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TRICHLOROETHYLENE: METABOLISM AND OTHER BIOLOGICAL DETERMINANTS OF MOUSE LIVER TUMORS

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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.

FOR THE COMMANDER

TERRY A. CHILDRESS, Lt Col, USAF, BSC
Director, Toxicology Division
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Trichloroethylene (TCE) is one of the most commonly found groundwater contaminants at DoD facilities due to its widespread use in degreasing and as a solvent in other operations. Trichloroethylene has been the subject of extensive study including a large number of lifetime studies in laboratory rodents exposed by inhalation and oil gavage (oral bolus dosing). Several toxicities have been identified, including both noncarcinogenic and carcinogenic effects. This review considers both pharmacokinetic and pharmacodynamic factors that could act as determinants of TCE carcinogenesis. The implications of these data are to suggest that alternatives to the current risk assessment for TCE-induced cancer should be seriously considered.

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PREFACE

This report describes the results of an extensive review of the literature available on trichloroethylene, a chemical of concern to the U.S. Air Force for the health risks associated with occupational exposure and environmental contamination. The literature review was a collaborative effort conducted by ManTech Environmental Technology, Inc., Toxic Hazards Research Unit, located at Wright-Patterson Air Force Base, and by the Occupational and Environmental Health Directorate, Toxicology Division. That part of the research by ManTech Environmental was conducted under Department of the Air Force Contract No. F33615-90-C-0532 (Study Nos. F23, F32, and F35). That part of the research by the Toxicology Division was conducted under Project or Work Unit No. 4223OT01. Funding was provided to the Toxicology Division by the Strategic Environmental Research and Development Program (SERDP). This effort began in July 1993 and was completed in April 1994. Lt Col Terry A. Childress, Director of the Toxicology Division, served as Contract Technical Monitor.

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ABBREVIATIONS

^{14}C	Carbon-14
Alb	Albumin
AHF	Altered hepatic foci
ALCDH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALRED	Aldehyde reductase
BEI	Biological exposure index
BUN	Blood urea nitrogen
CBI	Covalent binding index
CCl_4	Carbon tetrachloride
CH	Chloral hydrate
CO_2	Carbon dioxide
DCA	Dichloroacetate
DCA	Dichloroacetic acid
DCE	1,1-Dichloroethylene
DCVC	Dichlorovinylcysteine
DEN	*N, N*-Diethylnitrosamine
EA	Ethyl acrylate
F-344	Fischer 344
G6Pase	Glucose 6-phosphate dehydrogenase
GGT	γ-Glutamyltranspeptidase
GI	Gastrointestinal
GSH	Glutathione
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HCs	Hepatocellular carcinomas
HNs	Hyperplastic nodules
HPLs	Hepatic proliferative lesions
i.p.	Intraperitoneal
ICC	Intrinsic clearance capacity
LD_{50}	Median lethal dose
MW	Molecular weight

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
p.o.	Per os
P-450	Cytochrome P450
PB	Phenobarbital
PBPK	Physiologically based pharmacokinetic
PCE	Perchlorethylene (tetrachloroethylene)
PcoA	Palmitoyl-coenzyme A oxidation
PPAR	Peroxisome proliferator activated receptor
SSBs	Single strand breaks
TBARS	Thiobarbituric acid-reactive substances
TCA	Trichloroacetate
TCE	Trichloroethylene
TCOG	Trichloroethanol-glucuronide
TCOH	Trichloroethanol
TFOH	2,2,2-Trifluoroethanol
TLV	Threshold limit value
UDP	Uridine diphospho glucuronic acid
UDPGT	Uridine diphospho glucuronyl transferase
USAF	U.S. Air Force
VOC	Volatile organic compound

SECTION 1

INTRODUCTION

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Trichloroethylene (TCE) is one of the most commonly found groundwater contaminants at Department of Defense facilities due to its widespread use in degreasing and as a solvent in other operations. Trichloroethylene has been the subject of extensive study, including a large number of lifetime studies in laboratory rodents exposed by inhalation and oil gavage (oral bolus dosing). Several toxicities have been identified, including both noncarcinogenic and carcinogenic effects. This review considers both pharmacokinetic and pharmacodynamic factors that could act as determinants of TCE carcinogenesis. The implications of these data suggest that alternatives to the current risk assessment for TCE-induced cancer should be seriously considered.

The cancers seen have been highly species- (rat versus mouse), strain-, and route-dependent. Recent years have seen increasing focus on metabolites of TCE, particularly the minor chloroacids. Like the parent, trichloroacetate (TCA) and dichloroacetate (DCA) have been shown to cause mouse liver tumors, leading to the hypothesis that they could be the ultimate carcinogens. Chloral hydrate (the product formed by cytochrome P450 [P-450] metabolism of TCE) also causes mouse liver tumors, but again this may arise from subsequent metabolism to TCA and DCA. Other tumors seen in multiple studies have included lung tumors in mice exposed to TCE by inhalation and kidney tumors in rats exposed to TCE by gavage. Several tumors have been reported in only a single study.

Trichloroethylene does not appear to be genotoxic, nor do its metabolites, TCA and DCA. A minor glutathione conjugation pathway does lead to mutagenic and cytotoxic metabolites formed from 1,2-dichlorovinylcysteine. It is hypothesized that this may be important to the kidney tumors that are found at a very low incidence (0 to 10%) in exposed rats while being extremely rarely seen in control animals. However, this area is not considered in depth because it does not appear to be significant for the liver tumors, whereas TCA and DCA clearly are significant.

Although there is a general sense that TCE must act through a nongenotoxic mechanism, there is no clear hypothesis for what that mechanism is. Several possibilities are discussed in this review. Peroxisome proliferation may play a role in TCA carcinogenesis, but is not required for DCA's effects

based upon recent work (A. DeAngelo, personal communication). Peroxisomal proliferation does appear to increase the multiplicity of tumors found with high DCA exposures, suggesting that oxidative damage may play a role. Changes in cell cycling manifested either as altered apoptosis (programmed cell death) or altered proliferation may also play a role. However, TCE appears not to fit easily into the widely discussed mechanism involving cell death (necrosis) and a reparative hyperplastic response by the liver. There are some indications of necrosis, but nothing as extensive as that seen with other chemicals such as chloroform. This mechanism may, however, be significant in explaining the rat kidney tumors because extensive necrosis is seen in kidneys.

Estimates of risk from exposure to TCE have, until now, used the default U.S. Environmental Protection Agency methodology. The linearized multistage model was originally developed from assumptions about chemicals acting as radiomimetic agents for use in analyzing cancer epidemiology studies. As it became apparent that some cancer-causing chemicals may act through nongenotoxic pathways, the use of this model for risk assessment has been increasingly questioned.

This review considers what is known about the metabolism and biological effects of TCE to establish a basis for evaluating potential methodologies for estimating risks. Because risk estimates have been based upon the liver tumors, this tumor was the focus of the literature reviews reported herein. A complete reanalysis of the risks of TCE exposure would, of course, have to consider other tumors that are less completely addressed in this report. The evidence strongly suggests that the current assessment of TCE-induced cancer risks is not consistant with the biology of the compound. This indicates that a research and development effort to propose alternatives would be highly beneficial.

SECTION 2

TRICHLOROETHYLENE METABOLISM

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INTRODUCTION

The goal of this review is to discuss the relevant factors involved in the conversion of trichloroethylene (TCE) to its metabolites *in vivo*. An important aspect of the assessment involves experiments performed *in vitro*. The distribution of pertinent drug metabolizing enzymes between the subcellular compartments of cytosol, microsomes, and mitochondria will be reviewed. This discussion provides an overview of the metabolic scheme regarding the identified and postulated metabolites as well as the enzymology involved.

The initial rate-limiting step in TCE metabolism is microsomally catalyzed, reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent conversion of TCE to chloral hydrate (CH) (Byington and Leibman, 1965; Ikeda et al., 1980). This step occurs within the active site of P-450 (particularly IIE1). Chloral hydrate is metabolized to trichloroethanol (TCOH) and trichloroacetate (TCA). The TCOH is conjugated to form TCOH-glucuronide (TCOG). Dichloroacetate (DCA) is also a minor metabolite. Although the exact origin of DCA has not been conclusively demonstrated, Dekant et al. (1986) hypothesized that saturation of metabolism to trichloroacetic acid (TCA) and TCOH may result in alternate steps in the pathway that result in the formation of DCA. It has been postulated that metabolites of DCA enter the amino acid pathways (Stevens et al., 1992). It has been has hypothesized that conjugation of TCE with glutathione (GSH) may account for a genotoxic and nephrotoxic metabolite, dichlorovinylcysteine (DCVC). This might occur when the P-450 pathway becomes saturated. These steps are reviewed in detail in subsequent sections.

COMPARATIVE METABOLISM: SPECIES, ABSORPTION ROUTES, AND ORGAN SPECIFICITIES

As with any metabolism study, most of the metabolic activity of the animal is assumed to reside in the liver. Trichloroethylene is no exception and, as such, most of the metabolism discussed will have been studied in hepatic tissue. Hobara et al. (1986) demonstrated that extrahepatic metabolism of CH in the dog (using liver bypass hemodynamics) accounts for less than 20% of the total metabolism of CH. In the liver of experimental animals, the metabolism of CH yields either TCOH or TCA, both of which are at least primarily cytosolic reactions. The metabolism of CH to TCOH is catalyzed by aldehyde reductase, whereas TCA is the product of CH metabolism by aldehyde

dehydrogenase.

In the isolated perfused rodent lung, Dalbey and Bingham (1978) have shown that up to 30% of a TCE dose may be converted to TCOH, but that CH and TCA were not observed. Data from Larson and Bull (1992a) indicate that gastrointestinal (GI) uptake of a dose of TCE administered in Tween 80 is slower in rats than in mice, based on time to peak blood levels. These data may impact metabolism/elimination results across species following exposures via the GI route. It is apparent that the liver is not the only site of metabolism.

Larson and Bull (1992a,b) and Green and Prout (1985) separately have hypothesized that species differences in metabolism of TCE may account for species differences in susceptibility to carcinogenesis produced by TCE. Both laboratories have reported no major species-related qualitative differences in metabolism, but both have identified higher rates of metabolism in the mice examined. Such higher rates result in significantly higher blood levels of potentially toxic metabolites, which also occur at earlier times than in the rats. Trichloroethylene was dosed per os (p.o.) at 1.5 to 23 mmol/kg and blood areas under the curve were calculated for TCA and DCA in the Sprague-Dawley rat and B6C3F$_1$ mouse. Peak blood concentrations of TCOH, TCA, and TCE were much higher in mice than rats, while urinary elimination of TCA and TCOH (percent of dose) were comparable. Dichloracetic acid was detected in mouse blood, but not in rat blood. It is thought that the net metabolism of TCE to TCA and TCOH is similar in rats and mice, but that rats do not seem to form DCA. Levels of carcinogenic acid metabolites (TCA and DCA) in circulating blood are similar in rodents exposed to carcinogenic doses of the metabolites themselves and rodents exposed to carcinogenic doses of parent TCE.

The distribution and elimination of TCE in the rat have been assessed. Physiologically based pharmacokinetic models exist. The elimination of TCE occurs thus: approximately 35% is eliminated unchanged from alveolar ventilation, and urinary elimination of metabolites accounts for 49% of the TCE dose. The urinary metabolites identified are TCOH/TCOG, TCA, DCA, and oxalic acid; the TCOH forms account for >80% of the urinary metabolites (40% of the TCE dose) (Dekant and Henschler, 1983). When administered as ^{14}C-TCE, the urinary metabolites account for 41% of the rat's dose and 76% of the mouse's dose. Enterohepatic circulation of TCA has been postulated, with a fecal route of elimination.

Of a 200 mg/kg dose of TCE, mice eliminate 11% of the dose as parent TCE in breath in 72 h, as opposed to rats' elimination of 52% of the dose in breath (Dekant, et al., 1984). The primary

metabolites identified in urine of ^{14}C-TCE-exposed mice are TCOH (free + conjugate) 94% and only traces of DCA and TCA (as compared to DCA representing 2%, TCA 15%, unconjugated TCOH 12%, and TCOG 62% of the total ^{14}C-TCE dose in rat urine) (Dekant et al., 1984). Rats eliminate much more TCA in urine than do mice. Rats eliminate 2% of a TCE dose as DCA in urine, whereas mice do not seem to eliminate DCA in urine.

The metabolism of TCE in humans and experimental animals is governed by the concentration of TCE present. The concentration of TCE may have a significant impact on the rate at which TCE is metabolized. Inhibition of metabolism at high doses may be possible. Therefore, the rates at which TCE is absorbed via the several common exposure routes becomes important. If the exposure is via inhalation, TCE may be diluted into systemic blood before entering the hepatic compartment. If the exposure is via the gut, portal circulation will carry 100% of an absorbed dose directly to the liver before entering the systemic circulation.

Numerous reports indicate that humans exposed to TCE metabolize between 40 and 90% of an absorbed dose. Inhalation data were collected from humans exposed to TCE and correlated with the urinary elimination of total trichloro compounds (Nomiyama and Nomiyama, 1979). These data indicate that in the human, metabolism is saturated above 315 ppm for 3 h (an estimated absorbed dose of 25 mg/kg). Their data also indicate that a single pass through the liver accounts for total removal of TCE from blood. If this is true for rodents, then special care should be given to the determination of the rates at which TCE is absorbed from the gut. Data in rats and mice indicate that dose-dependent metabolism occurs and that saturation in the mouse occurs at approximately 2000 mg/kg and in the rat at 500 to 1000 mg/kg. Inhalation kinetics in the Fischer 344 (F-344) rat indicate saturation point at 65 ppm (Filser and Bolt, 1979) and 1000 ppm. The rate of TCE metabolism at V_{max} was calculated to be 185 mmol/kg/h. Data from Prout et al. (1985) indicate that TCE is handled quantitatively (at least) differently in rats than in mice. In Davidson's review (Davidson and Beliles, 1991), the difference is shown plainly. Rats metabolize only 400 mg TCE/kg compared to mice metabolizing 1700 mg TCE/kg at 2000 ppm TCE.

METABOLIC SCHEME

Trichloroethylene (possibly under conditions which saturate the P-450-mediated metabolism) is enzymatically conjugated with GSH. This may occur in both the liver and the kidney. The GSH conjugate is metabolized via *γ*-glutamyl transpeptidase and aminopeptidase to yield (finally) the cysteine conjugate. The cysteine conjugate then may be metabolized via cysteine beta-lyase or *N*-acetyl transferase to yield DCVC or the mercapturic acid, respectively.

In most of the several proposed metabolic pathways, TCE is converted to the epoxide, and the epoxide directly yields chloral (trichloro aldehyde) and CH. Ikeda et al. (1980) report a Km for the conversion of TCE to CH in rat liver microsomes of 1.75 mM. Dichloroacetyl chloride and *N*-(hydroxyacetyl)aminoethanol are also derived from the epoxide intermediate. The path that involves the initial formation of dichloroacetyl chloride is the mechanism through which DCA is hypothesized to be produced. Dichloroacetic acid is thought to break down to yield glyoxylic acid, which then gives rise to oxalic acid and glycolic acid. Carbon dioxide (CO_2) has been identified as a metabolite of DCA.

Chloral hydrate gives rise to both TCA and TCOH; TCA is reported to arise from CH via the activity of aldehyde dehydrogenase (ALDH, EC 1.2.1.3) in cytosol using nicotinamide adenine dinucleotide (NAD) as cofactor. The mitochondrial conversion of CH to TCA has been examined by Ikeda et al. (1980) using a crude ALDH preparation and cofactors. These authors have demonstrated that NAD but not NADP was required for CH oxidation to TCA, and the apparent Km with the mitochondrial system is 63 mM for CH. No data on the kinetic constants for the cytosolic conversion of CH to TCA have been found.

The TCOH formation from CH was previously thought to arise from metabolism catalyzed by alcohol dehydrogenase (ALCDH, EC 1.1.1.1) using NADH as cofactor, but recent evidence and arguments have implicated aldehyde reductase (ALRED, EC 1.1.1.2) using NADPH as cofactor to be the mechanism by which TCOH is generated. Ikeda et al. (1980) report an apparent Km for the conversion of CH to TCOH of 6 mM. Once formed, TCOH undergoes conjugation with uridine diphospho glucuronic acid (UDP) via uridine diphospho glucuronyl transferase (UDPGT). Recent data (Stenner et al., 1994) seem to implicate the glucuronide in a significant amount of enterohepatic recirculation, which may contribute to prolonged elimination times for TCE metabolites.

Alternately to the formation of TCA and TCOH, CH can be oxidized (twice) to give rise to glyoxylic acid chloride, which is further oxidized to produce oxalic acid. Dichloroacetyl chloride loses a chloride and is oxidized to produce DCA. The trichloro-epoxide loses all chlorines through reaction with phosphatidyl groups and is reduced to yield *N*-(hydroxyacetyl) aminoethanol. The oxide metabolite of TCE rearranges thermally to yield dichloroacetyl chloride, and TCE-oxirane yields chloral in the presence of Lewis acids (Fe^{+3}). Saturation of the Lewis-catalyzed pathway may yield higher levels of dichloroacetyl chloride as proposed in Hathway's spillover hypothesis (Hathway, 1980).

RESEARCH FINDINGS *IN VIVO*

In experiments performed by Dekant et al. (1986), female Wistar rats and NMRI mice were exposed

p.o. to ^{14}C-TCE at 200, 20, and 2 mg/kg in corn oil (2.5 g/kg). Ninety percent of the administered radiolabel was excreted in the first 72 h following administration. The ^{14}C-TCE and ^{14}C-CO_2 only were exhaled in breath, and TCE elimination in breath was virtually nonexistent after the initial 24 h following administration. Urinary elimination accounted for roughly the same proportion of dose (approximately 90% of a 2 and 20 mg/kg dose) in both species, but the metabolite profile in urine of the two species differed: urine of mice contained much less (0.1 vs. 6.5%) free TCOH and more conjugated TCOH (86 vs. 70%) than rat urine. Mice eliminated less radiolabel in urine as DCA (0.3 vs. 1.3%) and oxalic acid (0.9 vs. 1.8%) than did the rats. The elimination of TCA in urine was nearly identical in the species administered the same dose (20 mg/kg). *N*-(hydroxyacetyl) aminoethanol accounted for 5 to 7% of the urinary radiolabel in the species. Saturation of the metabolic pathway seems to occur in the rat at doses below 200 mg/kg, whereas these doses do not induce saturation in the mouse, as evidenced by the percentage of TCE exhaled unchanged in breath (0 to 24 h). At a dose of 200 mg/kg, urinary elimination accounted for 77% of the dose in the mouse and 38% of the dose in the rat.

Buben and O'Flaherty, (1985) examined the metabolism and hepatotoxicity of TCE in male Swiss-Cox mice. Mice were exposed via corn oil gavage to TCE at 0 to 3200 mg/kg/day for 6 weeks, and the metabolism of TCE was monitored by following the urinary elimination of TCOH and TCA. These data indicated saturation of metabolism at 1600 mg/kg/day. The metabolism of TCE was correlated with hepatotoxicity through the assessment of several indices of liver damage.

Larson and Bull (1992a) investigated species-related differences in the *in vivo* metabolism of TCE. Trichloroethylene administration p.o. in Tween-80 resulted in TCA, TCOH, and TCE in blood of rats and mice, but DCA was detectable only in the blood of the mice that received the highest TCE dose. There were significant differences in the blood profiles of metabolites. Time to peak TCE levels were markedly shorter in mice (15 min) than ir rats (2 h). These results may reflect possible species-specific gastric emptying times, different kinetic or physical parameters that influence TCE uptake from the GI system, or possible differences in enterohepatic circulation (Stenner et al., 1994).

Nomiyama and Nomiyama (1979) have examined the urinary fate of TCE metabolites. Rats were administered 10 mg TCE, CH, TCA, or TCOH intraperitoneally (i.p.) in saline. The urinary elimination of TCE and metabolites was examined over the following 3 days; total trichloro compounds (TCA and free and conjugated TCOH) were determined, and (total) TCOH was calculated by subtracting TCA from total trichloro compounds. Although CH was administered to the animals, no detection of CH in urine samples was reported. No discussion of the possibility of CH contributing to "total trichloro

compounds" was made. It was demonstrated that urinary elimination of trichloro compounds over 3 days accounted for 32 to 37% of the dose for TCE, CH, and TCA, but accounted for 27% of the TCOH dose. One percent of the TCA dose was eliminated as (total) TCOH; 1% of the TCOH dose was eliminated as TCA; and of the CH dose, 1.6% was eliminated as TCA and 30% was eliminated as (total) TCOH. Of the TCE dose, 2.6% was eliminated as TCA and 32% was eliminated as (total) TCOH. Metabolites other than TCA, total TCOH, and total trichloro compounds were not reported. These data indicate that, to a limited degree, the *in vivo* interconversion of TCA and TCOH is possible. Both TCE and CH *in vivo* were preferentially metabolized to TCOH.

Ogata et al. (1979) have exposed female dd mice and Wistar rats i.p. to 1 mmol (131 mg) TCE/kg in olive oil. Results indicate that urinary elimination of metabolites over 5 days accounted for 20% of the dose in mice and 15% in rats. Of this, 79% of the (total) TCOH eliminated by mice was eliminated in 0 to 24 h, whereas 92% of the (total) TCOH was eliminated in 0 to 24 h in the rat. Mice eliminated 1.67% of the dose as TCA over 5 days, whereas rats eliminated 4.7% of their TCE dose as TCA over the same period. These results indicate that there may not be significant species-related differences in the total urinary elimination of TCE metabolites, but that there are significant differences in the quantity of TCA formed. It is unfortunate that experiments exploring species-related differences have not been performed with the same strains of animals.

Larson and Bull (1992b) have investigated the metabolism of DCA and TCA in rats and mice. It was shown that DCA produces more severe lesions in the liver than does TCA. Male F-344 and male B6C3F$_1$ mice were administered TCA and DCA p.o. in water. Urinary elimination of parent compound in both species could account for more than 50% of TCA dose, but could account for only 2% of a DCA dose. Blood TCA concentration versus time curves were very similar in rats and mice; the DCA concentration versus time curve in rats was much greater than that in mice, whether DCA resulted from administration of parent compound or arose as a consequence of TCA metabolism. Dichloroacetic acid was found in TCA-treated animals and chloroacetic acid was found in animals treated with DCA, implying radical production from dechlorination *in vivo*. Carbon dioxide in breath accounted for 24 to 30% of DCA dose, but only 6 to 8% of TCA dose in the rat. The majority of the CO_2 following DCA dose was recovered in less than 2 h, whereas recovery of CO_2 from TCA occurred over 48 h. Comparing CO_2 exhaled following DCA dosing (100 mg/kg), mice eliminated only 2% of their DCA dose as exhaled CO_2, whereas rats eliminated more than 10-fold that level. Data on the combined elimination of ^{14}C-metabolites from DCA reveal that the mouse may be less able to metabolize DCA *in vivo*. This combined with the substantially increased elimination of $^{14}CO_2$ in the rat exposed to the same dose may indicate that much lower quantities of metabolites between DCA and CO_2 (such as

oxalic acid, glyoxylic acid, and glycolic acid) may be present in the mouse.

Experiments were performed by Lin et al. (1993) with F-344 rats administered ^{14}C-DCA p.o. in water. The major routes of elimination were breath (CO_2) and urine (glycolic acid, glyoxylic acid, and oxalic acid). Approximately 25% of a radiolabelled dose of DCA was eliminated as CO_2 in breath within 48 h. Radiolabel in urine accounted for approximately the same percent of dose (25%). At the lower DCA dose (28 mg/kg) a higher percentage was eliminated as CO_2, whereas at the higher dose (282 mg/kg), more of the dose was eliminated in the form of urinary glycolic acid, glyoxalic acid, and oxalic acid. These data seem to indicate that higher doses may overwhelm the potential for further conversion to CO_2, with the balance of metabolism shifting toward those metabolites eliminated in urine.

RESEARCH FINDINGS *IN VITRO* AND ENZYME DISTRIBUTION

Ikeda et al. (1980) have shown that 700 g supernatant of rat liver (containing cytosol, microsomes, and mitochondria) convert TCE to TCOH, CH, and TCA *in vitro*. Highest quantities of TCOH were formed, followed by CH and TCA. They conducted experiments with 9,000 g supernatant of rat liver (devoid of mitochondria) with either NAD or NADP present. Their work indicated that in the presence of NAD, the total metabolism of TCE to CH, TCA, and TCOH was 5.36 nmol/mg protein, and in the presence of NADP, the combined metabolism was 4.79 nmol/mg protein. Chloral hydrate was present in incubations containing NAD at concentrations that were double those observed in NADP-containing incubations. These data may indicate that NADP may stimulate, to a greater degree than NAD, the further metabolism of CH.

Examination of the cytosolic conversion of CH to TCA and TCOH over 60 min *in vitro* revealed that the addition of either NAD, NADH, NADP, or NADPH stimulated the net conversion of CH to (summed) TCA and TCOH at rates that were double those observed in cytosol without nicotinamide cofactor. There were no appreciable differences between the addition of either oxidized or reduced forms of cofactors, yet there were appreciable differences between NAD-based and NADP-based cofactors. Formation of TCA without further cofactor addition was 4.2 nmol/mg, and the formation of TCOH was approximately 43 nmol/mg. The presence of NAD-based cofactors resulted in TCA being formed at approximately 15.6 nmol/mg; and TCOH was formed at approximately 76 nmol/mg. The presence of NADP-based cofactor stimulated the production of TCA at rates approximating 2.3 nmol/mg, and TCOH was formed at approximately 92 nmol/mg.

Previously, it has been thought that ALCDH accounted for the conversion of CH to TCOH. Such

is apparently not the case, as it has been shown that it is ALRED that is responsible for this metabolic step. An elegant *in vitro* study was conducted that examined the roles of ALCDH and ALRED in the conversion of CH to TCOH. It is important to note that although ALCDH is exclusively distributed to cytosol, it is reported (Shultz and Weiner, 1979) to exist already bound to cofactor and the rate-limiting factor is the dissociation of cofactor. As the endogenous cofactor ratio favors NAD over NADH and the ALCDH reaction relies on the presence of NAD, the enzyme may already exist in complex with NAD. If this were the case, then the conversion to TCOH may involve ALCDH to a limited extent. On the other hand, the addition of NADH to the mixture may result in the favored formation of enzyme-NADH complex, thereby allowing ALCDH to contribute significantly to the reaction. Although ALRED catalyzes the conversion of CH to TCOH, its distribution to cytosol is in only limited quantities and, as such, this enzyme may not contribute significantly to the formation of TCOH. It would be valuable to have the results of similar experiments carried out with microsomes, and mitochondria. In an experiment involving cytosol, microsomes, and mitochondria in separate incubations containing CH, it was demonstrated that specific activity yielding TCOH was cytosol (71) >> microsomes (4.9) > mitochondria (2.2) and specific activity for the formation of TCA was mitochondria (34) >> cytosol (4) > microsomes (1.4 nmol/mg protein). From these data, the TCOH:TCA ratio in cytosolic incubations is 17.8; in microsomes it is 3.5; and in mitochondria, it is 0.06. These *in vitro* data agree closely with findings *in vivo* that demonstrate much more TCOH eliminated over TCA.

TRICHLOROETHYLENE TO CHLORAL HYDRATE

In the overall metabolism of TCE, the conversion to CH by microsomes is thought to be the rate-limiting step (Ikeda et al., 1980). Byington and Leibman (1965) examined the rat microsomal metabolism of TCE. Their work identified a metabolite undistinguishable from CH. Phenobarbital (PB) treatment *in vivo* (P-450 induction) has been shown to increase the metabolism of TCE (Moslen et al., 1977). The treatment of hepatocytes with PB produces a fourfold increase in the overall conversion of TCE to TCOH (Miller and Guengerich, 1983), but the effect of PB on the metabolism of TCE by microsomes (only) did not yield so dramatic an increase (PB has since been shown to impact several other enzymes, which may account for the dramatic increase in TCOH production contrary to only minor increases in microsomal TCE metabolism). Interesting species differences in the microsomal metabolism of TCE exist. It has been shown that microsomes from the human and the rat were equipotent in the conversion of TCE to DNA-reactive species, but that the mouse microsomes produced levels of DNA-reactive metabolites two- to threefold higher (Miller and Guengerich, 1983). By contrast, the adduction of protein across species showed no appreciable differences. The metabolism of TCE by mouse hepatocytes in culture was followed over 60 min, and the results indicated that early on, CH was the predominant metabolite, but by 60 min, the level of TCOH-glucuronide had increased to

reach the level of the then slowly increasing CH (4 nmol/mg). The level of total TCOH (5 nmol/mg) exceeded that of CH, whereas the level of TCA and CO_2 remained very low (<1 nmol/mg) (Miller and Guengerich, 1983).

The initial metabolism of TCE is reported to yield an epoxide intermediate that rearranges to yield DCA, formic acid, and carbon monoxide (Henschler et al., 1979). Studies by Miller and Guengerich (1982) produced data that indicated that the epoxide did not rearrange to chloral in the presence of cytochrome P-450. These authors hypothesize that chlorine migration occurs within the oxygenated enzyme-TCE complex, which is the defining step in the formation of chloral. Experiments with TCE-epoxide may implicate epoxide hydrolase activity in the metabolic scheme of TCE. Additional authors decline to support the role of epoxide hydrolase or the formation of the epoxide as an intermediate. A Lewis acid-catalyzed shift has been proposed to account for the conversion of the epoxide to chloral. Saturation of this pathway led Hathway to propose the spillover hypothesis (Hathway, 1980). This proposed mechanism accounts for the formation of DCA through saturation of the Lewis acid-catalyzed step, which leads to the increased formation of dichloroacetyl chloride, which undergoes dechlorination to yield DCA.

CHLORAL HYDRATE TO TRICHLOROACETIC ACID

Aldehyde dehydrogenase exists as several isoforms that characteristically utilize NAD or NADP. Some species differences exist between rats and mice, but in general ALDH activity is localized in mitochondria > microsomes >> cytosol (Lindahl and Evces, 1984).

Ikeda et al. (1980) have examined the conversion of CH to TCA and TCOH within cytosol, microsomes, and mitochondria. Mitochondria account for approximately twice the TCA formation than either cytosol or microsomes. Cytosol accounts for greater than 90% of the TCOH found in the *in vitro* incubations. If ALDH is the enzyme responsible for the conversion of CH to TCA, then these data agree closely with the subcellular distribution of ALDH demonstrated by Lindahl and Evces (1984). The subcellular distribution of these enzymes reveals some possibly important species differences. The subcellular distribution of ALDH has been reported by Lindahl and Evces (1984). The ALDH activity was studied using three different ALDH substrates in several rat strains. Data for the F-344 rat indicate that cytosol accounts for very little (<2.5% of the total) ALDH activity, and that microsomes and mitochondria account for the remainder. Using NAD and NADP as cofactors, it was demonstrated that 50 to 66% of the total NAD-dependent activity resides in the mitochondria, whereas 32% of the NADP-dependent activity is confined within the mitochondria. Microsomes account for 20 to 30% of NAD-dependent activity and 44% of NADP-dependent activity. Total activity towards benzaldehyde

using NADP as cofactor accounts for much less total activity than either of the other substrates coupled with NAD as cofactor. The same authors present data for the CD-1 mouse, which indicate that cytosol may account for roughly two times the activity of rat liver cytosol (still < 6% total activity). Sixty to 80% of NAD-dependent activity is located in the mitochondria, as compared to 40% of the NADP-dependent activity. Microsomes contain less than 23% of the total NAD-dependent activity and 37% of the NADP-dependent activity. Ferro et al. (1991) have examined the distribution of ALDH activity within the rat hepatocyte in terms of specific activity. They report that activity towards acetaldehyde with NAD as cofactor is 15, 2.5, and 5 nmol/min/mg protein in mitochondria, microsomes, and cytosol, respectively. Activity towards acetaldehyde with NADP as cofactor is 5, 1.2, and 3 nmoles/min/mg protein in mitochondria, microsomes, and cytosol, respectively. Activity towards benzaldehyde with NAD as cofactor is 2.4, 2.5, and 5.0 in mitochondria, microsomes, and cytosol, respectively; and activity towards benzaldehyde with NADP as cofactor is 1, 1.7, and 2.8 nmol/min/mg protein in mitochondria, microsomes, and cytosol, respectively. From these reports, it is clear that cytosol alone is not the *in vitro* preparation of choice in which to examine the conversion of CH to TCA.

Additional reports on the kinetics and activity of ALDH exist. Henehan and Tipton (1991) performed temperature-dependent kinetics experiments with NAD and acetaldehyde as substrate. Their findings explain the apparent complex kinetics of the reactions that are observed at 25 °C *in vitro* and may explain the protection from enzyme inhibition by disulfiram at concentrations of acetaldehyde in excess of 10 mM. It has been shown (Blackwell et al., 1989) that the order of the reaction catalyzed by ALDH is (1) binding of NAD to the enzyme, (2) binding of the aldehyde to the enzyme-NAD complex, (3) transfer of proton and release of acid, and (4) release of NADH. Blackwell et al. (1987) report that the release of NADH from enzyme is the rate-limiting step in the reaction. Given the cellular ratios of cofactors (much more NAD than NADH, much more NADPH than NADP), it seems that the ALDH reaction utilizing NAD as cofactor may be of more importance *in vivo* than the reaction involving NADP as cofactor. Under physiological conditions, more ALDH may exist bound to NAD than bound to NADP.

The zonal distribution of ALDH in the human liver acinus has been examined by Maly and Sasse (1991). Their results on two isoforms (low Km and high Km) indicate that minimal gender and age-related differences exist and that the low Km ALDH isoform is almost evenly distributed along the sinusoid with activities approximating 13 μmol/min/g liver (30 μM acetaldehyde as substrate). Distribution of activity including the high Km isoform (1.5 mM acetaldehyde as substrate) was evenly distributed along the sinusoid, with values ranging from 23 to 25 μmol/min/g liver. In women <50

years, there is a statistically higher activity (20.7 vs. 16.5 μmol/min/g liver, $p<0.001$) in the perivenous zone as compared to the periportal zone. Studies on the distribution of ALDH in the gastric mucosa of rats have indicated that the enzyme is present in significant amounts and is highest in distribution and activity near the surface (Maly et al., 1992). Results from these authors indicate that maximal activity of 6 μmol/min/g tissue are obtained near the surface and decline almost to nil at the basal area.

CHLORAL HYDRATE TO TRICHLOROETHANOL

Alcohol dehydrogenase is a cytosolic component dependent upon NAD for the conversion of ethanol to acetaldehyde with the concomitant production of NADH. Alcohol dehydrogenase exists as a family of enzymes divided into at least three classes. Class I ALCDH, which is primarily responsible for ethanol metabolism, is located exclusively in the cytoplasm, although Class III ALCDH (formaldehyde dehydrogenase) has been identified in the nucleus of the rat hepatocyte (Iborra et al., 1992). In the reaction scheme, the release of NADH from enzyme has been shown to be the rate-limiting step. The reaction step catalyzed by ALCDH is thought to be the mechanism by which the sedative effect of CH is potentiated by concomitant administration of ethanol (Sellers et al., 1972). Exposure to both CH and ethanol results in increased formation of TCOH, which has been attributed to either the increased pool of NADH resulting from the conversion of ethanol to acetaldehyde through ALCDH, or via stimulation of ALCDH activity itself. Shultz and Weiner (1979) postulated that the higher rates of metabolism of CH to TCOH in the presence of ethanol (34% increase) is a direct result on the recruitment of ALCDH into the reaction, through its being in complex with NADH upon release of acetaldehyde from the complex. Alcohol dehydrogenase exists *in vivo* in complex with cofactor. This is predicted to be NAD because the cellular ratio of NAD:NADH greatly favors NAD. The ratio of NADP:NADPH (approximately 0.02) greatly favors NADPH. As ALCDH is primed (through forming an enzyme-NAD complex) to effect the conversion of an alcohol to an aldehyde, it seems unlikely that ALCDH may be involved in the conversion of CH to TCOH.

Data derived from experiments with liver homogenate have indicated that ALCDH exhibits a Km for CH of 1.8 mM, whereas the Km of ALRED for CH is 6 mM (Shultz and Weiner, 1979). When 10 mM pyrazole was added to inhibit ALCDH, it was shown that ALRED activity exceeded that of ALCDH activity by 1.5-fold. It was demonstrated that pyrazole had no effect on the NADPH-dependent (ALRED-catalyzed) conversion of CH to TCOH (Shultz and Weiner, 1979). These investigators have clearly shown that the rate of TCOH formation decreases to virtually nil when NAD:NADH ratios are in the physiological range, whereas the NADPH-dependent activity (indicative of ALRED) does not significantly differ over the range of NADP:NADPH ratios that include those of physiological relevance.

The authors went on to describe their explanation for the increased activity in the conversion of CH to TCOH that occurs in the presence of ethanol. Ethanol is oxidized to acetaldehyde via ALCDH, with NAD as cofactor. This cofactor exists bound with the enzyme, awaiting the addition of suitable substrate. Upon the introduction of ethanol to incubations containing CH and other components, the ethanol is oxidized to the aldehyde and is released from the enzyme. At that point, the enzyme-NADH complex may bind CH and catalyze the reduction of CH to TCOH. The additional presence of ALCDH inhibitors prevented the increase in TCOH formation in the presence of ethanol.

As with ALDH, the distribution of ALCDH in the liver acinus has been examined (Maly and Sasse, 1991). Studies with liver from human males <53 years old have shown that the distribution of ALCDH along the acinus is not linear; statistically significantly higher activity is observed in the intermediate zone (12.6 μmol/min/g liver) than at the periportal beginning (7.1 μmol/min/g) or at the perivenous end (11.0 μmol/min/g). The distribution of ALCDH activity in women <50 years old was 7.9 and 16.7 μmol/min/g in the periportal and perivenous zones, respectively. Alcohol dehydrogenase also is distributed to the gastric mucosa; its presence there has prompted Maly et al. (1992) to postulate that it may play a significant role in the first-pass effect. Those authors have determined the activity of ALCDH in gastric mucosa of rats and have shown that the highest activity is at the mucosal surface and declines to nearly nil in the basal region. Male and female rats demonstrate an activity towards ethanol of 13 to 14 μmol/min/g tissue. It is generally well accepted that ALCDH is almost exclusively distributed to the cytosolic compartment.

Aldehyde reductase is similar to aldose reductase and catalyzes similar reactions through different mechanisms. Care should be used not to confuse these two enzymes. Aldehyde reductase is sensitive to several aldose reductase inhibitors (Bhatnagar et al., 1990) that are useful in diabetes mellitus. Aldehyde reductase expresses only 19% of the ability to catalyze alcohol oxidation (to the aldehyde) as it does for the reduction of aldehydes to alcohols (Bhatnagar et al., 1991). Therefore, this enzyme may be viewed as being active most predominantly in the conversion of CH to TCOH, especially because the NADP:NADPH ratio is shifted *in vivo* to favor NADPH. Aldehyde reductase has been shown to be the enzyme in brain responsible for the conversion of CH to TCOH (Tabakoff et al., 1974).

TRICHLOROETHANOL TO TRICHLOROETHANOL-GLUCURONIDE

Uridine diphospho glucuronyl transferase is microsomal and exists as a family of isoforms. These

isoforms are separable based on specificity of substrate conjugated with UDP-glucuronic acid. This glucuronidated metabolite may be passed from liver into blood or into bile. Trichloroethylene has been demonstrated to significantly increase rat UDPGT activity with *p*-nitrophenol as substrate *in vitro* and following exposure *in vivo* (Pessayre et al., 1979). It has been demonstrated that the intestinal mucosa (and microflora) exhibit appreciable quantities of beta-glucuronidase, which cleaves glucuronic acid, thereby leaving the aglycone available for intestinal reabsorption, a mechanism by which enterohepatic recirculation might occur. The distribution of beta-glucuronidase to the kidney allows for recycling of glucuronic acid and may result in potentially high renal concentrations of TCOH.

TRICHLOROETHANOL TO CHLORAL

Although no data have been uncovered that support the P-450-mediated conversion of TCOH to chloral (the aldehyde), it seems possible that microsomal P-450 may catalyze such a reaction *in vivo*. Data on the *in vitro* metabolism of 2,2,2-trifluoroethanol (TFOH) have indicated that cytochrome P-450 IIE1 plays a role in the conversion of TFOH to the aldehyde, but not in the conversion of trifluoroacetaldehyde to trifluoroacetic acid (Kaminsky et al., 1992). The potential role of such a metabolic step may have an impact on the overall metabolism of post-CH TCE metabolites.

SUMMARY

Trichloroethylene may be taken up from the GI tract of rats at lower rates than from the GI tract of mice. This may be a factor in the lower blood levels of metabolites of TCE in rats versus mice. A significant amount of an absorbed dose of TCE is excreted unchanged in the lungs of all of the animal species and humans studied. When exposure occurs via the inhalation route, a high proportion of TCE is absorbed, owing to its high blood:air partition coefficient. This absorbed dose does not undergo the first-pass effect by the liver as does the dose absorbed via the GI tract. In fact, some of this dose may be metabolized to TCOH due to the initial exposure of the chemical to drug metabolizing enzymes present in the lung (30% of a TCE dose in isolated perfused rat lung was converted to TCOH).

Trichloroethylene is a substrate for the P-450 system, and several forms of the enzyme metabolize TCE. The metabolism of TCE to CH may or may not involve the trichloro-oxide intermediate. Data from human experiments have shown that chloral hydrate is rapidly removed from blood, with a half-life of only minutes. Chloral hydrate as a hypnotic sedative likely has TCOH as its active compound. The metabolism of CH may be catalyzed by ALCDH, ALDH, and ALRED isoforms. Reports from the cancer literature (in Lindahl, 1992) indicate that CH exerts an inhibitory effect on ALDH, which might become important as we delve further into TCE metabolism. Trichloroethanol in urine of animals exists

largely as the glucuronide form. This accounts for almost all of the urinary metabolites in mice, but not in rats. Trichloroethanol may be metabolized to TCA, but no published record exists. I have been told that some have seen the conversion to TCA and DCA, but to date I cannot identify these metabolites. Recent evidence from the separate laboratories of Brashear (Wright-Patterson AFB, OH) and Stevens (Washington State University, Pullman, WA) indicated that the stability of DCA derived from trichloro precursors in biological matrices may be greatly compromised over a very short period of time, while the stability of authentic DCA in the same matrices is not in any manner compromised. Just as TCOH is derived from CH, so is TCA, supposedly. No dechlorination reaction governing the formation of DCA from TCA has been demonstrated. Dichloroacetic acid is postulated to arise from the epoxide intermediate formed from TCE in the P-450 active site, progressing through dechlorination of the dichloroacetylchloride to DCA. The further degradation of DCA has not been reported in the literature, but certainly occurs *in vitro*.

When engineering *in vitro* experiments, care should be taken to use appropriate subcellular fractions that contain the enzymes thought to catalyze the metabolic step under examination. Finally, the cellular concentrations of nicotinamide cofactors should not be taken for granted and should be factored into the development of *in vitro* experiments.

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SECTION 3

PHARMACOKINETICS OF TCA/DCA TRANSPORT

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Trichloroacetic acid (TCA) and dichloroacetic acid (DCA) are strong carboxylic acids, fully ionized at physiological pH; a mathematical description of the cellular uptake of these polar compounds is required for their pharmacokinetic modeling.

OVERVIEW - MEMBRANE TRANSPORT

The plasma membrane provides a relatively impermeable barrier to the diffusion of polar molecules. Hydrophobic (trichloroethylene, benzene) and small, uncharged polar molecules (water, ethanol, urea) readily diffuse across the plasma membrane, and integral membrane transport proteins provide membrane permeability to large polar (glucose, fructose) and charged molecules (ions, amino acids). Several transport mechanisms exist: channel proteins (ionophores) allow passive transport of inorganic ions along a concentration gradient, and carrier proteins provide either concentration-driven facilitated diffusion or active transport against a concentration gradient (adenosine triphosphate or co-transport driven).

TCA/DCA TRANSPORT

Although no data on the hepatocellular uptake of TCA or DCA were found in this review, inference can be drawn from studies of other water-soluble, polar compounds with similar molecular weight. Kinetic constants for uptake are usually estimated *in vitro* using isolated hepatocytes; liver plasma membrane vesicles; or isolated, perfused liver preparations. Uptake of these compounds generally occurs by carrier-mediated facilitated diffusion. Carrier-mediated transport of anionic compounds is saturable, pH-gradient stimulated, and temperature dependent (Hugentobler et al., 1987). Kinetic analysis of lactate (H_3C-CHOH-COOH) uptake by rat liver plasma membrane vesicles gave a Km value of 2.9 mM (Quintana et al., 1988). The Km values for lactate and pyruvate (H_3C-CO-COOH) uptake by isolated rat hepatocytes were 2.4 and 0.6 mM, respectively, and Km values for transport of lactate and pyruvate into rat erythrocytes were 3.0 and 0.96 mM (Edlund and Halestrap, 1988). The sulfate (oxalate)-bicarbonate anion exchange system in canalicular rat liver plasma membrane is saturable, with an apparent Km of 0.3 mM (Meier et al., 1987). In the isolated, perfused rat liver, distribution and transport of niflumic acid (an anti-inflammatory agent) is flow-limited at both 10 μM and 1 mM (Kelmer

et al.,1993), suggesting the Vmax for hepatocellular uptake is above 1 mM.

Transport proteins behave like enzymes - they bind and transfer a solute across a lipid bilayer - and exhibit saturation kinetics (Jmax, maximal transport rate). The carrier-mediated facilitated transport rate (J) is described by the equation

$$J = (Jmax)[C]/(Km + [C])$$

with Km describing the affinity of the carrier for the substrate and [C] describing the solute concentration (Graf, 1993). Carrier-mediated transport is usually symmetrical, with the same kinetics observed for transport in either direction.

MODELING TCA/DCA STUDIES

Assuming similar kinetics, what can we predict for TCA/DCA transport in reported exposures? The highest concentrations come from oral gavage studies. Larson and Bull (1992) delivered single bolus doses of up to 2000 mg/kg, resulting in a 54-mg exposure in mice (27 g average). We can use total extracellular water as the volume of distribution (0.345 x body weight), as used in Vinegar's trifluoroacetic acid model (personal communication), and for simplicity assume instantaneous and 100% gastric uptake.

This gives a maximum potential blood concentration of 5.8 g/L (0.345 x 0.027 kg = 9.3 mL fluid; 54 mg/9.3 mL = 5.8 g/L), which translates to 35 mM TCA (molecular weight [MW] = 163.4) and 45 mM DCA (MW = 128.9). Lower oral exposures used in this study yield maximal blood concentrations of 0.35 mM TCA/0.45 mM DCA (20 mg/kg exposure) and 1.8 mM TCA/2.2 mM DCA (100 mg/kg exposure). These rough calculations suggest that oral exposures of TCA/DCA could generate blood concentrations that saturate the hepatocellular transport capacity.

Peak blood plasma concentrations were reported in this study, which were three- to sixfold higher for TCA than DCA with equivalent doses. In mice, exposures of 20 and 100 mg/kg TCA gave peak values of 0.23 mM and 0.79 mM, respectively (close to the above predictions), whereas 20 and 100 mg/kg DCA gave peak values of 4 μM and 20 μM (presumambly due to its greater metabolism). No data were reported for higher exposures.

If required, the kinetics of hepatocellular transport for TCA/DCA can be determined using suspensions of isolated hepatocytes (Plaa and Hewitt, 1982), which allows the rapid sampling required to estimate initial rates of transport. The technique involves centrifugal separation of cells and incubation medium through a silicone oil interface, and subsequent quantitation of intercellular content

of the compound.

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SECTION 4

TRICHLOROETHANOL GLUCURONIDATION AND ENTEROHEPATIC CIRCULATION

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OVERVIEW - GLUCURONIDATION

Glucuronide formation is quantitatively and qualitatively the most important Phase II metabolism reaction, occurring in all mammalian tissues, generating a water-soluble, polar xenobiotic conjugate for excretion in urine or bile (Hayes, 1989). Hydroxyl, carboxyl, amino, and sulfhydryl functional groups can serve as substrates for glucuronidation; the cofactor for this reaction is uridine diphosphate (UDP)-glucuronic acid, a ubiquitous part of intermediary metabolism. Its formation is outlined below.

UDP-glucose (UDPG) + NAD^+ (nicotinamide adenine dinucleotide)

|

| UDP-glucose dehydrogenase (cytosolic)

V

UDP-glucuronic acid (UDPGA) + NADH (reduced NAD^+)

O-glucuronides are formed from alcohols and carboxylic acids - acids forming esters and alcohols forming ether-linked conjugates. The reaction, outlined below for trichloroethanol (TCOH), generates a *B*-linked glucuronide.

CCl_3CH_2-OH (the aglycone) + UDPGA (the glycone)

|

| UDP-glucuronyltransferase (microsomal; multiple forms)

V

CCl_3CH_2-O-Glucuronic acid + UDP

OVERVIEW - GLUCURONIDES AND ENTEROHEPATIC CIRCULATION

Glucuronic acid contributes a carboxyl group to the conjugate, predominantly ionized at physiological pH; this carboxyl moiety promotes excretion of the conjugate both from increased water solubility and participation in biliary and renal organic anion transport systems. The route of excretion is largely influenced by molecular weight (MW) of the xenobiotic conjugate MW of TCOH-glucuronide

is ~340). In the rat, small conjugates (less than 350 daltons) are excluded from bile and large molecules (over 450 daltons) are excluded from urine. Intermediate compounds (350 to 450 daltons) are excreted by both routes (Hirom et al., 1976). Biliary inclusion threshold molecular weights have been estimated at 325 +/-50 in rats, 440 +/-50 in guinea pigs, and 475 +/-50 in rabbits (Hirom et al., 1972). Molecular weight thresholds have not been reported for mice. Klaassen et al. (1986) report generically that renal tubular organic acid excretion into urine is favored for aglycones below 250 MW (MW of TCOH is ~150); over 350 favors biliary excretion, and intermediate 250 to 350 may be excreted by either route.

Biliary glucuronides pass into the intestine, where they can be excreted in feces or metabolized by microfloral *B*-glucuronidase and resorbed as parent (Gibson and Skett, 1986). Biliary excretion of xenobiotics shows species- and compound-specific variability. Mice and rats are generally "good" biliary excretors (Klaassen et al., 1986); this variation makes it difficult to predict human biliary excretion from rodent data. Glucuronides are both acid and base labile; this is an important point to remember in extractions from a biological matrix.

TCOH-GLUCURONIDE

Based on molecular weight, TCOH-glucuronide would be predicted to appear solely in urine; data from Bull's laboratory, however (Templin, et al., 1994), have been interpreted to suggest significant enterohepatic circulation in bile. This apparent contradiction requires a closer evaluation of Bull's bile data (not published).

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SECTION 5

THE POTENTIAL EFFECT OF VEHICLE ON THE KINETIC UPTAKE/TOXICITY OF TRICHLOROETHYLENE

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The following six articles address the potential effect a dosing vehicle may have on the kinetics of uptake and/or the toxicity of a chemical (See Table 5-1). Specifically, the study by Merrick et al. (1989) suggests that the type of oral gavage vehicle is an important factor in determining the nature of trichloroethylene (TCE) toxicity, whereas the simulation study by Fiserova-Bergerova et al. (1980) hypothesizes that the use of digestible oils as dosing vehicles may affect the uptake and deposition of chemical in tissues by changing the blood:air partition coefficient. It can be concluded from these studies that experimental design considerations should be given to the potential for a vehicle to influence the outcome of the end point of interest.

(1) Merrick, B.A., M. Robinson, and L.W. Condie. 1989. Differing hepatotoxicity and lethality after subacute trichloroethylene exposure in aqueous or corn oil gavage vehicles in B6C3F$_1$ Mice. *J. Appl. Toxicol.* 9:15-21.

Subacute toxicity of TCE was evaluated in male and female B6C3F$_1$ mice using corn oil or aqueous gavage vehicles. Male and female mice received oral doses of TCE five times per week for 4 weeks at exposure concentrations that ranged from 600 to 2400 mg/kg/day. Vehicle control mice (no chemical) were dosed with either corn oil or a 20% aqueous solution of Emulphor. During the first week of treatment, a dose-related increase in lethality occurred in male and female mice receiving TCE in Emulphor but not corn oil. After 4 weeks of exposure, liver to body weight ratios (not body weights) were increased by TCE administered in either vehicle in both sexes. Male mice treated with TCE in corn oil had elevations in serum enzyme levels, accompanied by liver histopathology (inflammation-associated focal necrosis in 30 to 40%) with increasing severity from low to high dose. Lipid accumulation was prevalent in male mice treated with TCE in corn oil but also occurred in animals receiving either gavage vehicle alone.

(2) Withey, J.R., B.T. Collins, and P.G. Collins. 1983. Effect of vehicle on the pharmacokinetics and uptake of four halogenated hydrocarbons from the gastrointestinal tract of the rat. *J. Appl. Toxicol.* 3:249-253.

The relative uptake of TCE using vegetable oil and aqueous dosing vehicles, as assessed by the area

under blood concentration-time curves, was examined after intragastric dosing in the rat. Uptake of equivalent doses of methylene, chloroform, and dichloroethane also were investigated. In general, the uptake from aqueous solution was more rapid and gave rise to higher peak blood levels than from oil-based solutions when equivalent amounts were administered intragastrically to rats. For TCE, the observed blood concentrations after the administration of aqueous solution were three orders of magnitude greater than those measured after the administration of the equivalent dose in oil. It also was demonstrated that uptake from oil-based solutions was generally more complex (biphasic/pulsed) than from aqueous solution. It also was observed that some of the oil was absorbed into the systemic circulation and may have increased the lipid content of the sampled blood. It was then speculated, but not proven, that this may reduce the effective vapor pressure of chlorinated hydrocarbons.

(3) Cheico, P., M.T. Molsen, and E.S. Reynolds. 1981. Effect of administrative vehicle on oral 1,1-dichloroethylene toxicity. *Toxicol. Appl. Pharmacol.* 57:146-155.

The relationship between the acute toxicity and biologic fate of 1,1-dichloroethylene (DCE) was examined in fasted and fed rats given chemical orally in a mineral oil, corn oil, and Tween-80 vehicle. The vehicle in which the chemical was administered affected the magnitude of liver injury in fasted rats. With mineral oil or corn oil, injury was massive, whereas with aqueous Tween-80 vehicle, injury was moderate. In contrast, liver injury in all fed groups was slight. It was speculated that the relative resistance of fed animals to hepatic injury is due to the capacity of the animals to detoxify DCE for a longer duration than the fasted animals.

In terms of uptake and elimination of chemical, the study findings indicate that the vehicle altered the rate of exhalation through an effect on uptake from the gastrointestinal (GI) tract. The administrative vehicle did not affect the total amount of DCE exhaled in the initial rapid exhalation phase (first hour), but in the later slow phase of exhalation, the amount of DCE exhaled was affected by the vehicle in which the chemical was administered. Mineral oil prolonged exhalation, with corn oil intermediate, and exhalation being the briefest with aqueous Tween. The aqueous Tween also was thought to substantially enhance the absorption of DCE, allowing for more rapid uptake and clearance of the chemical.

(4) Fiserova-Bergerova, V., J. Vlach, and J.C. Cassady. 1980. Predictable "individual differences" in uptake and excretion of gases and lipid soluble vapors simulation study. *Br. J. Ind. Med.* 37:42-49.

The following article discusses the effect that a postprandial elevation of plasma lipids may have on the blood:air partition coefficient. Lipid content in blood is higher postprandially than during fasting. Thus, the effect of a meal on blood:air partition coefficients of seven lipid-soluble vapors (enflurane,

halothane, methylene chloride, benzene, TCE, toluene, and methoxyflurane) was investigated in human volunteers. The blood:air partition values measured in samples collected after a meal were greater than after fasting. When plotted on a graph with the x-axis = after meal and y-axis = after fasting, the slope of the regression line for all chemicals was 0.61, versus 1.0 if the after meal and after fasting values had been equal.

Additionally, the effect of changes of blood:air partition coefficient on uptake was studied by modeling 8-h exposures to hypothetical lipid soluble vapors. Variations of partition coefficients in the range of 7.5 to 13 affected the uptake of vapors by the inhalation route by less than 10%. Deposition in fat was enhanced, but the deposition in lean tissues was lowered due to changes in the partition coefficient.

(5) Bull, R.J., J.M. Brown, E.A. Meierhenry, M.R. Jorgenson, and J.A. Stober. 1986. Enhancement of the hepatotoxicity of chloroform in B6C3F$_1$ mice by corn oil: Implications for chloroform carcinogenesis. *Environ. Health Perspect.* 69:49-58.

The differential effects of chloroform when it was administered by gavage using corn oil versus a 2% Emulphor suspension as the vehicle was examined. Chloroform increased serum glutamic oxaloacetic transaminase (no effect on lactic dehydrogenase) levels significantly only when administered in corn oil in both male and female mice. There was a small increase in blood urea nitrogen (BUN) when chloroform was administered in corn oil, but not when administered in 2% Emulphor. When administered in corn oil, chloroform significantly decreased serum triglyceride levels and there was a small increase in BUN. When administered in 2% Emulphor, there was no alteration in triglycerides or BUN. Mice administered chloroform in corn oil displayed a significant degree of diffuse parenchymal degeneration and mild to moderate early cirrhosis. Significant pathological lesions were not observed in the animals administered corn oil without chloroform nor in mice receiving chloroform in 2% Emulphor. The hypothesis that remains from this study is that the difference in carcinogenic response to chloroform observed with different vehicles may be attributable to interactions between the vehicle and chloroform.

(6) Frederick, C.B., D.W. Potter, M.I. Chang-Mateu, and M.E. Andersen. 1992. A Physiologically based pharmacokinetic and pharmacodynamic model to describe the oral dosing of rats with ethyl acrylate and its implications for risk assessment. *Toxicol. Appl. Pharmacol.* 114:246-260.

A physiologically based pharmacokinetic and pharmacodynamic model was developed to describe the absorption, distribution, and metabolism of orally dosed ethyl acrylate (EA). In this study, a reasonable effort was directed at attempting to describe and model the absorption kinetics of the

chemical administered in a corn oil vehicle. The regional deposition of a 5-mL/kg gavage dose of corn oil in the GI tract was determined prior to the dosing with chemical. Examination of the stomach contents following gavage dosing with the oil indicated two phases in the gut lumen, an oil phase and an aqueous gel of food matter. The bottom line is that the effort of describing the kinetics could be summed up as a "fitting exercise." A fully detailed description of the time-course of the partitioning of EA between the phases present in the gut lumen and the absorption into the gut wall was considered to be too complex. The process was approximated by using first-order transfer constants (from oily phase to aqueous phase and from aqueous phase into the gut wall) that do not vary with time. The values for these constants were varied manually to fit tissue glutathione depletion data. The values were found to change somewhat with dose.

TABLE 5-1. THE POTENTIAL EFFECT OF VEHICLE ON THE KINETIC UPTAKE/TOXICITY OF TRICHLOROETHYLENE

Author	Chemical	Species	Duration	Vehicle	Effect
Chieco et al., 1981	1,1-Dichloro-ethylene	Sprague-Dawley rats	Single Dose	Corn oil, mineral oil 0.5% Tween 80	Acute hepatotoxic effects
Merrick et al., 1989	Trichloro-ethylene	$B6C3F_1$ mice	5x/week for 4 weeks	Corn oil 20% Emulphor	Increase serum enzymes and liver histopath Dose-related increase in lethality
Bull et al., 1986	Chloroform	$B6C3F_1$ mice	90 Days	Corn oil 2% Emulphor	Increase serum glutam, BUN, liver histopath, decrease triglycerides
Withey et al., 1983	Trichloro-ethylene	Wistar rats	--	Corn oil Water	Complex, biphasic absorption Rapid Tmax, high Cmax (3 orders of magnitude)
Frederick et al., 1992	Ethyl acrylate	Fischer 344 rats	--	Corn oil	Two phases - Oil phase - Aqueous gel of food matter
Fiserova-Bergerova et al., 1980	Trichloro-ethylene	Humans	Simulation study	--	Increase in lipids causes increase in blood:air partition coefficients for lipid-soluble vapors

[a]Condie, L.W., R.D. Laurie, T. Mills, M. Robinson, and J.P. Bercz. 1986. Effect of gavage vehicle on hepatotoxicity of carbon tetrachloride in CD-1 mice: Corn oil versus Tween-60. Aqueous emulsion. *Fund. and Appl. Toxicol.* 8:199-206.

SECTION 6

TRICHLOROETHYLENE: REVIEW OF PHARMACOKINETICS AND IMPLICATIONS FOR MODE OF ACTION

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INTRODUCTION

U.S. AIR FORCE RELEVANCY

The health risks associated with occupational chemical exposure is a U.S. Air Force (USAF) concern, both for operational chemicals and for chemicals occurring as environmental contaminants at USAF installations. A broad, collaborative study on the reevaluation of trichloroethylene (TCE)-induced hepatocarcinogenicity has been initiated by the US AF. The study aims at the link between exposure and assessment of the local concentrations in target organs and subcellular mode of action. To ensure a realistic exposure assessment to TCE, and especially its local concentrations in the target organs, a review of the available literature on its pharmacokinetics and physiologically based pharmacokinetic (PBPK) models was carried out. To establish levels of exposure tolerable by humans, the literature search was focused on species differences in subcellular mode of action and protection against the deleterious effect on liver.

TECHNICAL BACKGROUND

Trichloroethylene is a solvent that has been used extensively for the degreasing of metals and as a solvent in adhesives, textile manufacturing, paint stripping, dry cleaning, etc. An estimated 3.5 million people are occupationally exposed to TCE. At least 100,000 people are thought to be exposed on a full-time basis. It is also a common environmental contaminant found in surface water, groundwater, ambient air, and soil. Up to 34% of water supplies contained TCE or its derivatives. The highest levels are leaching from landfill waste disposal sites. Trichloroethylene is one of the 10 most commonly detected chemicals (at 27.9% of hazardous-waste sites). Trichloroethylene is often found in groundwater at Air Force installations.

Trichloroethylene has a carcinogenic potential. Metabolism is believed to play an important role in its cytotoxicity and carcinogenicity. The U.S. Environmental Protection Agency calculates cancer risk based on metabolized dose-tumor incidence relationship. Quantitative differences among species may substantially alter the effective dose of reactive metabolite(s) that is delivered to the target organ. It is believed that metabolites of TCE, rather than the parent compound, are responsible for cytotoxicity and/or carcinogenicity in liver, kidney, and lung (Bruckner et al., 1989).

PHYSICAL PROPERTIES OF TRICHLOROETHYLENE:

Boiling point, °C		86.7
Melting point, °C		-87.1
Vapor pressure, mmHg, 20 °C		57.8
Vapor density, boiling point, 1 atm, g/L		4.45
Autoignition temperature, °C		410
Decomposition temperature, °C		700
Specific gravity, 20/4 °C		1.46
Surface tension, 30 °C, dyne/cm		29
Odor threshold, ppm		21.4
Solubility in water, g/100 g H_2O, 20 °C		0.11
Distribution coefficients of solubility, 20 °C and 37 °C:		
Water/air	3	1.6
Blood/water	18-22	8-10
Plasma/air	16-20	
Oil:water distribution		900:1

METABOLISM AND BIOLOGICAL EFFECTS OF TRICHLOROETHYLENE

First metabolic step involves oxidation of TCE to chloral hydrate (CH) (through an intermediate). An epoxide intermediate had been hypothesized but never proved. Chloral

hydrate has a short biological half-life in humans (5 to 30 min after ingestion of 30 mg/kg) and in mice (10 to 20 min in brain tissue). The principal urinary metabolites of TCE are trichloroethanol (TCOH), TCOH glucuronide, and trichloroacetic acid (TCA). Much of the absorbed TCE is exhaled unchanged. Excretion kinetics appeared to be biphasic, with an initial fast phase followed by a slow one. Both TCOH and TCA were detected for more than 16 days after exposure. Seventy-three percent of the retained TCE was excreted in urine. The metabolic profile in urine was 4% monochloroacetic acid, 19% TCA, and 50% TCOH.

Death resulted from ventricular fibrillation in most acute intoxications with TCE. After inhalation, lung hemorrhages and fatty degeneration of liver were described. Hepatorenal failure was considered the cause of death of a man who accidentally drank a large quantity of TCE. Before death, nephrosis and acute pancreatitis developed (there was, however, no necrosis to the liver). More than 60% of workers exposed to TCE exhibited increased cardiac output (attributed to elevated epinephrine levels).

Oral median lethal doses (LD_{50}s) have been established in rats (4,920 to 5,200 mg/kg) and in dogs (5,900 mg/kg). The lowest published toxic concentration in humans was 160 ppm/83 min, and 110 ppm/8 h gave central nervous system toxic effects (Waters et al., 1977).

In preliminary tests on mice, the National Cancer Institute (1976) implicated TCE as a cause of hepatocellular carcinoma (a previous study did not reveal any carcinogenicity, only chronic neuropathy at doses up to 0.1 to 0.2 LD_{50}). The percentage of hepatocellular carcinoma for females was significantly lower than for males. The criticism of this study was that the carcinogenic level was attained at doses equivalent to a person drinking 6 oz of TCE every day (a situation that has no relationship to actual exposure levels).

So far, no proven cases of human cancer due to TCE have been reported. A review comparing metabolism and pharmacokinetics of TCE and its metabolites in different species was published in Davidson and Beliles (1991). The American Conference of Governmental Industrial Hygienists (1993) has set the following threshold limit values (TLVs) and biological exposure indices (BEIs) for TCE [CAS #79-01-6]:

TLV - TWA 50 ppm = 269 mg/m^3; STEL 100 ppm = 537 mg/m^3

KEY: TLV = threshold limit value
TWA = time-weighted average
STEL = short-term exposure limit
BEI = biological exposure index

TCA in urine at end of workweek:

BEI 100 mg/g creatinine

TCA and TCOH in urine at end of shift at end of workweek:

BEI 300 mg/g creatinine

Free TCE in blood at end of shift at end of workweek:

BEI 4 mg/L

TRICHLOROETHYLENE AND FREE RADICAL DAMAGE IN LIVER

It was demonstrated, using a spin-trapping technique by Gonthier and Barret (1989), that further metabolism of TCOH, an intermediate product of TCE metabolism, may supply free radicals in the microsomal preparations *in vitro*. Larson and Bull (1992a,b) suggested that at least two metabolic reactions of reductive dechlorination (generating TCA and dichloroacetic acid [DCA]) may supply carbon-centered free radicals. The carbon-centered free radicals under aerobic conditions tend to interact rapidly with dioxygen, providing the reactive oxygen species (McCay et al., 1984).

Several free radicals and reactive oxygen species can initiate the lipid peroxidation process by abstracting the hydrogen with its single electron (hydrogen atom) from an unsaturated fatty acid molecule. Apparently, the most common initiators are: hydroxyl radical, singlet oxygen, alkoxyl, peroxyl, and perferryl (but not superoxide alone, Byczkowski and Gessner, 1988). Lipid peroxidation may be initiated also by carbon-centered free radicals (e.g.,trichlorocarbonyl radical). During the initiation, lipienyl free radical ($L^{\cdot}$) is formed from unsaturated fatty acid (Gardner, 1989):

$$LH + X^{\cdot} \dashrightarrow L^{\cdot} + XH$$

Where LH is unsaturated fatty acid, $X^{\cdot}$ is free radical, and XH is non-radical compound.

Lipid peroxidation is a unique form of hepatocellular injury implicated in the genesis of liver necrosis evoked by hepatotoxicants such as carbon tetrachloride (CCl_4), yellow phosphorous, and possibly ethanol. It is characterized by the formation of conjugated dienes, formation of thiobarbituric acid reactive material (malondialdehyde) and the exhalation of ethane (Riely et

all., 1974). Larson and Bull (1992a,b) proved that both TCA and DCA increased production of thiobarbituric reactive material in mice at doses above 300 mg/kg.

Free radical damage to the hepatic biomembranes results in accumulation of peroxidation products, which eventually leak into the bloodstream. Serum lipid peroxide levels (measured indirectly as malondialdehyde) increase 1.8- to 3.7-fold in patients suffering from different forms of hepatitis, fatty liver, and cirrhosis (Kulkarni and Byczkowski, 1993).

Subcellular membranes rich in unsaturated fatty acids are obvious targets of lipid peroxidation, resulting in the loss of both structural integrity and function of the affected organelles (Smith, 1991). In addition to this localized damage, the breakdown products of lipid peroxides, such as aldehydes (e.g., nonenal, octanol, hexanal, pentanal) migrate far from their production site and may cause damage at distant loci. Several lipid peroxides and hydroperoxides are known for their extremely high toxicity and carcinogenicity.

DIFFERENCES IN HEPATOCELLULAR ANTIOXIDANT DEFENSE

Several drugs and environmental chemicals inhibit hepatocyte antioxidant defenses against lipid peroxidation, thus increasing severity and duration of free radical damage. For example, azide, hydroxylamine, and aminothiazole inhibit catalase; dithiocarbamatic acid inhibits superoxide dismutase; and heavy metals bind sulfhydryl groups of glutathione, lipoic acid, sulfhydryl amino acids, and peptides (Kulkarni and Byczkowski, 1993). Also, diets low in selenium and natural antioxidants such as vitamin E, vitamin C, and carotenoids may result in decreased antioxidant defense. On the other hand, treatment with high doses of vitamin A depleted stored tocopherols (vitamin E) in liver and caused increased ethane exhalation in CCl_4-treated rats (ElSisi *et al.*, 1993).

Some animal species are less protected against the free radical attack and lipid peroxidation than are others. For instance the activity of superoxide dismutase, an antioxidant enzyme, is significantly lower in mouse liver than in rat liver (Sohal *et al.*, 1989). Therefore, it seems that susceptibility of mouse to the free radical liver damage may be higher than that of rat or human.

PBPK MODELING OF TRICHLOROETHYLENE

Assessment of toxic and carcinogenic risks of exposure to volatile organic compounds (VOCs) has become a subject of major importance over the last decade. Although it has been common practice to calculate risk estimates on the basis of administered dose-toxicity/tumor incidence data, it is now recognized that internal, or target organ dose, is a more accurate and direct determinant of the magnitude of injury. The dose of chemical actually reaching a target organ is dependent upon kinetic processes which may vary considerably with the administered dose, route of exposure, and animal species. Thus, recognition and use of pharmacokinetic data can substantially reduce uncertainties inherent in the route-to-route, high-dose to low-dose, and species-to-species extrapolation often necessary in risk assessment.

Time-course profiles for humans are increasingly limited by the ethical question of exposing humans to a potential human carcinogen. Thus, investigations utilizing laboratory animals must be largely relied on to provide such information (Dallas et al., 1991). The rat becomes the most used laboratory species in studies of pharmacokinetics of VOCs; although the rat blood:air partition coefficients for each VOC are significantly higher than those for humans (Gargas et al., 1989). Moreover, most of the positive carcinogenesis effects of VOCs were obtained with mice.

Physiologically based pharmacokinetic models have been formulated for a number of VOCs in an effort to better understand and forecast the dynamics of the chemicals in the blood and tissues of laboratory animals and humans. These models have utilized blood-flow-limited organ representations (Ramsey and Andersen, 1984; Angelo and Pritchard, 1984; Dallas et al., 1989). Also, PBPK models have been used with some success to describe the disposition of TCE in the rat.

In the most recent study, it was proved by Dallas et al. (1991) that a blood-flow-limited model was adequate to characterize the tissue distribution of TCE in rats exposed to 50 to 500 ppm by inhalation. In their model, compartmental volumes and organ blood flows were obtained from the values used by Ramsey and Andersen (1984) for rats and were scaled to 340 g, the mean body weight of rats utilized in their study. Tissue:blood partition coefficients that characterize the extent of tissue TCE uptake were obtained from Andersen et al. (1987).

The Michaelis-Menten parameters describing the rate of TCE metabolism, V_{max} and K_m, were initially estimated from Andersen et al. (1987) and were $K_m = 0.25$ mg/L and $V_{max} = 183.3$ μg/kg/min. When scaled to the 340-g rat used in the reported study, $V_{max} = 82$ μg/min. The final value of V_{max}, set equal to 75 μg/min, provided good agreement between observed and predicted blood TCE concentrations. Differential mass balance equations were numerically solved with an Advanced Continuous Simulation Language computer program (Mitchell and Gauthier, Concord, MA). The solution to the equations provided predicted blood and exhaled breath TCE concentrations as a function of time. Although 50 and 500 ppm were the target TCE inhalation concentrations in the Dallas et al. (1991) study, the actual concentrations inhaled by the animals were determined by analysis of air samples taken from the airway immediately adjacent to the breathing valve. Mean (± SE) inhaled TCE concentrations for the six rats in each group were 499.8 ± 12.7 ppm for the 500-ppm exposures and 50.7 ± 0.8 ppm for the 50-ppm exposures. There was a cumulative uptake of 8.4 mg/kg in rats inhaling 50 ppm TCE for 2 h (Dallas et al., 1991).

In the successful PBPK modeling study in rats, postexposure exhaled breath concentrations were well simulated for the 50-ppm group and only slightly underpredicted during the first 45 min for the 500-ppm group. The pattern of uptake of TCE into the blood of the 50-ppm animals was adequately described, although the TCE concentrations were slightly overpredicted (by about 0.1 mg/L). The model predicted a more rapid postexposure decline in blood levels than was observed during the first hour in the 500-ppm group. Predicted and observed postexposure blood concentrations compared favorably, however, for the 50-ppm rats (Dallas et al., 1991).

Another successful description of TCE pharmacokinetics using the PBPK model was provided by Fisher et al. (1989). They modified the Ramsey and Andersen (1984) model to simulate the kinetics of TCE and TCA in the pregnant rat following inhalation and ingestion of TCE. Additional compartments (i.e., mammary tissue, placenta, and fetus) were incorporated into the model, and allowance was made for certain physiological changes that occur during pregnancy. The PBPK model of Fisher et al. (1989) provided a good representation of TCE and TCA levels measured experimentally in maternal and fetal blood at a limited number of times postexposure. This model has been extended, consequently, to

predict the kinetics of TCE and TCA in lactating rats and nursing pups Fisher et al. (1990).

There is no report in the literature about successful description of TCE pharmacokinetics by PBPK in mice. The major routes of elimination of TCE are metabolism and exhalation of the parent compound, and they are apparently the same in rats and mice. The elimination of TCE in the exhaled breath generally paralleled elimination of the chemical from the bloodstream of rats (Dallas et al., 1991) but not necessarily of mice (Fisher et al., 1991). The plasma concentrations of TCA in rats were lower than in mice exposed to equivalent doses of TCE. Female rats and mice cleared TCA from systemic circulation more rapidly than males (Fisher et al., 1991). The developed PBPK model failed to describe TCE disposition in mice exposed to a broad range of concentrations, although it worked successfully in rats. On the other hand, it seems that TCA (the acidic metabolite of TCE) concentrations were adequately described in mice exposed to TCE either by inhalation (Fisher et al., 1991) or by gavage (Fisher and Allen, 1993).

PBPK MODEL VALIDATION IN MICE

Because the positive carcinogenesis studies of TCE were performed in $B6C3F_1$ mice (National Cancer Institute, 1976; National Toxicology Program, 1982), it is logical to validate the PBPK model of TCE metabolites using the same species/strain.

In the preliminary study, a modified code of the PBPK model used by Fisher et al. (1991) and Fisher and Allen (1993) that adequately describes the TCA concentrations in mice also was used to simulate data reported by Larson and Bull (1992a,b) for blood concentrations of DCA in mice exposed to TCE (Byczkowski, unpublished preliminary study). It was assumed that DCA follows the concentrations of TCA in mice blood with a mass conversion rate of 0.047 (4.7%). The modified model followed the DCA concentration in blood but, unfortunately, the description of parent compound (TCE) was unsuccessful using the same model. It seems that further modifications are necessary to include formation of other metabolites of TCE in this model (e.g., TCOH, its glucuronide, and CH). Possibly estimating more accurate kinetic constants for TCE metabolism (V_{max} and K_m) as well as balancing and accounting for all other metabolites of TCE may help in a more adequate simulation of the parent compound concentrations in mice blood than was possible with a model simulating

only one metabolite (TCA).

Obviously, the most crucial task, from a mechanistic point of view, would be to describe and simulate the kinetics of hypothetical carbon-centered free radicals produced as intermediates from metabolites of TCE. Production of such carbon-centered free radical intermediates was inferred from increased lipid peroxidation in rats and mice treated with TCA or DCA (Larson and Bull, 1992a,b). The carbon-centered free radicals (e.g., dichloroacetyl and monochloroacetyl) and their peroxyl derivatives (dichloroperoxyl acetate and monochloroperoxyl acetate) may be involved in both initiation of lipid peroxidation and interaction with macromolecules (protein and DNA), and thus, may ultimately be responsible for deleterious action of TCE on mouse liver. Better protection of human liver against free radical damage might explain a relatively lower sensitivity of humans to TCE-induced hepatocarcinogenicity when compared to mice.

Further examination of the scientific literature concerning TCE revealed only limited information on tissue TCE concentrations and biological effects resulting from test exposures in mice and humans.

The studies proposed by the USAF would fill this gap and provide data for further validation of the PBPK model for TCE metabolism, biological effects, and assessment of risk to humans.

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SECTION 7

MECHANISMS OF TUMORIGENICITY FOR TCE, TCA, AND DCA

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TRICHLOROETHYLENE

Mutagenicity/Genotoxicity

Numerous studies have been conducted to determine whether trichloroethylene (TCE) is directly mutagenic using *in vitro* protocols. These studies have been largely negative when pure compound has been used, but occasional equivocal or positive results have been obtained.

Bronzetti et al. (1978) used a yeast system to demonstrate both point mutation and gene conversion *in vitro*; however, microsomal activation was required. In an intrasanguineous host conversion assay, in which the mouse received a retro-orbital dose of yeast cells followed by 400 mg/kg TCE in corn oil gavage, gene conversion rates were increased; especially in the liver and kidney. A similar procedure using multiple gavage to a total dose of 3700 mg/kg yielded increased gene conversion in liver, kidney, and lung.

Also using a yeast system, Cerebelli et al. (1985) reported TCE to be weakly mutagenic; some disturbance in chromosomal segregation was observed - both effects again requiring endogenous metabolic activation. It is interesting to note that both trichloroethanol (TCOH) and chloral hydrate (CH), both major metabolic products of TCE, also were found to be weak mutagens. Both TCOH and CH induced higher frequencies of haploid and "nondisjunctional" diploid chromosomal events at both low (5 mM) and high (10 mM) concentration. Trichloroethylene, on the other hand, only demonstrated these effects at its highest concentration, 17,500 ppm as "vapour." In light of those results, the authors urge caution be used in attributing mutagenicity to TCE.

In a more standardized *E. coli* assay, Greim et al. (1975) tested several chlorinated ethylenes, including TCE, tetrachloroethylene (perchloroethylene, PCE), and vinyl chloride.

They report that no mutagenic activity of the chlorinated ethylenes was detected in the test system without microsomal (phenobarbital [PB]-induced mice) enzyme activity. With activation, TCE was twofold less mutagenic that VC for most end points.

In contrast, Waskell (1978) reported that TCE had no mutagenic activity on a standardized Ames test. However, CH was weakly mutagenic at 0.5 to 10 mg/plate (see also Henschler et al., 1977).

Macromolecular Binding/DNA Adducts/Strand breaks

Using carbon-14 labeled (^{14}C)-TCE, Bergman (1983) reported that covalent binding to calf thymus DNA *in vitro* occurred only in the presence of mouse liver microsomes. The binding was increased by 50% if PB-induced microsomes were used. This suggests that direct interaction between TCE and native DNA does not occur *in vitro*. Following repeated intraperitoneal ^{14}C-TCE injections in mice, radioactivity in RNA and DNA from selected organs followed similar patterns of accumulation. For RNA, the counts decreased in the order spleen > lung > liver > kidney > pancreas > testis > brain. With DNA, the pattern was spleen > liver > pancreas > lung > testis > kidney > brain. Chromatography revealed the entire radioactivity in liver and kidney RNA, and DNA from kidney, testis, lung, pancreas, and spleen was due to metabolic incorporation. The elution profile in liver DNA gave no direct evidence of the formation of TCE-DNA adducts *in vivo*.

The previous year, Stott et al. (1982) had calculated the maximum hepatic DNA covalent binding index (CBI) for B6C3F$_1$ mice dosed with a "tumorigenic" amount of TCE (1200 mg/kg, corn oil gavage). Because of the atypical peak retention times, the possibility of void volume spillover, and other analytical difficulties, the authors cautioned that the reported values must be viewed skeptically. They suggested that the CBIs for TCE, which ranged from 0.03 to 0.12, must be viewed as a maximum possible alkylation index and should be referenced to the CBIs of known genotoxins such as dimethylnitrosamine (5500) or aflatoxin B-1 (17,000). Following 6-h inhalation exposures at 10 or 600 ppm, the authors reported that mice activated more ^{14}C-TCE to a reactive, macromolecular-binding metabolite. On a picomolar equivalent ^{14}C-TCE per microgram protein basis, mice had three- to fourfold higher binding in renal and hepatic tissue following the 600-ppm exposure than did Osborne-Mendel rats,

presumably due to the saturation of TCE metabolic activation in the rats.

This work was refined by Stevens et al. (1992) in their study of hemoglobin (Hb) and albumin (Alb) adducts of ^{14}C-TCE metabolites in B6C3F$_1$ mice, Fischer 344 (F-344) rats, and isolated hepatocytes from Sprague-Dawley rats. Label associated with the male F-344 rat Hb and Alb is consistently higher than that of the B6C3F$_1$ mouse for TCE, trichloroacetic acid (TCA), and dichloroacetic acid (DCA). However, they demonstrated mice to have more label incorporated into proteins as ^{14}C-amino acids than do rats due to a higher metabolic rate. This is particularly interesting given their observation of the time course of Hb adduct levels. At the highest dose, 100 mg/kg gavage, all TCE is cleared from the system by 1 h in mice and 2 h in rats. However, the Hb adduct time course suggests an early plateau (4 h in the mouse and 8 h in the rat) followed by a slow rise to maximum at 120 h. The authors suggest this phenomenon may represent early adduction followed by a slower process of label incorporation via Hb synthesis in bone marrow. The level of Alb adduct, when corrected for metabolic incorporation, is much higher than that of Hb in both the rat and the mouse. They suggest that adducted Alb may serve as a biomarker of intrahepatocellular levels of reactive metabolites.

Proliferation/Oncogenes

In a 1993 study, Anna et al. discuss the mutational spectrometry within the *ras* oncogene family following a chronic 72-week study in B6C3F$_1$ mice. Dosing with TCE at 1700 mg/kg per os (p.o.) once per day, 5 days per week; PCE at 800 mg/kg p.o. (by gavage); and DCA at 5 g/L drinking water, the authors analyzed for point mutations within specific codons for *H-* and *K-ras*. Spontaneous tumors in control mice also were screened. Trichloroethylene and DCA were found to have a mutational frequency similar to spontaneous tumors. However the spectrum of *H-ras* mutations in tumors from these two agents suggests that they promote different precancerous hepatocytes to tumor than that which occurs in untreated controls. Perchloroethylene appears to involve a different mechanism altogether, which does not involve *H-ras* but does involve, to a limited extent, *K-ras*.

Elcombe et al. (1985) reported on the alteration of DNA synthesis and mitotic activity in B6C3F$_1$ and Alderly Park mice and Osborne-Mendel and Alderly Park rats gavaged with 500

to 1500 mg/kg once per day for 10 days. Significant species differences were observed in DNA and mitotic rates. In mice, a dose-related increase in [^{3}H]dT incorporation was seen with a concomitant increase in mitotic figures. Because there were no histopathological signs of overt cytotoxicity or necrosis, even at the high dose level, the increased incorporation appeared to be due to nonregenerative hyperplasia (cell proliferation). There was a dramatic increase in the number of peroxisomes, which was limited to hepatocytes immediately surrounding the central vein as measured by ultrastructural morphometry. Catalase and CN^--insensitive palmitoyl-coenzyme A oxidation (PCoA) were elevated 1.5- and 8-fold, respectively. In addition, TCE-treated mice showed a change in staining characteristics for the central vein hepatocytes but had no evidence of lipid accumulation.

In contrast, DNA synthesis was not impacted in either rat strain nor were biochemical indices of peroxisome activity elevated. In contrast to mice, rats had only slight, and nonsignificant, increased peroxisome numbers. The basal rate of DNA synthesis in rats was approximately 10 times that of mice, and TCE treatment actually decreased the number of mitotic figures observed. The large increase in DNA synthesis/mitosis in mice may potentially overwhelm repair mechanisms. If this is coupled by increased oxidative challenge, one may expect higher tumor incidence.

Species Differences/Metabolism

The striking differences in TCE-related effects that are observed between mice and rats is generally accepted to be due to species-specific metabolism. For example, mice gavaged with 50 to 2000 mg/kg had a dose-related increase in peroxisome number and associated enzymatic activity (Elcombe, 1985). Rats given the same treatment regime had no response. However, when each species was treated with similar amounts of TCA, both had dose-related PCoA oxidase activity. This observation was reported as being consistent with linear kinetics for TCA formation in mice and saturation kinetics in rats. The author discussed the concept of "intrinsic clearance capacity" (ICC) factors and referred to previous work that had determined that mouse hepatocytes had 30 times the ICC of rat hepatocytes. Furthermore, rat hepatocytes had 3 times the ICC of human hepatocytes.

These metabolic capacity rankings have been confirmed in a recent report that describes

the metabolism of TCE in rat and human hepatocytes (Knadle et al., 1990). These investigators found that rat hepatocytes metabolized TCE about 4 times faster than did human hepatocytes. Metabolite profiles also differed: human hepatocytes produced from 81 to 93% TCOH (total) and 2 to 5% TCA, rats yielded 57 to 76% TCOH (total) and 20 to 28% TCA at the lowest TCE concentration and 8% TCA at the highest TCE concentration. Moreover, rat hepatocytes greatly outproduced human hepatocytes for each metabolite, yielding 15 times more CH, 5 times more TCOH (total), and 20 times more TCA.

Still other studies have confirmed the much greater capacity of mice to metabolize TCE as compared to rats. Stott et al. (1982) reported B6C3F_1 male mice to metabolize 262% more TCE than male Osborne-Mendel rats. Another study showed that rats exhale 53.9% of TCE, given by gavage, as unchanged TCE + carbon dioxide (Dekant et al., 1984). Mice, however, excrete 76% of the dose as urinary metabolites.

TCA AND DCA

Tumorigenicity

Both TCA and DCA are tumorigenic in mice and act as complete hepatocarcinogens (Herren-Freund et al., 1987). In a well-designed study by Bull et al. (1990), fundamental differences in the effects of each metabolite were revealed. Using 1.0 and 2.0 g/L drinking water exposures, male and female B6C3F_1 mice were treated for a total of 52 weeks. A limited group of Sprague-Dawley rats was included to infer species-specificity of any effects. The dose-response curves, expressed as hepatic proliferative lesions (HPLs) per mouse, were markedly different. Trichloroacetic acid exposure produced a very linear increase of lesions with dose; however, the actual number of lesions was only about one-fourth that of DCA. To test the "reversibility" of these lesions, treatment was suspended in some groups at 37 weeks and they were allowed to recover out to the 52-week study end. The multiplicity of tumors predicted from the total dose at 37 weeks was significantly less than expected; however, the relative yield of hepatocellular carcinomas was more than twice that expected from continuous 52-week treatment.

Dichloroacetic acid, on the other hand, demonstrated a very nonlinear threshold-like-dose response, a finding that has been confirmed in other studies (DeAngelo et al., 1991). The

multiplicity of HPLs did not decline in the group allowed the 3 month recovery period, but unlike TCA, these mice did not develop hepatocellular carcinoma.

Interestingly, female mice were much less affected by the treatments; only 3 of 10 in the 52-week DCA group had hyperplastic nodules (no carcinomas), none of the females treated with TCA had such lesions. Similarly, the rats were much less affected by TCA or DCA, even when given a dose of 5 g/L for 12 months. The effects were limited to mild histopathological changes that were small and localized.

The authors concluded that the induction of hepatic tumors by DCA was strongly linked to the severe nontumor pathology it produces in male mice (see also, DeAngelo et al., 1993; Sanchez and Bull, 1990). The very sharp increase in the dose-response curve was closely associated with increases in liver weight. Hepatic proliferative lesions were present as early as 24 weeks at the high dose and these persisted, but did not progress, when treatment was suspended. Taken together with the TCA results, these findings suggest that DCA may be stimulating the growth of cells that are already initiated in this mouse strain.

Conversely, TCA does not produce the overwhelming hepatic cytomegaly of DCA. It does cause accumulation of large amounts of lipofuscin in a dose-dependent manner, which may suggest that TCA can generate a free radical species that initiates lipid peroxidation. The significant histopathological differences discussed so far have been confirmed in many studies (Sanchez and Bull, 1990; DeAngelo et al., 1993; Daniel et al., 1992).

In summary, the dose-response curves, marked differences in histopathology, progression to tumor, and relative potencies of TCA and DCA strongly suggest that they work through different mechanisms to produce tumors. Both compounds are substantially more potent than TCE as hepatocarcinogens in B6C3F$_1$ mice (for DCA see Anna et al, 1993). If compared by calculating doses necessary to increase cancer risk by 0.1 in mice, TCA and DCA can substantially account for the tumors produced by TCE (Larson and Bull, 1992).

Mutagenicity/Genotoxicity

Both TCA and DCA have been repeatedly negative for mutagenicity in *Salmonella* tester

strains when tested as the purified chemical (Waskell, 1978).

The case of direct DNA interaction is not as clear. A single oral dose of TCA or DCA was sufficient to produce DNA single strand breaks (SSBs) in mice and rats (Nelson and Bull, 1988). Dichloroacetic acid (0.23 mmol/kg) was significantly more potent than TCA (0.6 mmol/kg), but neither produced observable hepatotoxic effects at the doses necessary for SSBs to occur; doses that were significantly lower than those required of TCE (11.4 mmol/kg) for the same effect. Furthermore, the SSBs were clearly independent of peroxisome proliferation and quickly reversed within 8 h after dosing (Nelson et al., 1989). The order of potency in rats parallels tumorigenic effects; rats required a 100 times greater TCA dose than mice to increase the rate of DNA alkaline unwinding.

A more recent study conflicts with the earlier reports by finding no DNA SSBs with mouse *in vivo* exposures except at comparatively high levels, 5 and 10 mmol/kg for DCA and 10 mmol/kg for TCA (Chang et al., 1992). Rats were immune to the effect at all doses. Further, a variety of cell types were exposed *in vitro* and no DNA SSBs were detected in dosages that did not give clear evidence of cytotoxicity. Chronic *in vivo* exposures of mice (7 and 14 days) and rats (30 weeks) to DCA in the drinking water (5 g/L) did not induce appreciable DNA damage despite dramatic increases in peroxisomal enzyme activities.

Measurement of SSBs can detect both direct and indirect DNA effects. What seems clear is that this effect is rapid and reversible and does not seem to depend on peroxisomal proliferation. It has been argued that glyoxylate, resulting from metabolism of TCA or DCA, may form Schiff base adducts with purine and pyrimidine bases (Stevens et al., 1992). The instability of these adducts under acidic conditions may account for the inability to detect nucleic acid adducts following *in vivo* exposure. This does not explain the more recent conflicting report, however, because similar extraction protocols were used and no adducts were found.

Macromolecular (non-DNA) Binding

Stevens et al. (1992) reported that both TCA and DCA are capable of producing adducted Alb and Hb in rat and mouse. For rats, there is a lack of early metabolic TCA incorporation

into Alb, which contrasts with an approximately 60% incorporation from TCE. They suggest that the route to incorporation for the rat must be different for the two chemicals. Consistent with their higher rate of metabolism, mice have correspondingly greater portions of TCA and DCA incorporation than do rats. Significantly, the authors report an inability to demonstrate glyoxylate Schiff bases with any exposure.

Oncogenes

Following a 52-week drinking water exposure to 1.0 and 2.0 g/L DCA and TCA, *c-myc* expression was elevated within liver hyperplastic nodules (HNs) in male $B6C3F_1$ mice (Nelson et al., 1990). Hepatocellular carcinomas (HCs) resulting from DCA treatment had equivalent levels of *c-myc* as HNs. However TCA-induced HCs had significantly higher *c-myc* expression than TCA-induced HNs or DCA-induced HNs/HCs. In HCs from animals whose TCA treatment was suspended at 37 weeks, *c-myc* expression remained high relative to control and surrounding tissue at 52 weeks. Furthermore, the expression of *c-myc* in carcinomas from both treatments was heterogeneous; focal areas could be located that expressed oncogenes at varying levels.

Carcinomas derived from either TCA or DCA treatment had significantly increased *c-H-ras* levels relative to controls. A similar study using 5 g/L DCA in drinking water for 72 weeks confirmed that the expression of *H-ras* was elevated in both adenomas and carcinomas (Anna et al., 1993). In addition, the mutational spectrum of the DCA-associated tumors differed from those of both TCE and control groups. Most of the spontaneous tumors had a C to A transversion in codon 61 (CAA) and no point mutations in codons 13 (GTC) or 11 (AAT). DCA tumors had approximately equal frequencies of first and second base transversions (AAA and CTA, respectively) or second base transitions (CGA).

Elevated *H-ras* seems clearly linked to malignancy. Ras oncogenes are associated with elevated levels of guanosine triphosphate-dependent membrane proteins associated with transmembrane signaling. Disruption of this signaling process may account for the decreased intercellular communication that is observed following DCA/TCA treatment in rodent hepatocytes (Klaunig et al., 1989). The difference in *c-myc* expression between TCA- and DCA-induced carcinomas was due to an overall increase in *c-myc* expression throughout the

TCA-induced carcinomas, because there were no indications that focal areas within carcinomas that expressed even higher levels of *c-myc* differed either in size or number whether they were induced by DCA or TCA. The *c-myc* expression in the overall tumor can thus be associated with the earlier progression of TCA-induced neoplasms to hepatocellular carcinoma. In most cells, the consequence of *c-myc* expression is loss of cellular differentiation. The above study (Nelson et al., 1990) was unable to detect foci of altered cells in TCA-treated mice as late as 37 weeks into treatment, despite the fact that significant numbers of easily recognizable HCs could be seen at 52 weeks. The authors suggest that TCA directly or indirectly induced *c-myc* expression in initiated hepatocytes or selectively stimulated the growth of cells that expressed *c-myc* at high levels.

Richmond et al. (1991) report on the progression of DCA-induced lesions, from HN to hepatic adenoma to HC, and their associated pattern of expression of a series of tumor markers: p21 ras, p39 c-jun, p-tyro, BALDH, and (α-fetoprotein). The staining pattern for HNs was distinct in that only small nests of cells were positive for a particular marker among a field of normally appearing marker-negative hepatocytes. As lesions progressed, successively more of the markers were co-expressed, resulting in a significant phenotypic heterogeneity in HA and HCs. Except for a few BALDH-positive centrilobular cells (which have been reported in other studies as well), control sections were all marker-negative. This suggests that areas of transformed hepatocytes were occurring within hyperplastic nodules and that these hepatocytes could develop into later-appearing adenomas or carcinomas. This is generally held to be a general feature of transformation. Given an initiated cell and a favorable environment, clonal expansion can lead to unregulated growth with decreasing fidelity of DNA synthesis and chromosomal segregation. The expanding transformed cells become more and more aneuploid and could be expected to express higher numbers of tumor- associated proteins because of gene recombination. It is not difficult to see how DCA may establish a selective environment given the striking histopathological changes it produces (Sanchez and Bull, 1990; DeAngelo et al., 1993; Daniel et al., 1992). In the case of TCA, however, it is not clear at this point how the early progression of transformed cells comes about.

Proliferation and Histopathology

Increased [^{3}H]thymidine incorporation was seen by Day 5 in livers from male B6C3F$_1$ and Swiss-Webster mice drinking following 0.3, 1.0, and 2.0 g/L TCA or DCA (Sanchez and Bull, 1990). Both showed a dose response; however, only the highest dose was significantly increased for either metabolite. By Day 14, the incorporation level with DCA treatment had fallen by about one-half, while that for TCA remained steady. In autoradiographs of the 14-day treatment groups, DCA had produced a dose-related increase of labeled cells that were localized around necrotic areas. Labeling indices in these proliferative areas were as high as 30% and mitotic figures were reported, a finding similar to that reported by DeAngelo et al. (1993). An overall decrease in hepatic DNA content clearly demonstrated that the treatment-related liver enlargement is largely due to increases in cell size rather than replication (i.e., hypertrophy rather than hyperplasia). In the 1.0- and 2.0-g/L DCA groups, hepatocytomegaly was very evident, and large amounts of glycogen were seen in perilobular cells. Both the localized coagulative necrosis and glycogen deposition were mirrored in the Swiss-Webster mice, indicating these effects are not specific to the B6C3F$_1$ strain.

Trichloroacetic acid treatment produced a uniform distribution of labeled cells, which was identical to control animals. Even at the highest dose level, by Day 14 necrotic areas were of such low frequency (2/20 sections) that is was impossible to determine if they were treatment related. Although cytomegaly was observed, it was mild compared to DCA.

The effects of both compounds at 5 days and TCA at 14 days did not correlate with replicative DNA synthesis measured autoradiographically. Increased repair synthesis is one possible explanation of this phenomena. This explanation would be consistent with the production of DNA strand breaks (see previous discussion) and is consistent with results from this laboratory that showed a prolonged G2M cell cycle phase in rat hepatocytes under TCA exposure (Channel and Hancock, 1992).

Using bromodeoxyuridine labeling, Phelps and Pereira (1993) confirmed these findings. These authors report that both TCA and DCA can induce cell proliferation in the liver of B6C3F$_1$ mice, but the effect is of short duration. They suggest that precancerous (initiated) cells may remain sensitive to TCA or DCA for a longer period, which could result in their

clonal expansion. This is a sort of "promotion" effect that has been demonstrated for TCA, although it is much weaker than that of a classical promoter such as PB (Parnell et al., 1986).

Peroxisomes and Lipid Peroxidation

Trichloracetic acid and DCA do cause peroxisomes to proliferate; however, the effect is very dependent upon rodent species and strain (DeAngelo et al., 1989; Elcombe, 1985; Parnell et al., 1986; Odum et al., 1988). For DCA, the severity of hepatocellular toxicity was associated with enhanced liver cancer whereas peroxisome proliferation was not (De Angelo et al., 1993). For TCA, peroxisome proliferation and liver tumors show a similar dose response (DeAngelo, personal communication).

Lipid Peroxidation

Although it is not as necrotizing as DCA, TCA nevertheless can produce some interesting cellular changes of its own, which may point to its ability to alter cellular growth patterns. It produces a regionalized accumulation of glycogen, which is more prominent in periportal than centrilobular portions of the liver acinus (Bull et al., 1990). This distribution is unlike that of DCA, which causes a much greater, more generalized glycogen accumulation pattern. But more importantly, and quite unlike DCA, TCA produced considerable dose-related accumulations of lipofuscin after a 1-year drinking water exposure. The lipofuscin accumulated in large concentrations in areas surrounding hepatoproliferative lesions induced by TCA; however, it was completely absent from the lesion itself. The accumulation seems to be reversible because lipofuscin levels were no different from controls in mice whose exposure was suspended at 37 weeks. Lipofuscin is a complex of lipid-protein substances derived from lipid peroxidation of membranes and is unambiguously connected to lipid peroxidation.

The ability of acute doses of DCA and TCA to cause lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARS) (Larson and Bull, 1992). Single doses of TCA and DCA (gavage, in water) produced dose-dependent increases in TBARS formation in the livers of mice. The dose-response characteristics for TBARS are remarkably similar to the dose-response characteristics of tumor induction by both compounds. In the dose range effective for tumor induction, there is no significant difference in TBARS

production by mice and rats following DCA treatment (Bull, personal communication). Recent evidence suggests that DCA is hepatocarcinogenic in rats as well as mice; however, rats do not show associated nontumor pathology. This suggests that the histologic changes observed early in mice may not be related to subsequent tumor formation, whereas lipid peroxidation events may. The response to TCA treatment is linear over the 0 to 2000 mg/kg range, and although TCA produced a smaller response than DCA, there is a significant response at lower doses in mice than in rats. Only at doses that exceeded those used in the carcinogenesis bioassay by 4 times is there evidence of increased TBARS in rats treated with TCA. Trichloracetic acid clearly is not hepatocarcinogenic in rats, even at concentrations up to 5 g/L of drinking water for 2 years. Consequently, lipid peroxidation is closely correlated with the tumorigenicity of these two compounds.

Various pretreatment schemes were tried to determine the effect of repeated dosing on TBARS formation. Pretreating B6C3F_1 male mice for 14 days with DCA (1 g/L), TCA (1 g/L) or clofibric acid (250 mg/kg/day) was found to significantly alter TBARS production by acute doses of DCA or TCA administered by gavage on Treatment Day 14. The response to DCA was significantly enhanced by pretreatment with DCA, whereas the response to TCA was inhibited by TCA pretreatment. Clofibric acid pretreatment completely blocked TBARS response from both TCA and DCA, but had little effect on the control group. The author argues that peroxisome proliferation significantly alters P-450 isoform expression; in particular an increase in IVA and decrease in IIE1 has been associated with a number of peroxisome proliferators. The latter isoform is highly associated with the production of carbon-centered free radicals and the induction of lipid peroxidation. This may account for the higher potency of TCA to induce DNA damage and is consistent with other reports of the genotoxic effects of lipid peroxidation products (Zhang and Sevanian, 1993).

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SECTION 8

PEROXISOME PROLIFERATION AND LIPID PEROXIDATION

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OVERVIEW - HEPATIC PEROXISOME PROLIFERATION

Over 60 xenobiotics or physiological states have been reported to produce a proliferation of hepatic peroxisomes in susceptible rodent species (Moody et al., 1991). These include a structurally diverse group of chemicals such as the fibrate hypolipidemics, phthalate ester plasticizers, certain perchlorinated biphenyls, azole antifungal drugs, salicylates, chloroacetic acids, and perfluorocarboxylic acids, as well as high fat diets and diabetes (Lock et al., 1989; Conway et al., 1989; Moody et al., 1991; Reddy and Lalwani, 1983). Peroxisome proliferators produce a characteristic pleiotropic response in the liver of susceptible species, first reported in rats exposed to clofibrate (Paget, 1963). These include marked hepatomegaly arising from both cellular hypertrophy and hyperplasia, a striking proliferation of peroxisomes and smooth endoplasmic reticulum (microsomes), and increased cytochrome P450 content and activity (Cohen and Grasso, 1981; Moody and Reddy, 1978). Peroxisome proliferation has been demonstrated in most animal models tested; however, the dose required and degree of proliferation obtained vary widely. In terms of capacity to proliferate peroxisomes, the rat and mouse are most responsive; the hamster has an intermediate response; and the guinea pig, marmoset, and nonhuman primates are only weakly responsive even at high doses (Eacho et al., 1986; Lake et al., 1989).

PEROXISOME PROLIFERATORS AND HEPATOCARCINOGENICITY

Peroxisome proliferators, including trichloroacetic acid (TCA) and dichloroacetic acid (DCA), score uniformly negative in a wide range of *in vitro* and *in vivo* genotoxity tests in both prokaryotic and eukaryotic systems (DeAngelo et al., 1991). When evaluated in long-term cancer bioassays in rats and mice, however, most (if not all) are nevertheless complete hepatocarcinogens (Reddy and Lalwani, 1983; Moody et al., 1991). The consistent correlation between peroxisome proliferation and nongenotoxic carcinogenicity supports the

original hypothesis of their causal relationship (Reddy and Lalwani, 1983). Several models for the mechanism of their nonmutagenic carcinogenesis have been proposed. The oxidative stress hypothesis proposes that excess hydrogen peroxide (H_2O_2) produced through accelerated peroxisomal *B*-oxidation (induced 20- to 30-fold) exceeds the capacity of catalase and other peroxide degrading enzymes (induced twofold) and results in oxidative damage to the genome (Rao and Reddy, 1991). A number of laboratories have demonstrated DNA damage associated with proliferated peroxisomes both *in vivo* and *in vitro* (Fahl et al., 1984; Kasai et al., 1989) and increased hydroxyl radical formation (Elliot et al., 1986) following exposure. Alternatively, it has been proposed that peroxisome proliferators act through promotion of spontaneously initiated liver foci (Cattley and Popp, 1989; Glauert et al., 1986). This model is supported by the increased DNA synthesis and cell replication seen with peroxisome proliferators in some species (Moody and Reddy, 1978), which may or may not coincide with organelle proliferation (Bieri et al., 1988; Styles et al., 1988). Some peroxisome proliferators have liver tumor incidence rates approaching 100%, however (Reddy and Lalwani, 1983), which has not been reported for any other "pure" promoter (Moody et al., 1991). More recently, increasing evidence supports the existence of a peroxisome proliferator activated receptor (PPAR) proposed to activate the many functionally diverse genes regulated by peroxisome proliferators (Isseman and Green, 1990; Green, 1992). This receptor belongs to the steroid hormone receptor superfamily and contains both DNA-binding and ligand-binding domains (Green, 1992). An attractive hypothesis proposes the correlation between peroxisome proliferation and hepatocarcinogenisis in susceptible species involves a common DNA recognition motif shared by PPAR-activated genes and one or more oncogenes (G. Gibson, personal communication). It appears that in the nonsusceptible species examined (including humans), the PPAR is expressed at very low levels, perhaps in a mutated or truncated form (G. Gibson, personal communication).

TCE, TCA, DCA AND PEROXISOME PROLIFERATION

Trichloroacetic acid, a major metabolite of trichloroethylene (TCE), appears to account for peroxisome proliferation in susceptible species following TCE exposure. Trichloroacetic acid has been shown to be responsible for peroxisome proliferation in TCE-dosed mice (Elcombe, 1985). Trichloroethylene is a peroxisome proliferator in mice but not in rats (Elcombe et al. 1985). Quantitative differences in the metabolism of TCE in rats and mice, and hence in

circulating levels of TCA, may lead to this species difference in peroxisome proliferation. Peroxisome proliferators can be identified as weak, moderate, or strong based on the relative molar concentration required to induce proliferation in susceptible species. Trichloroacetic acid is a weak proliferator in both rats and mice (Elcombe, 1985), with proliferator activity similar to aspirin and an order of magnitude less than a moderate proliferator such as clofibrate (Cannon and Eacho, 1991). Dichloroacetic acid also induces hepatic peroxisomes, but at concentrations significantly higher than required to induce hepatic tumors (DeAngelo et al., 1989).

TCE, TCA, DCA AND LIPID PEROXIDATION

Rao and Reddy (1991) propose that persistent oxidative stress resulting from marked proliferation of peroxisomes (in susceptible species) generates excess H_2O_2 (see above). Free oxygen radicals lead to cellular damage, both directly and through lipid peroxidation. Alternately, reductive dehalogenation of TCE appears to generate a carbon-centered free radical, which can react with oxygen to form a peroxyl radical (Cheeseman et al., 1985). The mechanism has been well defined for carbon tetrachloride (CCl_4): (a) the P-450-mediated cleavage occurs within the endoplasmic reticulum, (b) the product of the cleavage can bind to hepatic proteins and lipids, and (c) the CCl_4-derived free radicals can initiate a process of *autocatalytic* lipid peroxidation by attacking the methylene bonds of microsomal unsaturated fatty acids. Membrane diffusion of peroxidized lipids can damage more distant cellular targets such as mitochondria and plasma membrane via autocatalytic peroxidation. Other data suggest that soluble, stable (not free radicals) cytotoxic products are formed during lipid peroxidation (Comporti, 1985). Nephrotoxic effects of TCE may be linked to the formation of biologically reactive soluble haloalkenyl Cys-S-conjugates (Nagelkerke and Boogaard, 1991).

Dehalogenation of chloroacetic acid metabolites of TCE can generate additional carbon-centered free radicals, which may contribute to the cytotoxicity seen following exposure to these compounds. Larson and Bull (1992) demonstrate increased hepatic lipid peroxidation in rats and mice following acute TCA or DCA exposure, with DCA being the more potent of the two in both species. Lipoperoxidation by DCA does not appear to involve excess H_2O_2 or peroxisome proliferation. The minimum dose of DCA to produce peroxidative damage was 300 mg/kg in mice and rats, which approximates the dose required to induce focal necrosis

and hepatic tumors in mice (Larson and Bull, 1992).

PEROXISOME PROLIFERATION AND HUMAN RISK

Causal or casual, the coincidence of peroxisome proliferation and nonmutagenic hepatocarcinogenisis in rodents is clear. The cancer risk, if any, posed by peroxisome proliferators to relatively nonsusceptible species (such as humans) is less so. Most, but not all, investigators in this field believe that the preponderance of evidence suggests that humans are at limited risk from these compounds. This argument is supported by a lack of characteristic responses in primary human hepatocytes (Elcombe and Mitchell, 1986; Elcombe, 1985) and by epidemiological evidence from extensive therapeutic exposures, some at high dose levels, to hypolipidemic agents such as clofibrate, gemfibrozil, and ciprofibrate for over 30 years without evidence of increased hepatocarcinogenicity. Interim sacrifices (up to 10 years) from ongoing chronic carcinogenicity studies with fibrate hypolipidemics in marmosets have demonstrated no evidence of hepatic tumors (Graham et al., 1994).

The data suggest that TCE presents no significant human hepatocarcinogenic hazard based on peroxisome proliferation. Peroxisome proliferation by TCE can be attributed to its metabolite, TCA (with perhaps a minor contribution from DCA); biotransformation of TCE to TCA in isolated hepatocytes is markedly species dependent, with mouse > > rat > > human; and TCA is not a peroxisome proliferator in human hepatocytes (Elcombe, 1985). Observations reported here suggest a tight correlation between susceptibility to hepatic peroxisome proliferation and risk of hepatocarcinogenesis from peroxisome proliferators. The characteristic hepatic response to peroxisome proliferators and coincident hepatocarcinogenesis appears to be a unique response in rodents. Although the mechanism of the associated epigenetic hepatocarcinogenesis is open for debate, the coincidence of these events suggests that nonsusceptible species are at limited risk.

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SECTION 9

SUSCEPTIBILITY OF B6C3F1 MICE TO HEPATOCARCINOGENESIS

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INTRODUCTION

Trichloroethylene (TCE) increased the formation of liver tumors in $B6C3F_1$ mice following either gavage (approximately 1000 and 2000 mg/kg/day) or inhalation (100, 300, 600 ppm). An increase in liver tumors also was seen in Swiss mice (100, 300, 600 ppm) By contrast, exposure of Han:NMRI mice (100, 500 ppm) did not increase liver tumors.

$B6C3F_1$ mice are the first generation (F1) prodigy of C57BL/6J (B6) and C3H/He (C3) mice. The C3H mice are highly susceptible to hepatocarcinogenesis (males may exceed 50% spontaneous incidence), whereas the B6 are much less susceptible (<4% incidence of spontaneous tumors). Other inbred strains also vary in their susceptibility to formation of tumors in various tissues. Existing inbred mouse strains, as well as creation of inbred recombinant (traditional breeding) and/or transgenic (introduction of genes by molecular biological approaches) strains provide opportunities for studies at the genetic level.

Studies in the mid-1980s identified *ras* and *raf* oncogenes as being activated in spontaneous and chemically induced tumors in $B6C3F_1$ mice (Fox and Watanabe, 1985; Reynolds et al., 1987; Fox and Goldsworthy, 1993). It has been hypothesized that exposure to tumor promoters would give rise to tumors with the same distribution (qualitative and quantitative) of altered oncogenes (and by extension, tumor suppressor genes) as found in spontaneous tumors. Initiating chemicals, in contrast, would be expected to alter DNA in ways that do not occur spontaneously, giving rise to either alterations of different genes or different kinds of mutations in the same genes. This hypothesis may not be correct for either initiators or promoters, for instance, if the tumor promotion process involves selection of specific cell types.

Although mice have been the species most commonly developing liver tumors in chronic

bioassays, hepatocarcinogenesis in rats has been more commonly studied. In particular, much work has focused upon putatively preneoplastic lesions (altered hepatic foci) often using an initiation-promotion protocol. This area will be reviewed first, followed by the putative *Hcs* (hepatocarcinogen sensitivity) locus, and intercellular communication through gap junctions. There are many other areas of research in tumor promotion such as the involvement of regulators of cell cycling (growth factors, etc.) or secondary messengers (diacylglycerol) that will not be considered here.

Altered Hepatic Foci

It has long been a focus of the field of cancer research to identify cells undergoing the cancer process prior to the formation of frank tumors. Foci of cells that stain differently have been identified during histological studies of liver tissues. A variety of terms have been used, including neoplastic nodules, enzyme-altered foci, and altered hepatic foci (AHF) (reviewed in Pitot, 1990; Pitot, 1993).

Morphologically, cells in foci are relatively similar to normal hepatocytes. A long list of differences in enzyme activities, immunohistochemistry, and other markers (e.g., *in situ* hybridization of oncogene sequences) has been identified. Common markers have been deficiencies in glucose 6-phosphate dehydrogenase (G6Pase) and increases in placental glutathione-*S*-transferase and *γ*-glutamyltranspeptidase (GGT). It has been found that the changes occurring in mice and rats are different, with G6Pase being the best for mice (Buchmann et al., 1992). It should also be noted that it is unclear whether the various markers (other than oncogenes) have anything directly to do with the cancer mechanism or if they simply arise as a correlation with that process.

Quantitative stereology has been developed to measure both the number and size (volume) of the AHF. Subsequent studies have focused on correlations of the number of foci with the dose of initiator and the size with promotional effects. The most commonly used protocol in studies of AHF has been initiation with a single dose of *N,N*-diethylnitrosamine (DEN) followed by exposure to a promoter (often phenobarbital or a selected chemical of interest such as TCE). In other cases, partial hepatectomy prior to the DEN administration provides promotion through cell proliferation. Partial hepatectomy is not a universal promoter, however, because

it works in C57BL/6 (B6) but not the more liver cancer-prone C3H/He mice (C3H) (Hanigan et al., 1990).

One study in which several chemicals were used in the promotion stage has found that different populations of preneoplastic lesions resulted (Hanigan et al., 1993). Glucose 6-phosphate dehydrogenase deficient lesions were found in all treatments, but independent populations of GGT+ lesions, more common in rats, were found in livers of animals treated with certain of the promoters.

Another potential indicator of the development of the foci is the complexity of their phenotypic expression (Buchmann et al., 1992). Those foci expressing more markers appear to be later in the tumor process, at least in rats. Several strains of mice (B6, C3, $B6C3F_1$), by contrast, had no change in phenotypic complexity of AHF over time, showing altered expression of multiple (six or more) markers at every time point studied in a protocol using a single injection of DEN and no promoter. No studies were identified during this literature review in which *in situ* hybridization for oncogene expression was carried out at the same time as the enzymatic/immunohistochemical techniques.

One utility of this assay is that it is easier to look at the dose-response relationship than in a 2-year chronic assay. Studies with phenobarbital have shown a no-effect level when the total volume of foci was accounted for (Goldsworthy et al., 1984 in Pitot, 1990). This contrasts with the linear response seen for initiators used in this protocol.

The major outstanding difficulty has been demonstrating that these foci are early stages in the cancer process. An estimate of the number of initiated cells in a liver following DEN exposure is 106 (Pitot, 1993). Following promotion with phenobarbital, there are only 104 AHF per liver. Finally, progression refers to the development of the tumor characterized by aneuploidy, invasiveness, metastasis, etc. This is the period when many oncogene or tumor suppressor gene changes occur. Pitot suggests that only about 10 neoplasms finally develop during progression. This suggests that biological effect modeling for cancer must account for the processes by which altered cells are eliminated from the body. These processes would include apoptosis and immune surveillance.

A study of TCE in the rat liver system found that GGT+ foci were not increased with exposure to TCE, either as the initiator (replacing DEN) or as a promoter (subsequent to DEN) (Milman et al., 1988). This contrasted with a positive finding for tetrachloroethylene (perchloroethylene) and several chlorinated ethanes. Trichloroethylene was perhaps slightly positive in an *in vitro* BALB/c-3T3 neoplastic transformation assay. It should be noted that the *in vivo* assay was carried out in rats, whereas all the liver tumors seen in chronic assays were in mice only. No promotion-type studies of liver foci have been carried out for TCE in mice.

The involvement of AHF in TCE carcinogenicity is unclear. Oral exposure to dichloroacetic acid (DCA) increased hyperplastic nodules, but produced very few AHF (Bull et al., 1990; Richmond et al., 1991). No enzyme-altered foci were identified in mice treated with DCA, nor were basophilic foci seen in trichloroacetic acid (TCA), phenobarbital, or control mice (Bull et al., 1990). It was within the hyperplastic nodules that nests of oncogene-positive cells were identified (Richmond et al., 1991). These studies may indicate that TCE and its metabolites do not involve a cancer mechanism involving formation of AHF or that the appropriate marker has not been identified. These exposures also did not utilize the initiation-promotion protocol, which might be positive for mice.

***Hcs* (Hepatocarcinogen sensitivity) locus**

The meaningfulness of the B6C3F$_1$ liver tumors when no similar tumors are seen in rats has long been a matter of great debate. The arguments, however, have not been enough to convince the Environmental Protection Agency to discontinue use of these tumors as the basis for quantitative cancer risk estimates. Thus, understanding the mechanism of the spontaneous tumors and TCE chemically induced tumors would be very important to better addressing TCE risk assessment.

Genetic studies with the B6, C3H, and B6C3F$_1$ strains led to the identification of the *Hcs* locus by Drinkwater and coworkers at the McArdle Laboratory for Cancer Research, University of Wisconsin (reviewed in Drinkwater et al., 1989). This single genetic locus appears largely responsible for the very high susceptibility of the C3H mice. (This does not mean it is the

only locus involved in the cancer process.)

The *Hcs* locus was identified using tumor induction at 32 weeks by treatment of 1-day-old male mice with a single dose of DEN (Drinkwater and Ginsler, 1986). *N,N*-diethylnitrosamine is a genotoxic initiator of tumors due to the formation of ethyl adducts of DNA bases. It is commonly used as the initiator in two-step rat liver initiation-promotion protocols.

Based upon this study, *Hcs* was estimated to account for 85% of the increased sensitivity of the C3H mice, whereas the remainder was due to a second locus. *Hcs* is autosomal and the C3H and B6 alleles are semidominant such that the $B6C3F_1$ heterozygotes have intermediated susceptibility. At a DEN dose of 0.1 μmol/g body weight, mean tumor multiplicities were about 30, 15, and 0.5 tumors per mouse for C3H, $B6C3F_1$, and B6 mice, respectively (Drinkwater and Ginsler, 1986). Further studies are required to determine if *Hcs* explains the greater sensitivity of males over females, or the higher spontaneous incidence of tumors in C3H mice.

Comparison of the development of G6Pase-deficient hepatic foci (thought to represent initiated cells early in the cancer process) in C3H and B6 mice found the same number of foci in each, suggesting that *Hcs* did not affect the initiation process (Hanigan et al., 1988). On the other hand, foci growth rates were higher in the male C3H mice. In female mice, the rates of foci growth were the same in both strains. Tritiated thymidine labeling also was higher in both normal and G6Pase-deficient hepatocytes. This suggests that the locus affects promotion through increased cell growth of both normal and preneoplastic cells and that it may be dependent upon male hormone.

The *Hcs* locus has not been identified yet, though the chromosomal location may have recently been identified (M. Bennet and N.R. Drinkwater, unpublished results). Until it is identified more concretely, some skepticism about this work has been expressed among cancer researchers (N. Gorelick, personal communication).

Gap Junction Mediated Intercellular Communication

Gap junctions are structures of the plasma membrane between adjacent cells that permit

diffusion of small molecules. Inhibition of the normal functioning of the gap junctions has been implicated in the mechanisms of several kinds of toxicity, including carcinogenesis (Klaunig et al., 1989). In particular, this affect appears associated with tumor promotion. It has been hypothesized that inhibition of the intercellular communication may play a role in releasing preneoplastic cells from controls on cellular proliferation.

The effects of TCE and several metabolites [TCA, trichloroethanol (TCOH), chloral hydrate (CH)] on gap junction mediated intercellular communication was studied in cultured hepatocytes from Fischer 344 rats and $B6C3F_1$ mice. No effects were seen in rat cells, which correlates with the lack of tumorigenicity in rat liver. Both TCOH and CH had no effect on intercellular communications, whereas both TCE and TCA did inhibit mouse cell communication. Generally, only the highest dose of TCE was effective, though occasionally lower doses were also effective. There was somewhat less inhibition when SKF-525A was used to inhibit P-450 metabolism, suggesting that the TCE metabolites may be involved.

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