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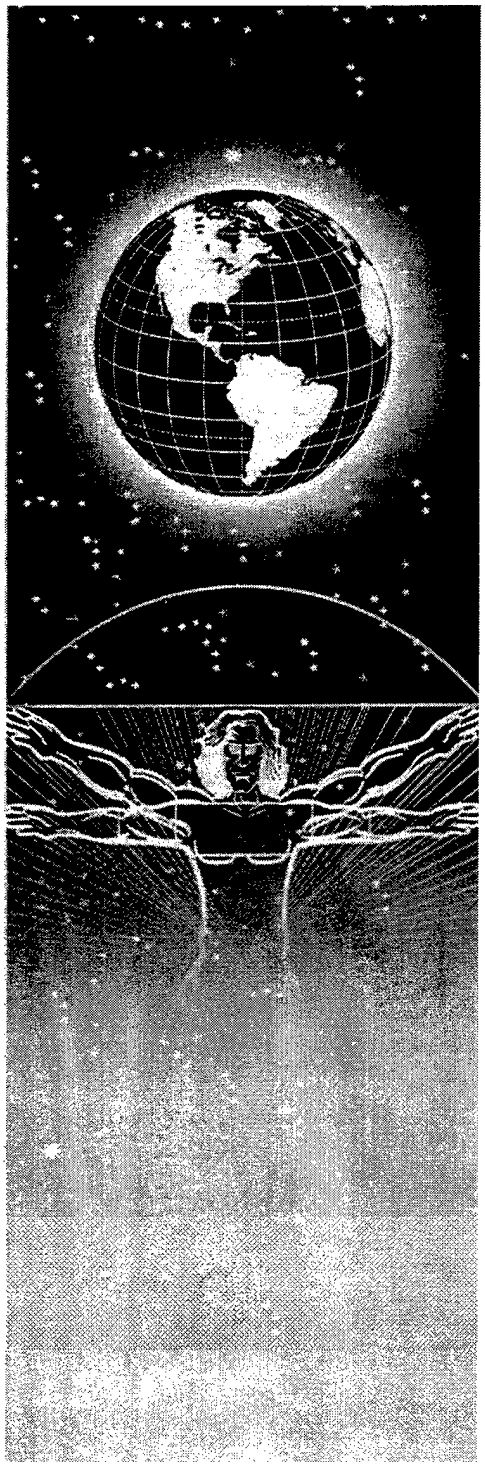

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

CHLORAL HYDRATE AND TRICHLOROETHANOL METABOLISM IN B6C3F1 MOUSE LIVER SLICES

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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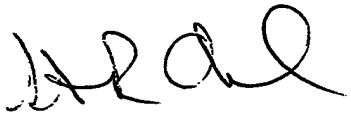

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Chloral hydrate (CH) and trichloroethanol (TCOH) are two major metabolites of the common groundwater pollutant trichloroethylene (TCE). TCE is presently regulated based on its ability to induce hepatocellular carcinomas in B6C3F1 mice, a species and strain specific effect that has been linked to differences in metabolism. Present whole animal exposure estimates of TCE degradation capacity make tissue specific rate calculations problematic. We determined B6C3F1 mouse liver-tissue rate constants for two initial TCE metabolites, chloral hydrate (CH) and trichloroethanol (TCOH), using a precision cut tissue explant system. The generally proposed metabolic scheme of TCE to CH, TCOH and subsequent metabolites was investigated. Metabolic rate constants (Vmax) of 274 nmoles CH removed/mg protein*min-1 and 20 nmoles TCOH removed/mg protein*min-1 were calculated. The results of the study confirm that CH is metabolized to trichloroacetic acid (TCA) and TCOH, and that TCOH can form CH via a "reverse" pathway.

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Preface

This non-peer-reviewed report summarizes the work done to investigate the in vitro metabolism of chloral hydrate and trichloroethanol in mouse liver slices. This research was supported in part by Air Force contract number F41624-96-C-9010 and was funded by the Strategic Environmental Research and Development Program (SERDP), project CU-115. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

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INTRODUCTION

Chloral hydrate (CH) is a byproduct of water chlorination, softwood pulp bleaching, pesticide and plastic manufacture. Although it has been used for many years as a clinical sedative, CH is reported to be mutagenic/clastogenic in some in vitro assays (1). Male B6C3F1 mice exposed to 1 g/L CH in drinking water for 104 weeks had increased liver weights, hepatocellular necrosis and an increased incidence of liver tumors (2-4). Trichloroethanol (TCOH) is a product of CH metabolism via either an NADPH-dependent alcohol dehydrogenase (5) or an NADPH-dependent aldehyde reductase activity (6), followed by uridine diphosphate glucuronyl transferase conversion to TCOH-glucuronide. Like CH, TCOH increases haploid / non-disjunctional diploid frequencies in Aspergillus nidulans (1). To date, TCOH has not been demonstrated to be a rodent hepatocarcinogen.

Both CH and TCOH are intermediate products of the metabolic degradation of trichloroethylene (TCE), a major groundwater contaminant (Figure 1). Significantly, TCE induces hepatocellular carcinoma in mice, but not in rats, following chronic oral gavage (7,8). This effect has been suggested to be strongly linked to production of trichloroacetic and/or dichloroacetic acids (TCA and DCA, respectively) (9-11). Both TCA and DCA are hepatocarcinogens in B6C3F1 mice yet they are consistently negative on standard mutagenesis assay (reviewed in: 12,13). TCA results from the oxidation of CH by aldehyde dehydrogenase, whereas DCA is putatively formed from the reductive dehalogenation of TCA (14,15). Significant species metabolic differences exist. Gorecki, et al. (16) report that in humans, CH is reduced to TCOH which is then oxidized to TCA. However, rats do not form significant amounts of TCA following oral exposure to TCOH (17). Mice form both TCA and DCA from

exposure to the parent TCE, however recent work from this laboratory has called into question the historical estimates of DCA production (18).

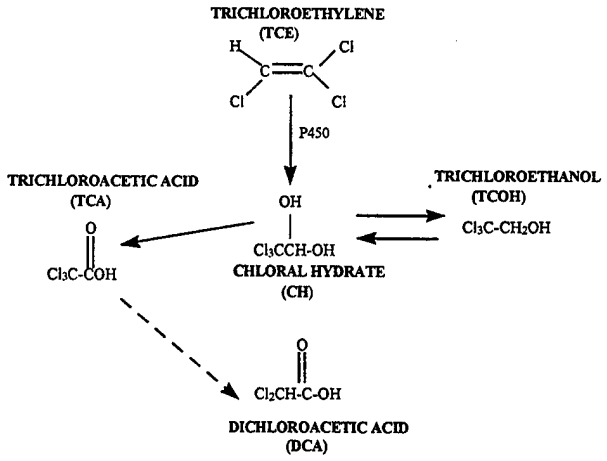

Figure 1. Trichloroethylene metabolism.

Proposed metabolic pathway for trichloroethylene (TCE). Arrows represent known biochemical transformations. Dotted lines indicate putative pathways. P-450 = cytochrome P-450 mediated metabolism. (After ref: 29-31).

As a major contributor to pollution remediation costs, TCE's tumorigenicity must be better understood to form a mechanistically based approach to assessing the risks to human health from environmental exposure. Clearly, one approach is to define the rate and abundance of metabolite production across species. The purpose of this study was to refine the proposed metabolic pathway for TCE in the B6C3F1 mouse liver and to develop tissue-specific estimates of metabolic capacity. We used a precision cut liver slice *in vitro* system to establish metabolic rate estimates for CH and TCOH disappearance and subsequent metabolite formation.

METHODS

Animals: Male B6C3F1 mice were obtained from Charles River Breeding Laboratories, Inc. (Raleigh, NC). Mice were provided with Purina Lab Chow #5008 and softened water *ad libitum*. They were housed 5 mice per plastic cage with hardwood chip bedding and maintained on a 12-hour light/dark cycle at constant temperature (22 ± 1°C) and humidity (40-60%). Animals were euthanized by CO_2 asphyxiation prior to liver excision.

Liver Slice Incubation: All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted and were of analytical grade. Slices were prepared from male B6C3F1 mice in Sacks preservation buffer, pH 7.4 ± 0.1, at 4°C, using a Krumdieck Tissue Slicer (Alabama Research and Development, Munford AL) and incubated under standard conditions as previously described (19-22). Briefly, the slices were loaded on rollers (two slices per roller) and placed in 37°C scintillation vials containing 1.7 mL Waymouths MB 752/1 media (Gibco Formula #78-5107EC, without phenol red), pH 7.4 ± 0.1, supplemented with (g/L): $NaHCO_3$ (1.3), HEPES (2.38), l-glutamine (0.350), gentamycin sulfate, 50 mg/mL (1.5 mL), and 10% Fetal Bovine Serum (Hyclone, Logan UT), and capped with a scintillation vial lid with a ¼" hole for gas exchange. Vials were placed in a Dynamic Roller Culture Incubator (Vitron, Tucson AZ) and gassed with 95% O_2/5% CO_2. After a two hour pre-incubation, rollers were removed from the vials and placed into pre-warmed vials containing the CH (0-5.7 mM) or TCOH (0-6.1 mM) dosed Waymouths media (pH 7.4) and returned to the roller culture incubator.

Viability was assessed for each experiment to assess cytotoxicity of CH and TCOH using standard enzyme and cation markers (19, 23, 24). Lactate dehydrogenase (LDH), aspartate

aminotransferase (AST), and alanine aminotransferase (ALT) levels in culture media and slices were determined using a DuPont acaV for LDH and a Kodak Ektachem Analyzer (model 700XR) for AST and ALT. Acceptable intracellular control enzyme leakage was established at less than 25% of total. Intracellular potassium content was measured using an AVL 982-S Electrolyte Analyzer (Roswell, GA). The acceptable control level of intracellular potassium content was set at greater than 35 mmoles K^+/g wet weight tissue. If the viability control samples did not meet these well accepted literature based standards, the experimental results were discarded.

Sample Analysis: Vials were harvested at multiple time points to 2 hours. Slices were weighed, sonicated in their own media, deactivated (detailed below), and frozen at -20°C pending metabolic analysis. For DCA and TCA analysis, samples were heat killed then derivitized using dimethylsulfate under acidic conditions, followed by hexane extraction as previously described (18, 25). The reaction efficiency (formation of methyl esters) for TCA and DCA was 75% and the hexane extraction efficiency was 67%. Samples for CH and free TCOH analysis were deactivated in 20% lead acetate and extracted into ethyl acetate (16, 18). The ethyl acetate extraction efficiency for CH and free TCOH was 98%. Total TCOH was determined after acid deactivation and hydrolysis using 18 M H_2SO_4 (200 µL sample + 500 µL acid), followed by ethyl acetate extraction. The difference between free TCOH and total TCOH measured was negligible. Metabolites were analyzed using a Hewlett-Packard 5890 II gas chromatograph (Avondale, PA) equipped with an electron capture device (GC-ECD) analysis (HP 5890) with data collected and integrated through a P.E. Nelson Turbochrome 4 data analysis system.

Metabolic Calculations: Metabolic rates of degradation were determined by measuring CH or TCOH removed over time, normalized by liver protein content for each sample. Liver tissue protein was determined using the Pierce BCA protein assay protocol (Rockford, IL) using bovine albumin as a standard. Zero hour samples were routinely prepared to confirm exposure levels. Rates estimates of CH and TCOH loss were calculated for each dose regimen by plotting a best fit line to the linear portion of a curve which described the amount of chemical removed per mg protein versus time (rate = slope) using SigmaPlot® software (Jandel Scientific; San Rafael, CA). Rate estimates were then graphed against CH or TCOH concentration and metabolic rate constants (Km and Vmax) calculated by non-linear Michaelis-Menten analysis using Enzfiter™ software (Biosoft, Ferguson, MO). Rates were also plotted as a double-reciprocal rate versus substrate concentration to verify Km and Vmax using SigmaPlot® software. TCA production from CH was calculated and presented in a similar manner using linear rate estimates.

RESULTS

Viability parameters for all experimental treatments concentrations of both CH and TCOH were well within acceptable limits (data not shown). Chloral hydrate metabolism was saturable with a Km of 1.1 mM CH and Vmax of 274 nmole CH removed/mg protein*min^{-1} (Figure 2). Comparable values were determined using double-reciprocal plot analysis (inset graph, Figure 2). Trichloroacetic acid production rate increased linearly with increasing CH concentrations up to 5.70 mM (Figures 3), with a maximum TCA production of 30 pmoles TCA produced/mg protein*min^{-1}. TCOH production appeared to plateau after the 1.84 mM CH dose,

with a maximum saturable TCOH production of 154 nmoles TCOH produced/mg protein*min^{-1} (Figure 4). DCA was not detected in any sample from CH exposure over the 0.137 mM to 5.70 mM experimental range.

Figure 2

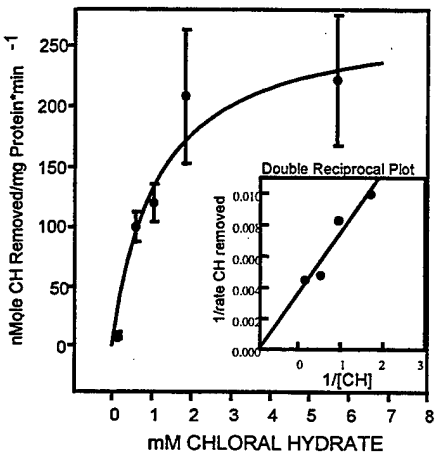

Figure 3

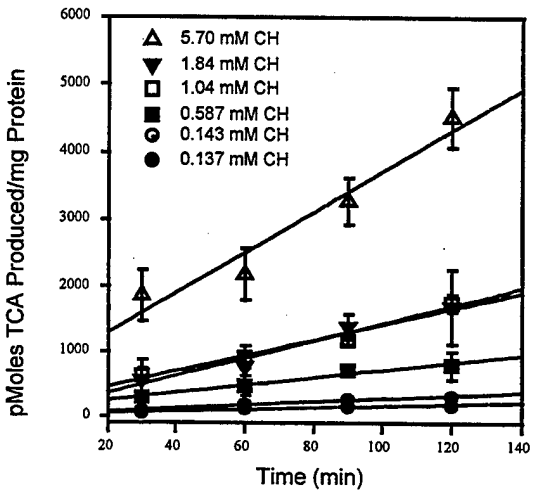

Figure 2. Chloral hydrate metabolism.

Rates of CH metabolized, expressed as nmoles removed/ mg protein * min^{-1} were plotted against concentration. Symbols represent best fit estimate ± S.E., n=15 data points as described in methods. Inset graph presents the double reciprocal plot of CH rate data (r^2 = 0.912). Michaelis-Menten constants: Km = 1.1 mM CH, Vmax = 274 nmole CH removed/mg protein*min^{-1}.

Figure 3. TCA produced from chloral hydrate metabolism.

Symbols represent pmoles of TCA produced per mg protein versus time for each concentration of CH, mean ± S.D., n=3. Rates of TCA formation were determined from the linear portion of each plot and used to determine metabolic rate constants. Similar calculations were performed for TCOH.

Free TCOH metabolism resulted in a Km of 0.14 mM TCOH and Vmax of 19.9 nmole TCOH removed/mg protein*min^{-1} (Figure 5). Formation of metabolites from TCOH degradation is presented in Figure 6. Trichloroacetic acid production reached its maximum saturable rate of 1.4 pmoles TCA produced/mg protein*min^{-1} at 2.6 mM TCOH treatment (data not shown). The rate of chloral hydrate production remained steady at about 0.13 nmoles CH produced/mg protein*min^{-1}. Dichloroacetic acid was not detected from 0 - 6.1 mM TCOH exposure.

Figure 4

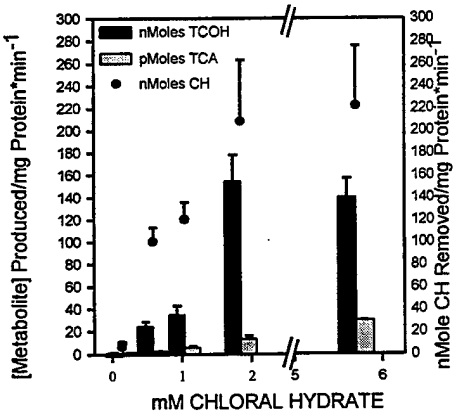

Figure 5

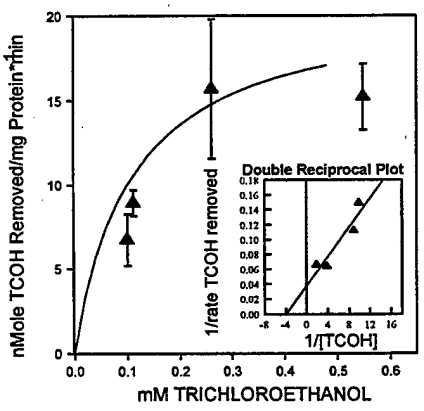

Figure 4. TCA and TCOH produced from CH metabolism.

Bar symbols represent production rates TCA and TCOH produced from CH metabolism. Amount of CH removed is also presented as circle symbols related to right y-axis. Each is the linear rate estimate ± S.E., of n=15 samples.

Figure 5. Trichloroethanol metabolism.

Rates of TCOH metabolized per mg protein*min^{-1}, mean ± S.E., n=15. Inset graph presents the double reciprocal plot of TCOH data (r^2 = 0.879). Michaelis-Menten constants: Km = 0.14 mM TCOH, Vmax = 19.9 nmoles TCOH removed/mg protein min^{-1}.

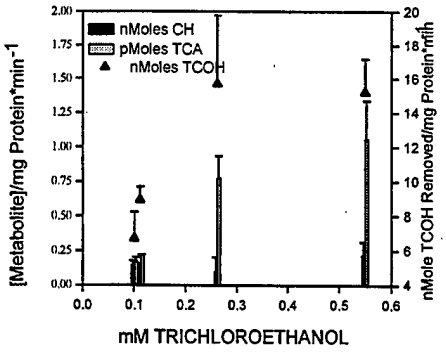

Figure 6. Chloral hydrate and TCA produced from TCOH metabolism.

Bar symbols represent rate of TCA and CH produced from TCOH metabolism. Amount of TCOH removed is also presented as the triangle symbol, referenced to right y-axis annotation). Data are presented as mean ± S.E., n=15.

DISCUSSION

Metabolic rate estimates in B6C3F1 male mouse liver tissue for CH, TCOH degradation and production of metabolites were determined using the precision cut liver slice *in vitro* system. We detected no DCA formation from the metabolism of TCOH or CH in mouse liver slices. This may indicate that DCA is not formed, or that any DCA formed is rapidly metabolized (18). Of the metabolites measured, TCOH was the major product of CH degradation in this system. It appears in quantities at least four orders of magnitude greater than does TCA at every CH concentration tested. The CH to TCOH path may be saturable, as evidenced in the dose response

seen in figure 4. By comparison, TCA production did not plateau over the 0-5.70 mM CH range, although CH removal clearly had. At the 5.70 mM CH exposure, TCA production was 30.02 pmoles TCA produced/mg protein min^{-1}.

Metabolism of TCOH does produce CH, presumably by the "reverse" metabolism of TCOH as suggested in Figure 1. The rate of conversion appears to be independent of dose in the range of TCOH concentrations we used. A fairly consistent rate of CH formation was observed that was at least three orders of magnitude greater than the rates of formation for TCA. However, TCA production rates began to plateau at TCOH concentrations greater than 2.6 mM. In addition, the maximum observed rate of TCA production from TCOH was at least 20-fold lower than was the rate from CH, and the later pathway had not plateaued. This suggests that the major contributor to TCA formation is via oxidation of chloral hydrate. If literature suggestions that TCA is largely responsible for the tumorigenicity of TCE are correct (10), our results suggest that reaction rates of CH to TCA would be critical for interspecies extrapolation.

TABLE 1. COMPARISON OF TCA:TCOH RATIOS IN RAT PERFUSED LIVER AND MOUSE LIVER SLICES. Metabolite values represent amount of TCA or TCOH, in pmoles per mg liver tissue protein, following a two hour exposure to the indicated concentrations of CH or TCOH in each system.

	Perfused Rat Liver Biliary Excretion		**Mouse Liver Slices**	
	CH (0.15mM)	TCOH (0.17mM)	CH (0.14mM)	TCOH (0.11mM)
TCA (pmoles)	37	2.4	56.5	26.2
TCOH (pmoles)	222	296	264	19400
RATIO TCA:TCOH	**0.17**	**0.008**	**0.21**	**0.0014**

Modified from Kawamoto, et al., 1987 (26). Data conversion based on TCA (F.W. = 163.4 g/mole), TCOH (F.W. = 149.4 g/mole) and 0.15 mg protein/ mg liver tissue after Pravecek and Channel, 1995 (24).

Understanding such species differences in metabolism will help us to understand the species-specific tumorigenicity of TCE. As mentioned previously, whole animal exposure estimates lack the ability to ascribe metabolic capacity to a specific tissue, such as liver. When employed in physiologically based pharmacokinetic (PBPK) computer models, metabolic rates are estimated for the target tissue as illustrated in Table 1 which presents data from metabolism of TCOH and CH in rat perfused liver as reported by Kawamoto, et al., 1987 (26). The ratio of TCA:TCOH resulting from a two hour exposure to equivalent concentrations of CH was in good agreement rat vs. mouse liver slice in our study. Similarly, the TCA:TCOH ratios from TCOH exposure were in fair agreement between the two species. However, observation of the absolute molar amounts of TCA or TCOH after two hours is interesting. Mice appear to produce significantly greater amounts of each metabolite following CH exposure. However, mice appear to be less able to clear TCOH than do rats, a factor that may become significant under chronic low-dose exposure conditions. Some have suggested that enterohepatic recirculation may contribute to sustained levels of both TCOH and TCA (27). This may force an incremental increase in TCA burden from the TCOH-CH-TCA pathway. Neither perfused live nor liver slice systems would be able to address this issue directly since each lacks functional enterohepatic recirculation. However, if sustained TCA correlate to the observed pattern of liver tumor formation (28), our data are consistent with one mechanism of sustaining those levels.

We have demonstrated tissue specific metabolism of CH and TCOH. The results of this study provide added verification of the putative TCE metabolic pathway presented in Figure 1. These liver-tissue specific kinetic parameters may be incorporated in the liver compartment of a physiologically based pharmacokinetic (PB-PK) model for TCE metabolism to refine the model

predictions, which at present are based on fitted estimates derived from whole animal exposures. The liver slice in vitro system has provided an excellent tool in this instance to study metabolic and mechanistic problems of toxicity.

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