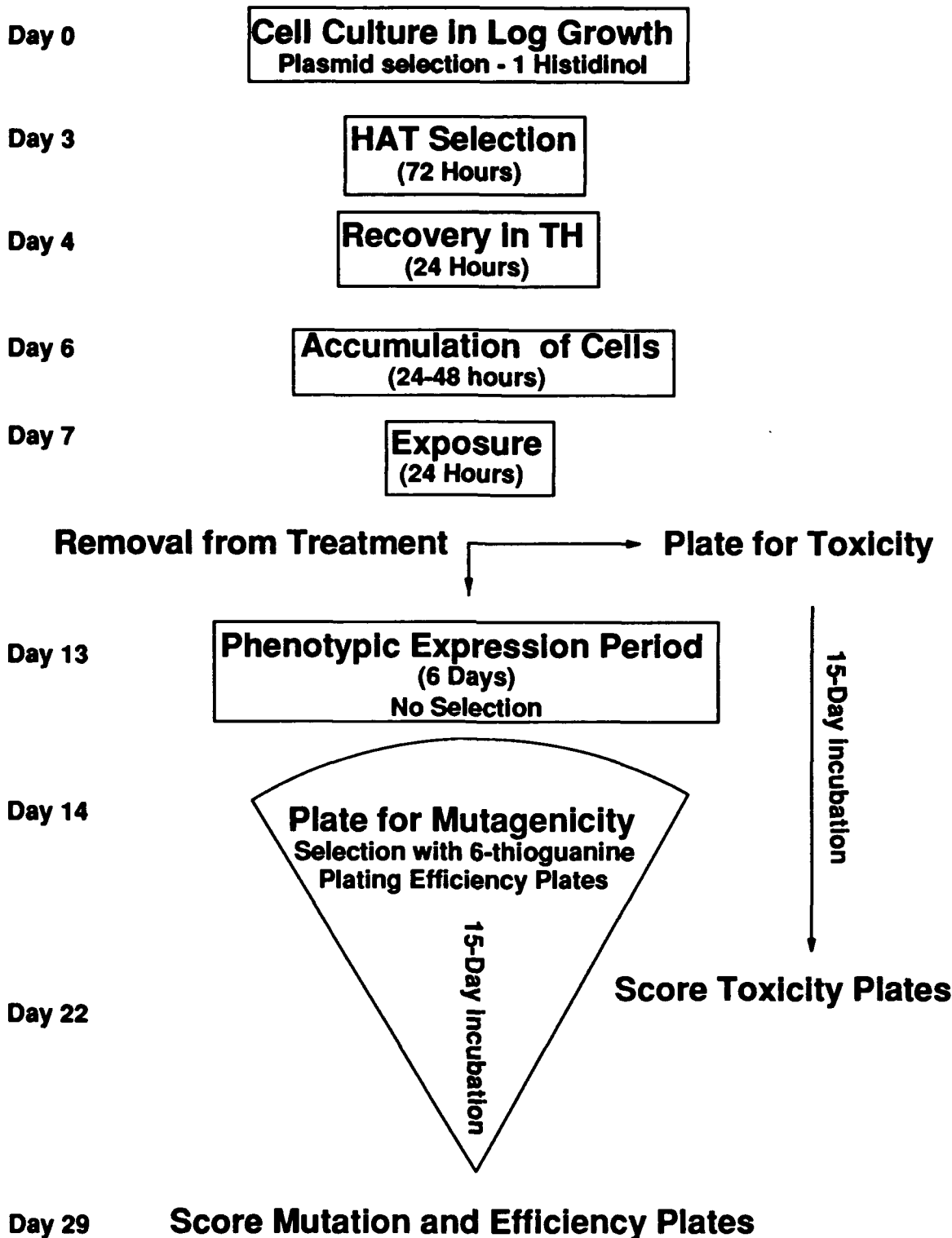


Figure 4.1: See text for details

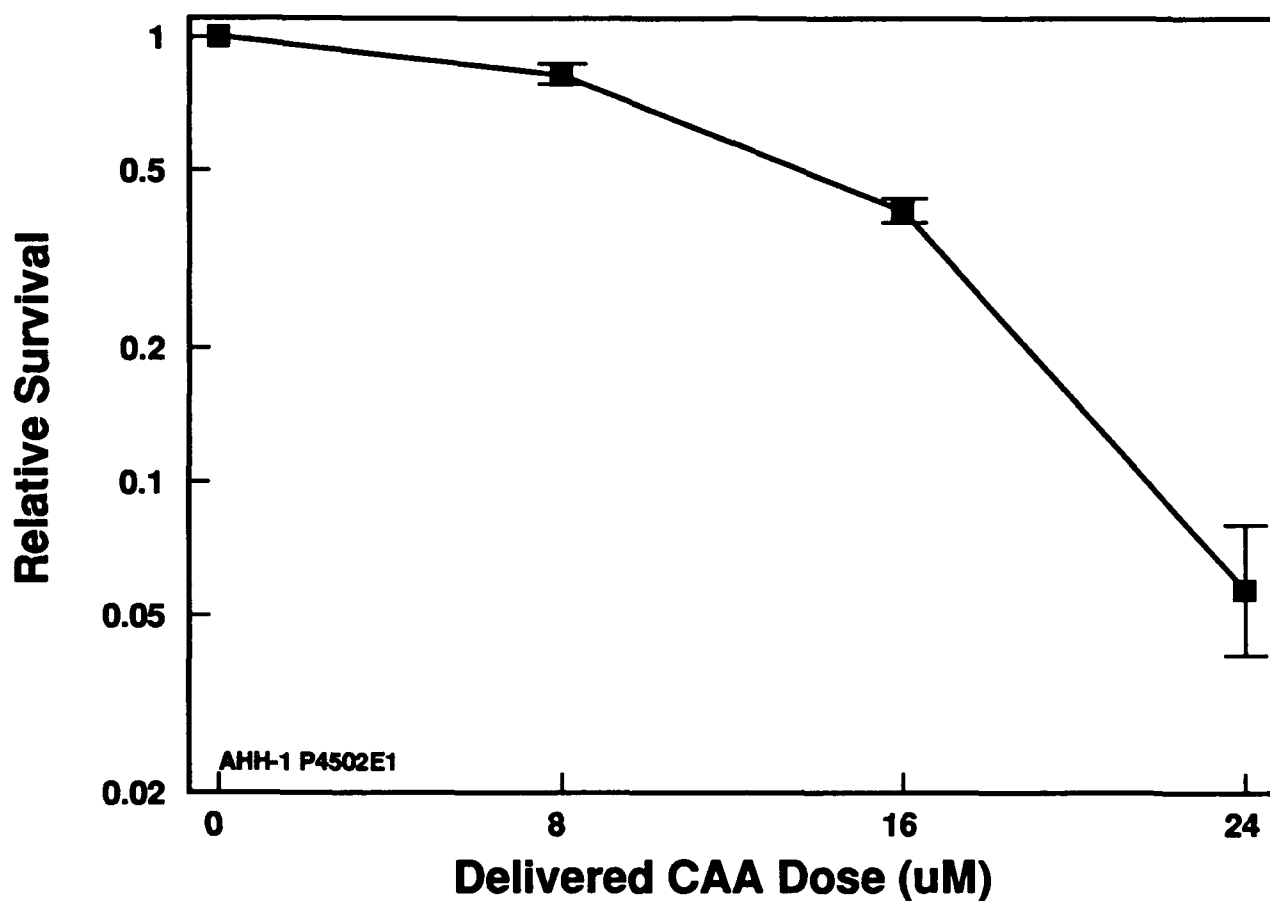


Fig 4.2. Survival of AHH-1, P450IIE1 cells after 24 hour exposure to various concentrations of chloroacetaldehyde. Cells were treated with CAA, returned to fresh treatment media, and a sample was taken immediately and plated in microtiter plates at a density of 2, 4, or 30 cells per well for 8  $\mu$ M, 16  $\mu$ M, and 24  $\mu$ M treatments respectively. Relative survival was calculated as a fraction of the plating efficiency obtained from the treated cells relative to the plating efficiency of the controls. Each point represents the mean of four independent experiments each containing 3 (8  $\mu$ M), 6 (16  $\mu$ M), or 15 (24  $\mu$ M) independently treated cultures. Error bars indicate the SEM of the four experiments.

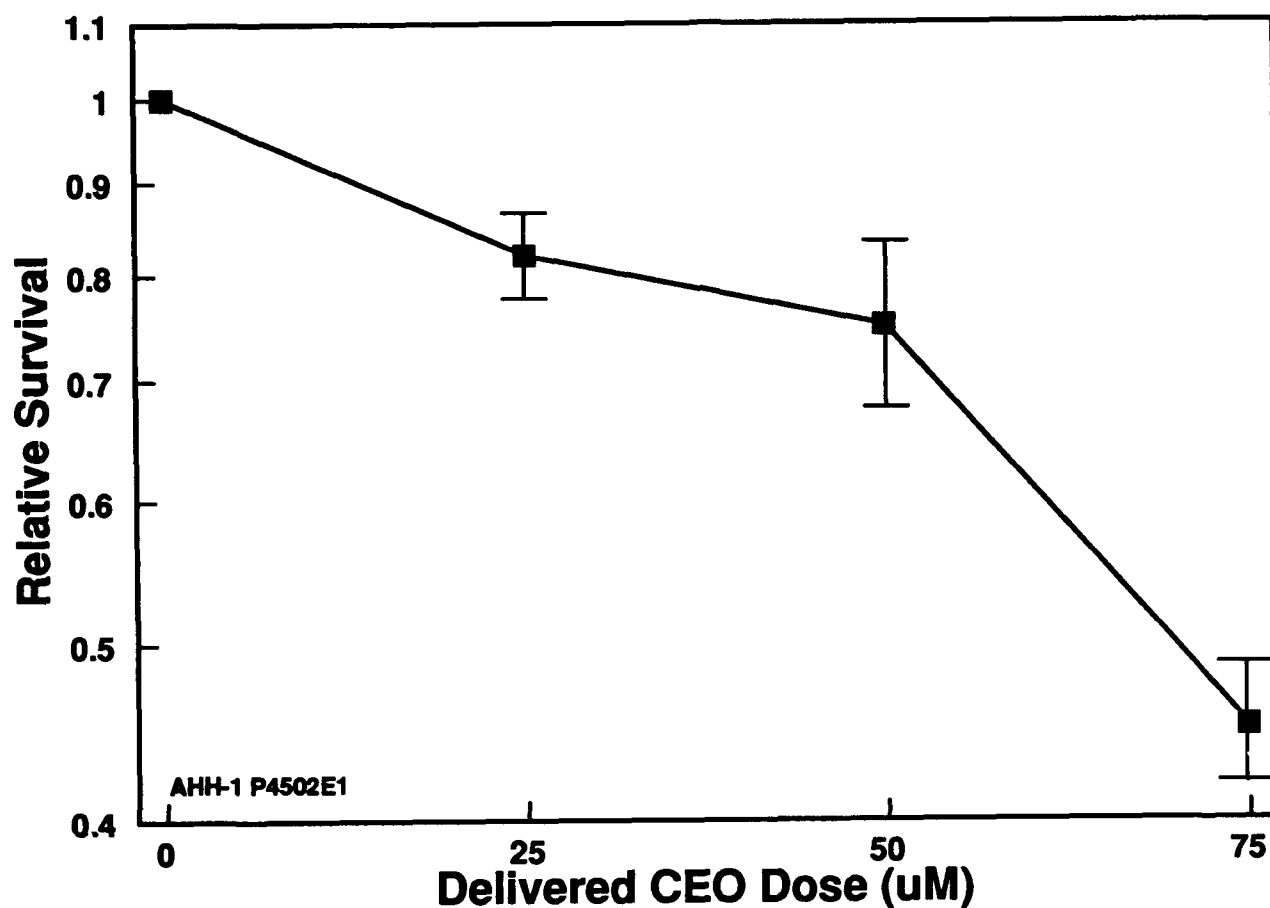


Fig 4.3. Survival of AHH-1, P450IIE1 cells after 24 hour exposure to various concentrations of chloroethylene oxide. Cells were treated with CEO, returned to fresh treatment media, and a sample was taken immediately and plated in microtiter plates at a density of 2. Relative survival was calculated as a fraction of the plating efficiency obtained from the treated cells relative to the plating efficiency of the controls. Each point represents the mean of four independent experiments each containing three independently treated cultures. Error bars indicate the SEM of the four experiments.

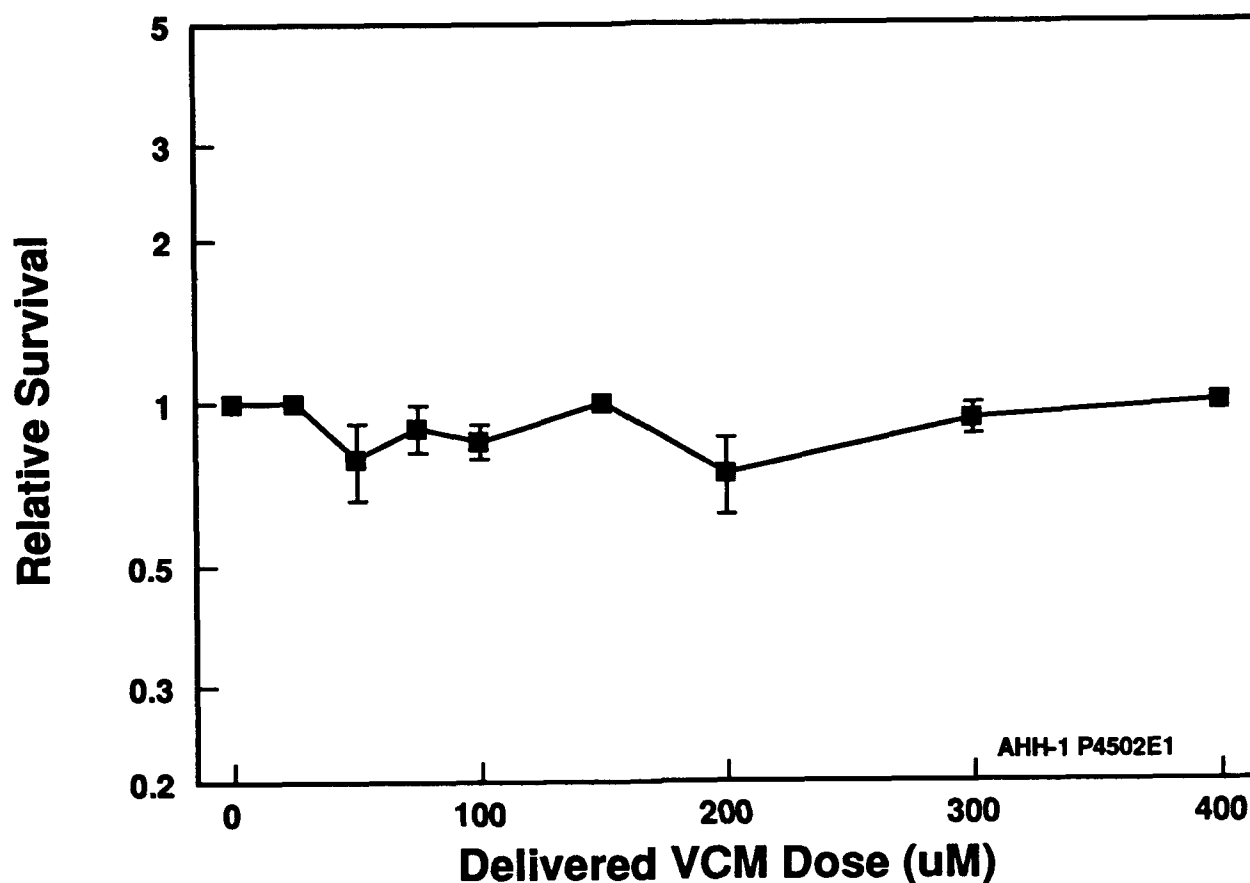


Fig 4.4. Survival of AHH-1, P450IIE1 cells after 24 hour exposure to various delivered concentrations of vinyl chloride. Cells were treated with VCM, returned to fresh treatment media, and a sample was taken and plated in microtiter plates at a density of 2 cells per well. Relative survival was calculated as a fraction of the plating efficiency obtained from the treated cultures relative to the plating efficiency of the controls. Each point represents the mean of three to five independent experiments each containing three independently treated cultures. Error bars indicate the SEM of the experiments.

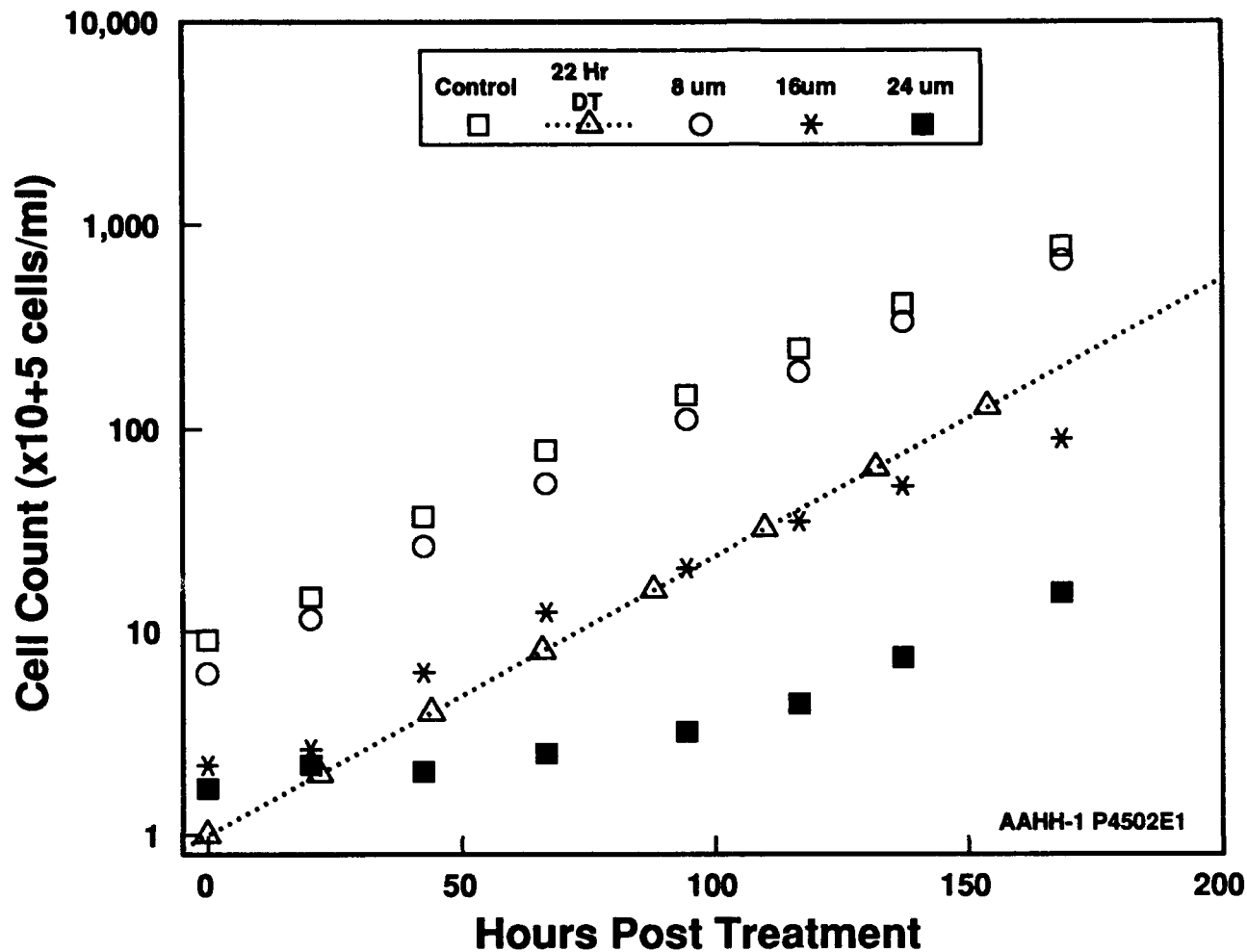


Fig 4.5. Growth delay of AHH-1, P450IIE1 cells after 24 hour exposure to various concentrations of chloroacetaldehyde. Cells were treated with CAA, returned to fresh media and cell density determined. Cell counts were based on daily cell counts of individual exposure cultures. Each treatment dose represents the mean of three independently treated cultures. Calculated relative survival for the individual treatment doses for this experiment as determined through plating trials was 93%, 48% and 2% for 8  $\mu$ M, 16  $\mu$ M, and 24  $\mu$ M respectively. Growth delay is represented by the non-linear portion of the graph. The theoretical 22-hour doubling time is represented by the dotted line.

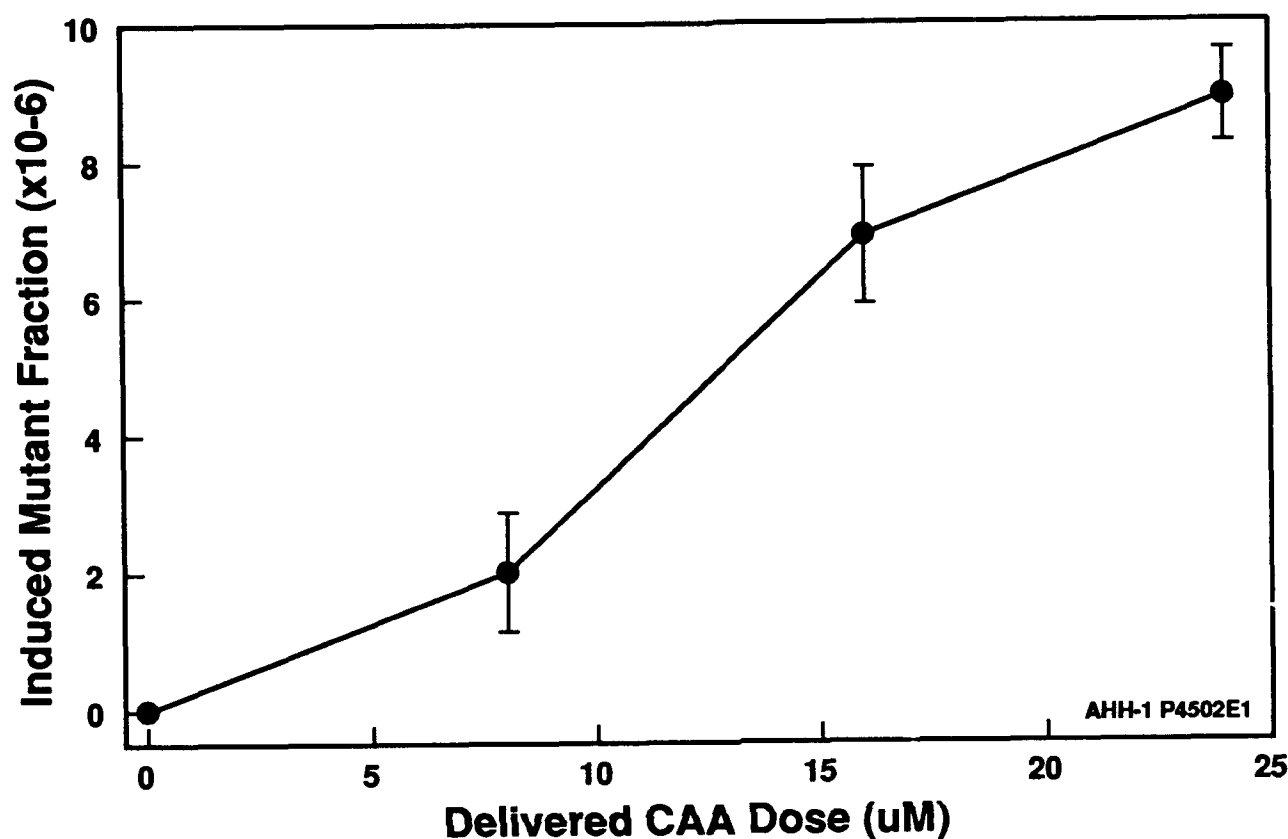


Fig 4.6. Rates of mutation at the *hprt* locus in CAA treated cells. Cultures were treated in HAT to lower the background levels of spontaneous mutants. The cells were exposed to CAA for 24 hours and returned to fresh media. After a six day phenotypic expression period cells were plated in microtiter plates in the presence of 6TG and scored for *hprt*<sup>-</sup> mutant colonies fifteen days later. Each point represents the mean of three independent experiments each containing 3 (8  $\mu$ M), 6 (16  $\mu$ M), or 15 (24  $\mu$ M) independently treated cultures. In each experiment, induced mutant fraction was obtained by subtracting average background MF seen in concurrent control cultures from average MF measured in treated cultures. Error bars indicate the SEM of the three experiments. Background mutant fractions for these studies ranged from 3.8 - 5.6x10<sup>-6</sup>.

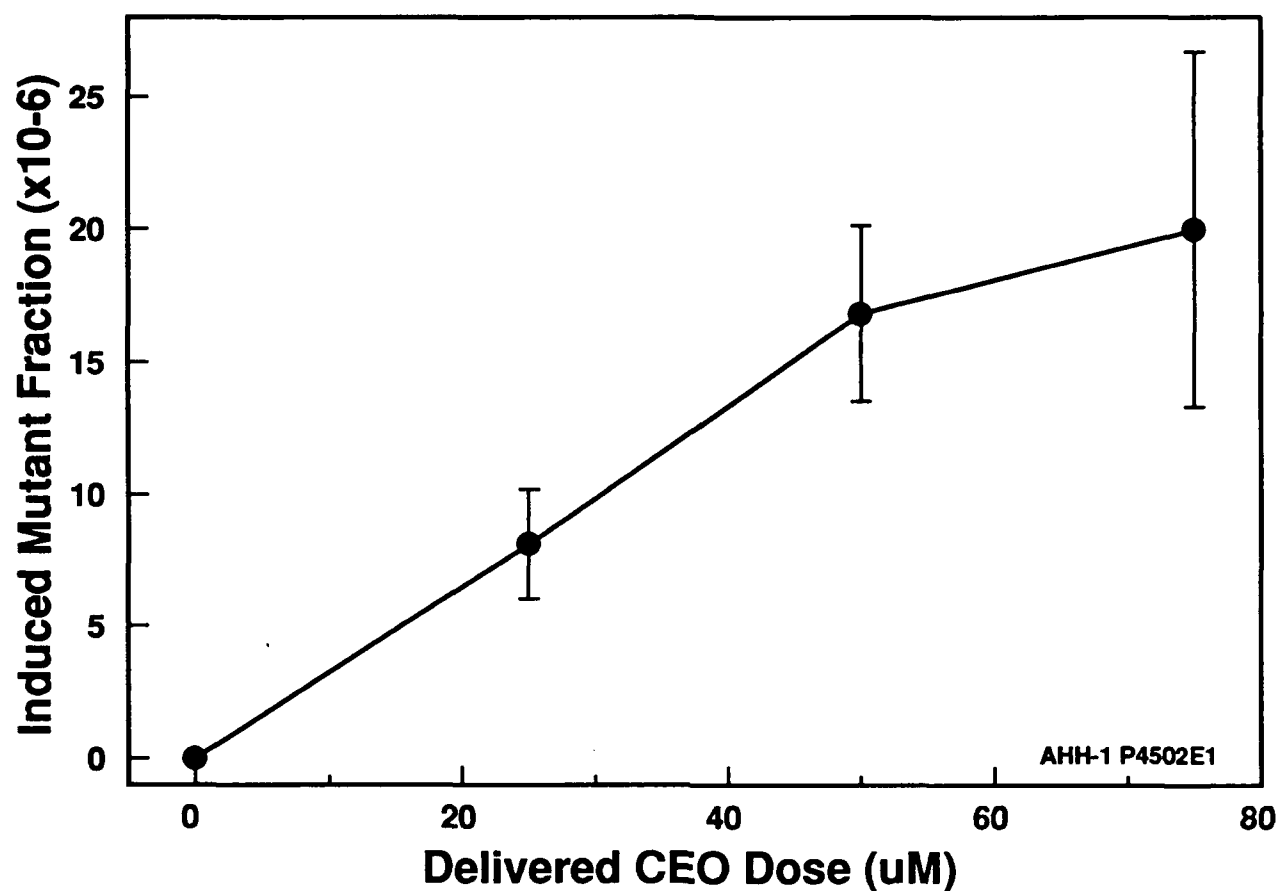


Fig 4.7. Rates of mutation at the *hprt* locus in CEO treated cells. Cultures were treated in HAT to lower the background levels of spontaneous mutants. The cells were exposed to CEO for 24 hours and returned to fresh media. After a six day phenotypic expression period cells were plated in microtiter plates in the presence of 6TG and scored for *hprt*<sup>-</sup> mutant colonies fifteen days later. Each point represents the mean of four independent experiments each containing three independently treated cultures. In each experiment, induced mutant fraction was obtained by subtracting average background MF seen in concurrent control cultures from average MF measured in treated cultures. Error bars indicate the SEM of the four experiments. Background mutant fractions for these studies ranged from 2.1 - 10.8x10<sup>-6</sup>.

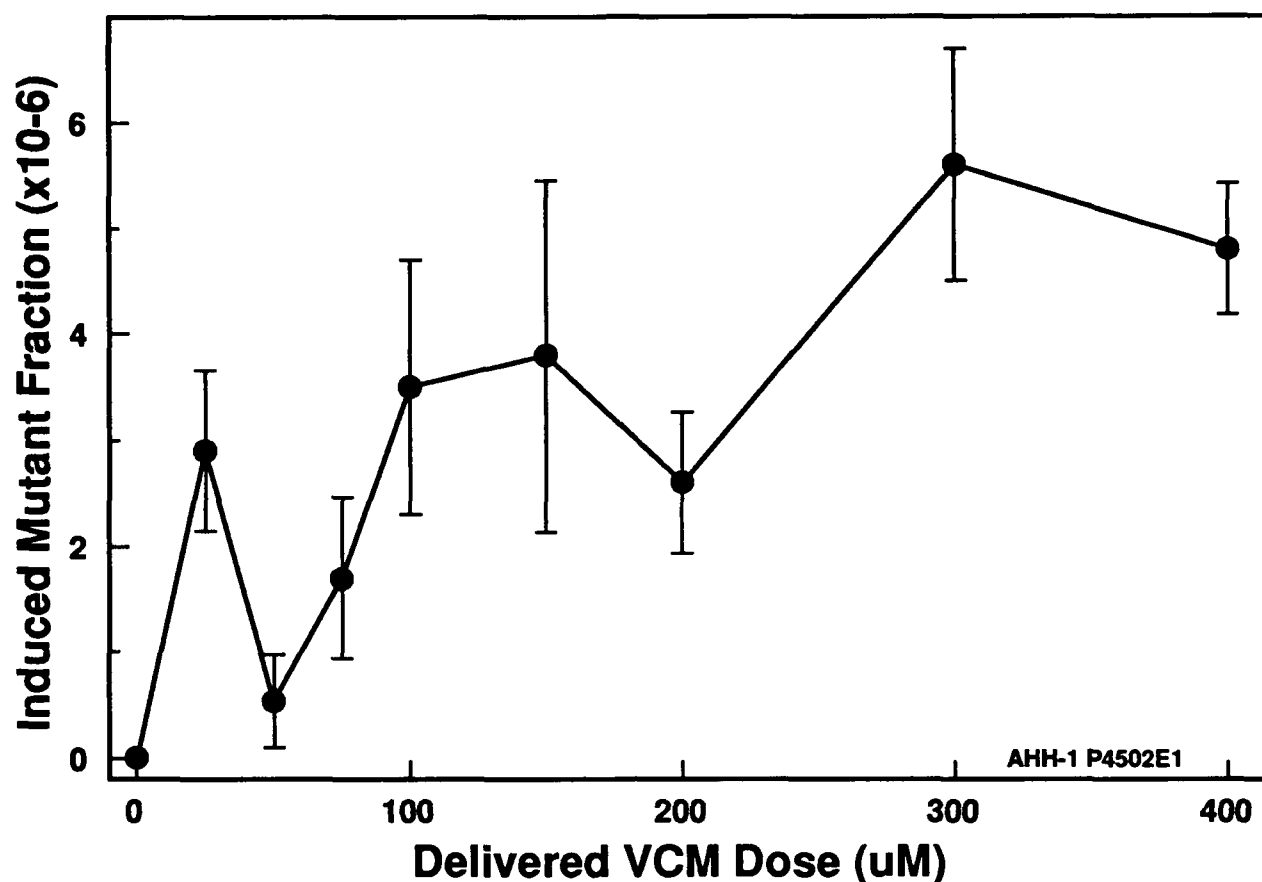


Fig 4.8. Rates of mutation at the *hprt* locus in VCM treated cells. Cultures were treated in HAT to lower the background levels of spontaneous mutants. The cells were exposed to VCM for 24 hours and returned to fresh media. After a six day phenotypic expression period cells were plated in microtiter plates in the presence of 6TG and scored for *hprt*<sup>-</sup> mutant colonies fifteen days later. Each point represents the mean of three to five independent experiments each containing 3 independently treated cultures. In each experiment, induced mutant fraction was obtained by subtracting average background MF seen in concurrent control cultures from average MF measured in treated cultures. Error bars indicate the SEM of the experiments. Dose represents the calculated cell culture concentration based on the delivered dose. Background mutant fraction for these studies ranged from  $2.1 - 5.8 \times 10^{-6}$ .

## **CHAPTER V**

### **MOLECULAR ANALYSIS OF MUTANTS**

#### **INDUCED BY VCM, CEO, AND CAA**

## INTRODUCTION

In order to decipher the true nature of mutation induced by the parent compound vinyl chloride monomer (VCM) and its metabolites within the AHH-1 P450IIE1 cell line, the mutated cells must be analyzed at the DNA level, investigating the unique pattern of changes in the DNA. This complete evaluation of mutation at a particular genetic locus is termed the mutational spectrum of a compound and indicates the location, frequency, and nature of changes to the DNA resulting from exposure to chemical compounds (Benzer, 1961, Benzer, 1958, Coulondre, 1977). Mutagenic compounds show unique mutational spectra that are different from spontaneous mutational spectrum (Schaaper et al., 1986, Liber et al., 1989, Giroux et al., 1988). Unique spectra have been identified for many mutagenic compounds (Skopek et al., 1982, Schaaper et al., 1986, Fuchs et al., 1981, Duane et al., 1986, Richardson et al., 1987, Giroux et al., 1988, Drobetsky et al., 1987, Vrielynck et al., 1988, Liber et al., 1989). The evaluation of the unique changes in the DNA can be used as a powerful tool to provide information as to the causes of DNA point mutations in human cells (Cariello et al., 1990).

## MATERIALS AND METHODS

An overview of the process involved in analyzing DNA from VCM, CEO, and CAA mutants is provided in figure 5.1.

### Chemicals, Enzymes and Primers

DNA preparation from mutant cells: Proteinase K, ultrapure, was obtained from Bethesda Research Laboratories (BRL) Gaithersburg MD. Tween 20 came from lab stock.

PCR: Ample Taq DNA polymerase 5 U/ $\mu$ l and 25mM MgCl<sub>2</sub> were purchased from Perkin Elmer Cetus. TEMED (N,N,N',N'-tetramethylethylenediamine), Acrylamide (ultrapure), N,N' methylene bisacrylamide (BIS) (ultrapure) were obtained from Gibco BRL, Gaithersburg Md. Tris Hydroxy-methyl Aminomethane Hydrochloride (Tris-HCL) (Electrophoresis Grade), and Ethidium Bromide were purchased from Fisher Biotech, Fair Lawn, N.J. Ultrapure dNTP'S from Pharmacia, LKB Biotechnology AB, International Biotechnologies, New Haven CT. Standard used in 8% PAGE (PBR322 DNA, MspI digest) from New England Biolabs Beverly, Ma. Boric acid from EM Service, Gibbstown N.J. EDTA from Mallinckrodt, Paris Kentucky. PCR primers were obtained from the Pathology Department, University of North Carolina, Chapel Hill and were HPLC purified. The oligonucleotide sequence and the annealing sites of the primers are given in table 5.1. Primer concentrations were quantified on a model UV160U Shimadzu spectrophotometer (Shimadzu Scientific Instruments, Norcross, GA) by absorbance at 260nm.

DGGE: Phenol, N,N'-methylethylene bisacrylamide (BIS), and urea (enzyme grade) were obtained from Gibco BRL, Life Tech. Inc., Gaithersburg MD. 2-mercaptoethanol, chloroform, and formamide (molecular biology grade) were obtained from Fisher Scientific, Fair Lawn N.J. Xylene cyanole FF; Sigma Chemical Company, St.Louis MO. Bromophenol blue was purchased from U.S. Biochemical Corp, Cleveland, OH. Analytical grade mixed resin (20-50 mesh) was purchased from Bio Rad Labs. Richmond, Va.

**DNA Sequencing** All the enzymes and buffers specific for the sequencing reactions were obtained from United States Biochemical Corp., Cleveland, OH. Ultrapure dNTP's were from Pharmacia. The fluorescently labeled sequencing primers were prepared according to procedures specified by Applied Biosystems.

### **Methods**

**Isolation of Mutants** Treatments were completed for VCM, CEO, and CAA at doses shown to induce mutant fractions significantly greater than background with minimal toxicity. For isolating both VCM and CAA mutants, two consecutive treatments were accomplished at the same treatment dose in order to achieve greater numbers of mutants. The concentrations for VCM were 300 and 500  $\mu\text{M}$ , and 16  $\mu\text{M}$  for CAA. The second treatment for VCM was accomplished immediately after the first, while for CAA, the cell cultures were allowed time to recover prior to the second treatment. Mutant frequencies for the consecutive treatments were shown to be at least double that measured when a single treatment was completed at the same dose. CEO mutants were isolated from a single mutation experiment of 50  $\mu\text{M}$  or 75  $\mu\text{M}$ . A large enough number of mutants was achieved in a single CEO treatment. After MF plates were scored, the cells from the colony were pipetted into eppendorf tubes, pelleted and washed with phosphate buffered saline, and stored at  $-20^{\circ}\text{C}$  until needed. Each colony isolated was labeled as an individual mutant for purposes of DNA isolation and sequencing. Genomic DNA was isolated from the cell pellet by adding "tween" cocktail, containing 1xPCR buffer (2.75 mM  $\text{MgCl}_2$ , 60mM KCl, 15.0 mM Tris HCl (pH 8.7) titrated to pH 8.75), 0.5% v/v Tween 20, and 0.1 mg/ml proteinase K, in sterile distilled water. The cell pellet was

resuspended in 20  $\mu$ l of this mixture, incubated for one hour at 55°C and then boiled (98°C) for ten minutes to inactivate the proteinase K.

PCR Conditions The first PCR reactions accomplished initiated the focus on exon 3 of *hpri* from genomic DNA. This was facilitated through the use of primers that annealed at the intron sequences flanking exon 3 (Cariello et al., 1988). To facilitate the processing of the large number of mutants, samples were pooled in groups of two mutants each. Unless noted, all PCR reactions were accomplished in 50  $\mu$ l volumes overlaid with 30  $\mu$ l mineral oil (Perkin Elmer Cetus) and contained 1xPCR buffer (as described above),  $1.3 \times 10^{-3}$   $\mu$ g/ml of each primer, 1.5 U AmpliTaq DNA polymerase. An additional 0.5-1mM  $MgCl_2$  was added, based on PCR optimization studies. Final concentration of  $MgCl_2$  in the PCR reaction was between 3.25 and 3.75 mM. All PCR manipulations (less addition of DNA template) were performed in a "clean-room" using material and equipment dedicated solely for PCR use and assumed free of HPRT contamination. All eppendorf tubes and pipette tips were autoclaved prior to use. Initial PCR amplification used 2  $\mu$ l total DNA template, 1  $\mu$ l from each of two mutants (this was the level of pooling mentioned previously). All PCR reactions were accomplished in a Perkin Elmer Cetus DNA Thermal Cycler (Model 480). The PCR thermal cycle used varied based on the primers and concentration of template, and end amplification product. The initial PCR cycle, for amplification of genomic DNA, consisted of one minute at 94°C, a quick ramp to 47°C and then one minute at 47°C, a two minute ramp to 72°C and then 30 seconds at 72°C. Thirty-two cycles were performed. The primers used in the initial amplification from genomic DNA were the  $P_1$  and  $P_2$  primers, as indicated in table 5-1. The results from this PCR reaction would serve as both template for the high temperature domain PCR reaction as

well as the product for analysis on the first low temperature domain denaturing gradient gel (see Figure 5.1). Oil was removed from the sample and 1/10 of the sample plus 2  $\mu$ l 6x loading buffer per sample was loaded into wells on an 8% polyacrylamide gel (37:1, acrylamide:biscrylamide) in 1xTBE buffer (10xTBE prepared as 108g Tris Base, 55g Boric Acid, 9.3g Na<sub>2</sub>EDTA in 1l total volume dH<sub>2</sub>O). The samples were run with a size standard, PBR322 DNA-Mspi Digest (2.0  $\mu$ l of 0.1  $\mu$ g/ml). The electrophoresis conditions were 275-300 volts for approximately one hour. The gel was removed, stained for ten minutes in ethidium bromide and destained briefly in water. A photograph was quickly taken of the gel and exon 3 bands were carefully excised using sparing long-wave UV light. The gel pieces were individually crushed with a pipette tip and 100  $\mu$ l elution mixture added (12.5 mM EDTA and 1/2xPCR buffer). The gel mixtures eluted at least 24 hours, at 4 °C before they were used as templates for the high temperature PCR reaction. The exon 3 PCR reaction was extracted twice with equal volumes of phenol (equilibrated as per Maniatis et al., 1989) and then twice with equal volumes of chloroform. The tubes were allowed to sit open in the lab hood for one hour or were spun briefly in a Speed Vac Concentrator (Model SVC 100H) for five minutes, without heat to remove excess chloroform. Mutant/wild type heteroduplexes were formed by heating the samples for ten minutes at 98 °C to denature the DNA, and then incubating for more than one hour at 37 °C to reanneal. The heteroduplex formation for high temperature melting domain PCR reactions was carried out for ten minutes at 98 °C and then at 67 °C overnight to reanneal (tubes were wrapped in parafilm to preclude evaporation). After heteroduplex formation, the samples are dried in the speed vac, with heat. The samples are

resuspended in 7  $\mu$ l, DGGE loading buffer (20% Sucrose, 0.3% each bromophenol blue and xylene cyanol in autoclaved distilled H<sub>2</sub>O).

DGGE conditions The detailed methodology for preparing the denaturing gradient gel is similar to those described in Myers et al., (1987). The glass plates are prepared, as described using petroleum jelly on the spacers to ensure an adequate seal. The glass plates with spacers in between are held together with large binder clips. These also facilitate leveling of the plates which stand upright when the gel is poured. The gradient maker (SG series, Hoeffer Scientific, San Francisco, CA) is leveled atop a stirrer plate, with the high percentage denaturant well centered over the stirring apparatus. This whole apparatus is placed on a ring stand above the level of the plates. The denaturants are prepared as follows: 150ml volumes: 18.75g polyacrylamide, 0.5 g Bis, formamide is added at 10.8 ml (18%) 21.6 ml (36%), or 31.8 ml (53%) and urea is added at 11.34g (18%), 22.68 g (36%), or 33.42 g (53%), where values in parenthesis represent percent denaturant. The volume is brought to slightly less than 150 ml with distilled H<sub>2</sub>O, 5 grams mixed bed resin are added and the mixture stirred for 30 minutes. Resin is filtered, 3.0 ml of 50x TAE, pH 8.3 (1.89M Tris-Base, 100mM Tris-HCl, 0.57% v/v glacial acetic acid) are added and the volume brought to 150 ml with H<sub>2</sub>O. This mixture is filtered through a 0.2  $\mu$ M filter and kept at 4°C until ready for use. 15.5 ml of the denaturant mixture is pipetted into two separate Erlenmeyer flasks on ice. 100  $\mu$ l of 10% ammonium persulfate (APS) are added to each flask, the solution mixed gently and thoroughly degassed. All components for pouring the gel are gathered including the appropriate size teflon comb, N,N,N',N'-tetramethylethylenediamine (TEMED), and pipettors with tips. A small volume (2 ml) of low percent denaturant is added to the low well and the fluid is forced with thumb

pressure into the tube. The same is done for the high percentage denaturant. The high percentage solution is carefully poured into the high side, 3.5  $\mu$ l TEMED added and the stir plate turned on. The same volume of TEMED is added to the low percent solution, swirled gently and added to the low side of the gel former. Both stopcocks are opened and the gel is allowed to drip through a very small (diameter) section of tubing between the plates to form the gradient. Additional TEMED and APS (1.5  $\mu$ l and 10  $\mu$ l respectively) are added when the gel solution is near the top of the plates to aid in polymerization around the comb. The comb is gently pushed in place and clamped and the gel is allowed to polymerize in the vertical position for two hours. For this present study one millimeter thick, 12.5% polyacrylamide 137.5:1 acrylamide: bisacrylamide 1 gels containing a linear denaturing gradient parallel to DNA migration were used. For the low temperature domain, an 18% to 36% gradient of denaturant was used. For resolving mutants in the high-temperature domain a 36% to 53% gradient of denaturant was established. (100% denaturant is defined as 7 M urea plus 40% v/v Formamide.)

After polymerizing, the gel is prepared for electrophoresis. The bottom spacer and comb are removed, wells immediately flushed with water, and excess petroleum jelly removed with hot water. The plates are clamped into a plexiglass frame and the frame and gel submerged in a rectangular plexiglass tank with 14 l of 1xTAE which is maintained rigorously at 60.0°C. Drastic changes in buffer pH in the upper well are prevented through circulating the TAE buffer from the tank into the smaller well at the top of the gel with a peristaltic pump (Myers et al., 1987).

Immediately before loading the samples, the wells are thoroughly flushed with TAE from the tank to remove urea that migrates out of the gel. The samples are loaded, and the graphite cathodes placed in the top chamber. The gel is run at 150 volts with the peristaltic pump connected to circulate the TAE overnight (approximately 15 hours). Gels were stained with ethidium bromide and bands visualized under sparing long-wavelength UV light. Thin slices of the gel that contained mutant/wild-type (wt) heteroduplexes and suspected mutant/mutant homoduplexes were carefully excised and eluted as described previously. Heteroduplexes are usually contaminated with wild-type DNA and suspect homoduplexes need to be verified. This is accomplished through an additional round of PCR amplification, using 2  $\mu$ l elution mixture as the template, and decreasing the number of cycles to only 20-25 because of purity and high copy number of the template (i.e. purified through the first DGGE) versus genomic DNA. If the template from the elution of the DGGE band was a mutant/mutant homoduplex, it will resolve on the second DGGE as only a single band in the same location as the first. The mutant/wt heteroduplexes, cut from a single band should resolve on the second DGGE as the full complement of bands observed from the first gel (mutant/wt heteroduplexes, wt homoduplexes, and mutant homoduplex if resolved from wt homoduplex). This demonstrates that the band contained mutant and wild-type DNA. However on the second gel there will be much less wt DNA in each band. The strongest intensity bands for each mutant are selected from the second gel, excised, crushed and eluted. Preparation of samples for analysis in the high temperature domain are essentially similar. The first PCR reaction uses the elution supernatant from the excised and crushed bands of the first 8% PAGE. This PCR reaction uses the P3HI primer as the downstream, (3') primer and

the GC5' primer for the upstream GC clamp, which makes up 40 nucleotides of the 57 nucleotide long primer. These primer sequences are given in table 5.1. The high temperature-domain PCR reaction contains 3  $\mu$ g of each of these primers, 2.5 U of Taq, and 0.75 mM of dNTP's. No additional  $MgCl_2$  is added and the template volume is 1  $\mu$ l. The PCR cycle is also slightly different: 96 °C for one minute, fast ramp to 42 °C and 42 °C for one minute, two minute ramp to 72 °C and 30 seconds at 72 °C. Fifteen cycles are performed and a single additional cycle at the same conditions but with a six minute extension time (i.e. 72 °C for 6min) is included to extend all attached primers. These samples are prepared for DGGE in a similar manner but with heteroduplex formation as previously mentioned for GC-clamped molecules. Mutant homoduplexes from either melting domain can be taken directly to single strand template generation for sequencing. However, for heteroduplexes the mutant strand must first be enriched. To accomplish this eluted heteroduplex was diluted 1:50 in two separate PCR reactions, one containing only the upstream primer (P<sub>2</sub> for low temperature and GC5' for high temperature analysis) and the other the appropriate down stream primer. Fifteen PCR cycles were performed (same cycling parameters as for the appropriate high or low first round PCR) to bias the concentration of one strand over the other. The appropriate second primer was added and the reaction subjected to fifteen more cycles. The products of these reactions were prepared for analysis on a denaturing gradient gel (i.e. phenol and chloroform extractions). In order to determine which reaction had been enriched for mutant homoduplex, an equal amount of wild-type DNA was added to each strand bias reaction prior to heteroduplex formation. The wild-type DNA was obtained from tween prepared mutant mixtures that clearly resolved on the denaturing gradient gel with only a wild-type band.

After heteroduplex formation the samples were dried, and run on another denaturing gradient gel in tandem. When the resultant gel was irradiated with UV light, the reaction that resulted in the more pronounced (i.e. bright) heteroduplex bands was the reaction containing the most mutant homoduplex. This difference could be seen as subtle differences in the intensity of the bands or in the complete lack of a band suspected of containing mutant/mutant homoduplexes depending on the efficiency of the biasing reaction.

Resolved homoduplexes, in the supernatant over the crushed denaturing gel band or in the strand-bias PCR reaction are diluted 50-fold into a 50  $\mu$ l PCR reaction containing the appropriate downstream primer ( $P_1$  for low temperature domains and an upstream primer (5'+C, see table 5.1) which contained a universal sequencing primer sequence attached to the 5' end of primer sequence. The GC clamp, required to analyze the sample in the high-temperature domain is no longer needed and is removed through the use of the 5'+C primer. The PCR reaction for the attachment of the sequencing primer used the same concentrations of primer, buffer, dNTP's and Taq,  $MgCl_2$ , and same volume template as in the initial high temperature or low temperature exon 3 PCR respectively. Since the 5'+C primer was designed for mouse exon 3 amplification there is a one base pair mismatch with the human sequence annealing site; therefore annealing temperatures could not be as stringent and a 42 °C temperature was used. For the low temperature domain reaction, the number of cycles was optimized at twelve, with the same cycle parameters as for the first exon 3 PCR. The high temperature domain cycle was the same as the initial  $P_3H_1$ , GC5' reaction (with the extended annealing cycle at the end of fifteen cycles). After the PCR reactions were run, the oil from the reaction was removed and one-tenth of the reaction mixture was run on an 8%

polyacrylamide gel to confirm the presence of product DNA. The remaining 45  $\mu$ l of the reaction was reduced by one-half in volume (in the speed-vac) combined with 5  $\mu$ l loading buffer and the entire sample was run on a 1.5 mm preparative polyacrylamide gel. This gel purified the DNA product for single strand generation by removing the large molar excess of 5'+C primer. If this was not removed, the excess primer would compete with the fluorescent primer for binding to the ssDNA template. Bands from the preparative gel were visualized with ethidium bromide and sparing UV light, excised from the gel and electroeluted.

Electroelution of the DNA was accomplished with an IBI unidirectional electroeluter, (model 46000), using procedures specified by the manufacturer. Briefly, up to six gel slices were placed in the horseshoe wells of the electroeluter and almost covered with running buffer "A" (20mM Tris HCL (pH 8.0), 0.2mM Na<sub>2</sub>EDTA, 5mM NaCl). A salt cushion of 75  $\mu$ l of saturated NH<sub>4</sub>OAc was added to the "V"-shaped column. Electroelution was done at 100 volts for one hour. The salt cushion containing the DNA was pipetted into 1.5 ml eppendorf tubes. The volume was reduced to 0.1 ml and 100% cold EtOH was added to 1.5 ml. The mixture was agitated and the samples were placed at -20 °C overnight (or -70 °C for 30 minutes). The samples were ethanol precipitated, 30 minute cold centrifugation, followed by a 70% EtOH rinse and 95% EtOH rinse. The sample was gently dried in the speed vac (no heat) and resuspended in 10  $\mu$ l H<sub>2</sub>O. One-tenth of the reaction was run on an 8% polyacrylamide gel to estimate the concentration of DNA. The amount of the remaining 9  $\mu$ l resuspended template to use in the single strand DNA reaction was determined empirically, comparing intensity of ethidium bromide stained gels with photographs of amounts used previously to yield successful sequencing reactions. The low temperature melting domain ssDNA PCR

reactions were 50  $\mu$ l in volume with the same buffer, dNTP, Taq and MgCl<sub>2</sub> concentrations. The final concentration of P1 primer used in these reactions was 0.01  $\mu$ g/ml, which is ten times greater than the 0.001  $\mu$ g/ml concentration used in preliminary low temperature PCR reactions. The cycling was fast, to prevent reannealing of the template strands: 94 °C for one minute, fast to 44 °C; then 44 °C for fifteen seconds, and fast to 72 °C which was maintained for 30 seconds. The PCR reactions were removed from the thermal cycler soon after the reaction was complete; the oil was removed and the volume of the reaction brought to 100  $\mu$ l. This was followed by two, 100  $\mu$ l phenol and two, 100  $\mu$ l chloroform extractions. 100  $\mu$ l of 7.5 NH<sub>4</sub>OAc and 500  $\mu$ l, 95% EtOH were added and the tube was inverted to mix. The sample was placed at -70 °C for 20-30 minutes or -20 °C overnight. A twenty minute centrifugation at 4 °C was followed by 2, 200  $\mu$ L, 95% EtOH rinses. The sample was dried gently, resuspended in 30  $\mu$ l H<sub>2</sub>O, and 3  $\mu$ l of the suspension was run with 2  $\mu$ l loading buffer (30% glycerol, 0.6% SDS, 0.6% xylene cyanol, 0.06% BBPh, and 60mM EDTA) on an 8% polyacrylamide gel. Single-strand DNA bands were visualized above the double stranded bands. Sequencing reactions were accomplished using an empirically determined volume of single strand suspension (determined through estimation of ethidium bromide/UV band intensity). Sequencing procedures were identical to those suggested by Applied Biosystems for use with their automated sequencer.

## RESULTS

118 VCM induced hprt<sup>-</sup> mutants from two independent experiments were analyzed by DGGE in both the low and high temperature domains. 3.3% (4 isolates) in the high

temperature domain displayed by the same banding pattern. This pattern was not identified in any of spontaneous mutants analyzed. 8.5% (10 isolates) in the low temperature domain had the same banding pattern, while only 1.2% (1 isolate) of the VCM spontaneous had the same pattern. 196 CEO mutants were analyzed in the low temperature domain and 5.6% (11 isolates) of the CEO mutants resolved with a banding pattern identical to VCM. None of the CEO spontaneous mutants (15 mutants total) and only one of the CAA spontaneous mutants (43 spontaneous mutants total) had this banding pattern.

The VCM "hotspots" in the low- and high-temperature melting domain were sequenced. Four different mutants that gave identical DGGE banding patterns in the low temperature domain were sequenced. The sequence change identified in all of these mutants was a G→T transversion at base pair 292 (see Fig 5.2). Three different mutants that gave identical DGGE high temperature domain banding patterns were also sequenced. The sequence change identified in all three of these mutants was a G→A transition at base pair 197.

## DISCUSSION

We have used the polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and automated dideoxy termination DNA sequencing to analyze *hprt*<sup>-</sup> exon 3 mutants from VCM, CEO, and CAA treated AHH-1 cells expressing cytochrome P450IIE1 activity. Analysis of all mutants expressed within exon 3 was made possible through the PCR attachment of G+C rich sequence (GC-Clamp) during amplification of exon DNA. This clamp allowed for complete analysis in both the high and low temperature melting domains of exon 3.

DGGE analysis of VCM-induced 6TG<sup>r</sup> cells revealed potential mutational "hotspots" in both the high and low temperature domain in hprt exon 3. The "hotspot" in the low temperature domain was identified in 8.5% of the VCM mutants analyzed, and 5.6% of the CEO mutants. In analysis of the high temperature domain, the potential "hotspot" was identified in 3.3% of the VCM, mutants. The analysis of the spontaneous mutants from the three different experiments in the low temperature melting domain indicates that these two mutational "hotspots" are enriched in treated cultures. The fact that the M.F. in treated culture were 2 to 3.5 fold higher than in concurrent controls makes the difference in hotspot percentages even more significant. The mutational events at bp 292 and 197 affected a single amino acid, aspartate and cysteine respectively. The G→T transversion at bp 292 and the G→A transition at bp 197 would result in the insertion of the amino acid tyrosine at each of these locations.

The two groups of VCM induced mutants that were analyzed were from different treatment concentrations from the same experiment. Each of these treatments had similar induced MF and the average background MF was  $5.5 \times 10^{-6}$ . The experiment had two consecutive (equal) delivered doses of 300  $\mu$ M or 500  $\mu$ M from which mutant cells were collected for analysis.

The G→A transition at base pair 197 in the high temperature domain is consistent with the observed miscoding of N<sup>2</sup>,3  $\epsilon$ G seen *in vitro*, and is most likely the responsible adduct. The G→T transversion at base pair 292 in the low temperature melting domain is not consistent with the miscoding potential of N<sup>2</sup>,3  $\epsilon$ G. It may be due rather to the formation of an apurinic site from 7-OEG and the misinsertion of adenine in the daughter strand (Svenberg et al.,

1990). The responsible adduct could also be  $\epsilon$ Cyd, causing a C→A transversion (as demonstrated *in vitro* in the opposite strand, appearing like a G→T transversion. However, the possibility does exist that N<sup>2</sup>,3 $\epsilon$ G has miscoding potential in human cells other than the G:T pairing seen *in vitro* which gives rise to a GC-AT transition (Singer et al., 1987).

# Mutation Analysis Summary

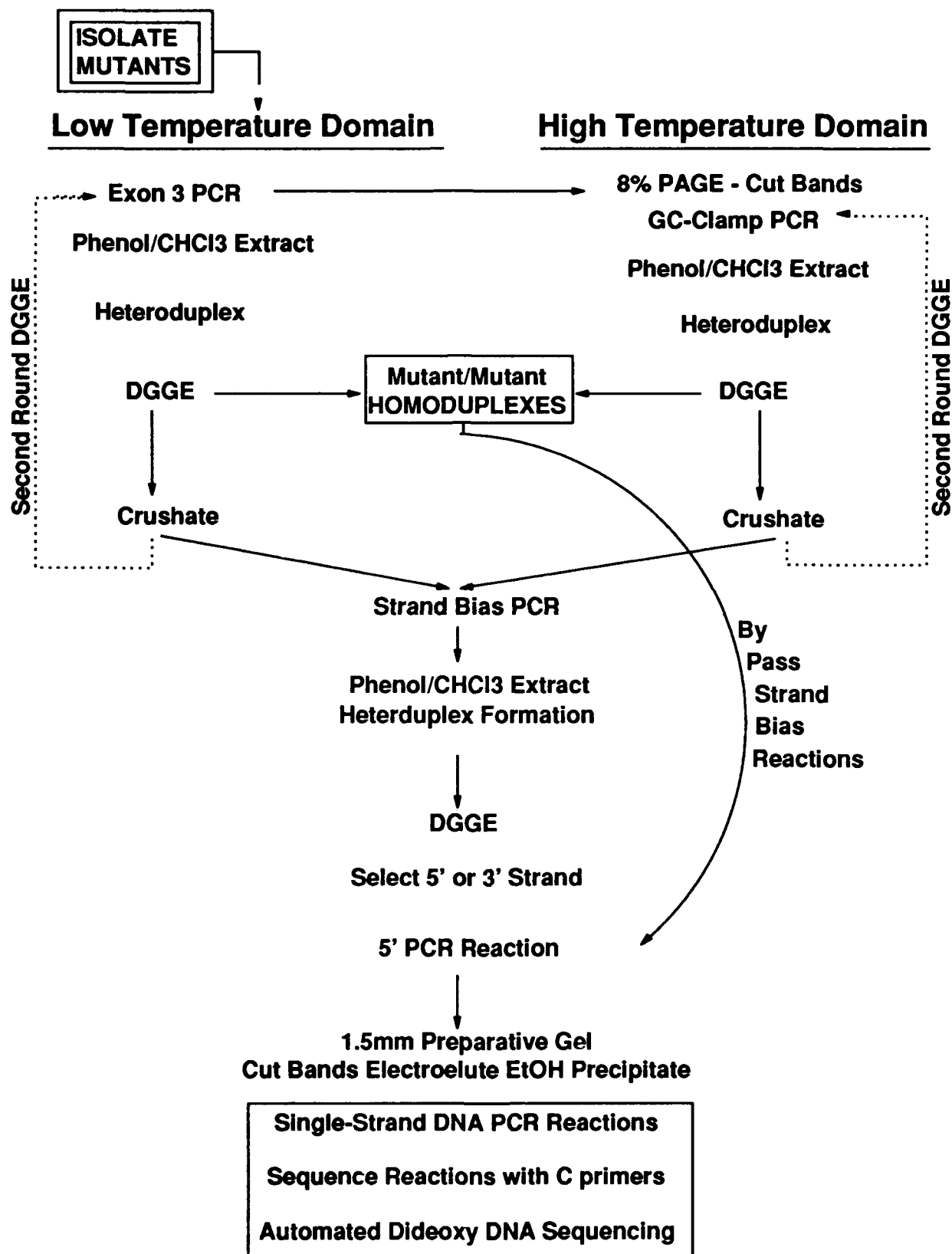


Figure 5.1 See text for details

## Sequences of Primers Used for PCR and Sequencing

Designation of human hprt annealing sites are as published (Edwards, 1990)

| <u>Primer</u>      | <u>Length</u> | <u>Sequence (5' to 3')</u>                                        | <u>Annealing Site</u> |
|--------------------|---------------|-------------------------------------------------------------------|-----------------------|
| PCR Primers:       |               |                                                                   |                       |
| P1                 | 20mer         | CATATATTAAATATACTCAC                                              | 16806-16787           |
| P2                 | 20mer         | TCCTGATTTTATTTCTGTAG                                              | 16583-16602           |
| P3Hi               | 17mer         | TCCAGCAGGTCAGCAAA                                                 | 16691-16707           |
| GC5'mus            | 57mer         | CCGCGGGCGGGCCTCGCGC<br>CGCGGGCCGGGACCCGCGG<br>CCTGATTTTATTTCTATAG | 16583-16602           |
| Sequencing Primer: |               |                                                                   |                       |
| C                  | 22mer         | CAGGAAGACAT<br>TCTTTCCAGTT                                        | -----                 |

Table 5.1

## Sequencing printout

This is a place holder for figure 5.2

## **CHAPTER VI**

### **CONCLUSIONS**

#### **AND**

### **SUMMARY**

We analyzed toxicity, mutagenicity and the mutational spectra in AHH-1 cells with IIE1 metabolizing capabilities following treatment with VCM, CEO and CAA. A dose related toxicity was evident from both CAA and CEO treatments, however, no toxicity was evident with VCM treatments at levels limited by solubility. In this study the relative toxicities of the three compounds were CAA>CEO>>VCM.

A dose related increase in MF was observed for CAA and CEO. In the human cells used in this experiment the mutagenic potency of CAA was found to be almost equal to CEO, an observation not demonstrated in other systems. Mutant fraction response did not monotonically increase for VCM, however, increased concentrations of VCM typically led to an increased MF measured with no toxicity measured at any dose. MF in the VCM experiments reached a plateau at approximately  $5 \times 10^{-6}$ . Comparison of mutation frequency/survival ratios for CEO and CAA at a MF of 5 suggest the majority of mutations induced by VCM must be produced by CEO.

Analysis of these mutants led to the identification of a suspected VCM mutational "hot spot" in both the low and high temperature domains of hprt exon 3. The DNA sequence changes identified in the VCM mutants that exhibited "hot spots" revealed a G→T transversion in bp 292 in the low temperature melting domain and a G→A transversion at bp 197 in the high temperature melting domain. *In vitro* studies suggest the transversion in the high temperature domain was caused by the N<sup>2</sup>,3εG adduct. The transversion identified in the low temperature domain may be the result of an apurinic site from 7-OEG, a C→A transversion in the opposite strand from εCyd, or a yet unidentified change caused by N<sup>2</sup>,3εG.

Two pieces of experimental data obtained from this study support the hypothesis that CEO is the more important mutagenic metabolite of VCM. 1) The comparison of induced MF from VCM, CEO and CAA indicated that only CEO could produce the ratio of mutagenicity and toxicity seen with VCM. 2) One of the mutational "hot spots" identified in VCM was seen at a significant rate in CEO mutants.

During the course of this study, the VCM treatment regime was constantly modified. This reflects the complexities involved in the VCM treatment, not the least of which was optimizing MeOH concentrations. All references to VCM treatments (and the others as well) referred to a delivered dose of the compound. The reliability in delivering a calculated concentration of CAA was high, since CAA is water soluble and treating the cell cultures was relatively straight forward. These conclusions cannot be said about the VCM treatments. As the experimentation progressed with VCM, we found it crucial to keep the VCM stock and dosing solutions very cold. During several early treatments when this was not done, the dose of VCM measured in the syringe was completely expelled by VCM gas coming out of solution. This was a problem that was effectively countered only in the last two treatments. Keeping the VCM solutions cold should also allow different MeOH concentrations to be optimized for the stock solution in future work involving VCM treatments with this cell line, precluding any possibility of MeOH inhibition of IIE1 activity. We analyzed MeOH concentrations at the highest delivered VCM dose and found a tendency for MF to be inversely related to MeOH concentrations in the cell suspension. Comparing the effect of MeOH concentrations on another test substance with known MF may not be appropriate because of the potential for different metabolic efficiencies between the test substances. The

reported level of MeOH inhibition of P450IIE1 was 0.03% (Gentest, 1990). Treatments with CEO were also complex as the pure CEO needed to be diluted in dosing solutions at the appropriate concentrations and administered quickly before it could rearrange to CAA.

One future course of study from this project would be the sequencing of all the VCM, CEO, and CAA mutants that exhibited different DGGE banding patterns to obtain the complete mutational spectra for these three compounds and to validate the mutational "hotspots". Additionally, the direct quantification and comparison of adducts resulting from VCM, CEO, and CAA would be important to characterize the adducts responsible for the observed mutations. Cells from each of the three analyzed treatments were collected, pelleted and frozen directly after treatment for such a purpose.

The analysis of mutation frequency, toxicity and mutational spectra in the AHH-1 cell line with P450IIE1 metabolic activity exposed to VCM, CEO, and CAA gives additional evidence as to the relevant metabolite potentially responsible for the known carcinogenic activity of VCM. This information may be useful in the future development of biomarkers of human VCM exposure.

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