CYP2E1-DEPENDENT BIOACTIVATION OF 1,1-DICHLOROETHYLENE TO REACTIVE INTERMEDIATES IN MURINE AND HUMAN LUNG AND LIVER MICROSOMES

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ABSTRACT

1,1-Dichloroethylene (DCE), a chemical used in the manufacturing of flexible films and a widespread water contaminant, has been shown to be pneumotoxic and hepatotoxic in animal studies. Although results of previous investigations have showed an important role for cytochrome P450-dependent metabolism in mediating these toxic effects, the ultimate toxic intermediate(s) and isozyme selectivity in DCE bioactivation in lung and liver are unknown. The metabolites of DCE formed in rat liver microsomes have been previously identified as 2,2-dichloroacetaldehyde, DCE-epoxide, and 2-chloroacetyl chloride. These were identified indirectly by trapping them as stable GSH conjugates. However, it was not completely clear which DCE-metabolites gave rise to these conjugates. In the present investigation, we have examined the reaction of these metabolites with GSH using chromatographic and spectrophotometric techniques. We have also identified the reactive intermediates formed in murine and human lung and liver microsomal incubations. The DCE-epoxide reacted efficiently with GSH and formed the mono- and di- glutathione adducts, 2-*S*-glutathionyl acetate [C] and 2-(*S*-glutathionyl) acetyl glutathione) [B]. The equilibrium constant between the hydrate of 2,2-dichloroacetaldehyde (acetal) and the GSH conjugate *S*-(2,2-dichloro-1-hydroxy)ethyl glutathione heavily favored the acetal, indicating that this metabolite does not react appreciably with GSH and will not likely contribute significantly to GSH depletion *in vivo*. The major metabolite formed in microsomal incubations was the DCE-epoxide, as estimated from formation of conjugates [B] and [C]. Lower levels of the acetal of 2,2-dichloroacetaldehyde were also formed. Levels of the DCE-epoxide formed in liver microsomal incubations were higher than those in lung. The DCE-epoxide was

produced at nearly 2-fold higher rates in murine lung, compared with human lung. In contrast, liver microsomes from some patients yielded levels of the DCE-epoxide that were 2.5 to 3-fold higher than those in mice. Our data supported a strong role for CYP2E1 in catalyzing the formation of the DCE-metabolites. The formation of the DCE-epoxide and the acetal of 2,2-dichloroacetaldehyde was inhibited by 50% by a CYP2E1 inhibitory monoclonal antibody. Induction of murine liver CYP2E1 with acetone caused a significant increase in DCE-epoxide formation, while inhibition of human liver CYP2E1 with diallyl sulphone ($DASO_2$) caused a 50% reduction in levels of this metabolite. Our results supported the proposal that DCE-induced toxicity is mediated by P450-dependent metabolism, that the DCE-epoxide may be the most important reactive species, and that CYP2E1 plays a role in this activation. Furthermore, our data suggested that humans exposed to DCE may be at potential risk to deleterious effects in both lung and liver, and humans possessing high expression of CYP2E1 may be particularly susceptible to DCE-induced hepatotoxicity.

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[A]	*S*-(2,2-dichloro-1-hydroxy) ethyl glutatione
[B]	2-(*S*-glutathionyl) acetyl glutathione
[C]	2-*S*-glutathionyl acetate
[D]	*S*-(2-chloroacetyl) glutathione
$DASO_2$	diallyl sulphone
DCE	1,1-dichloroethylene
DIBAL	diisobutylaluminum
DMSO	dimethyl sulphoxide
EI-MS	electron impact mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
GSH	glutathione
HPLC	high performance liquid chromatography
Mab	monoclonal antibody
MCPBA	*m*-chloroperbenzoic acid
NADPH	nicotinamide adenine dinucleotide phosphate
PNP	*p*-nitrophenol

CO-AUTHORSHIPS

Chapter 2. **Dowsley, T.F.**, Forkert, P.G., Benesch, L.A., Bolton, J.L. Reaction of glutathione with the electrophilic metabolites of 1,1-dichloroethylene. *Chemico-Biological Interactions.* **95**: 227-244, 1995.

The experimental contributions for the above manuscript were as follows: Lisbeth Benesch performed the original chemical syntheses and characterization of the DCE metabolites (see Materials and Methods). These synthesized standards were used to develop assays for identifying the metabolites in microsomal incubations. The reaction of glutathione with 2,2-dichloroacetaldehyde (Fig 2.5) was also performed by Lisbeth Benesch. The experimental work pertaining to all of the remaining figures was performed by Taylor Dowsley.

Chapter 3. **Dowsley, T.F.**, Ulreich, J.B., Bolton, J.L., Park, S.S., and Forkert, P.G. CYP2E1-dependent bioactivation of 1,1-dichloroethylene in murine lung: formation of reactive intermediates and glutathione conjugates. *Toxicology and Applied Pharmacology.* **139:** 42-48, 1996.

All of the experimental work pertaining to the above manuscript was performed by Taylor Dowsley.

Chapter 4. **Dowsley, T.F.**, Reid, K., Petsikas, D., Ulreich, J. B., and Forkert, P.G. Cytochrome P450-dependent bioactivation of 1,1-dichloroethylene to a reactive epoxide in human lung and liver microsomes. *Journal of Pharmacology and Experimental Therapeutics.* In press.

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CHAPTER 1

GENERAL INTRODUCTION

Living organisms are continuously exposed to a wide variety of foreign substances in their environment. The major route of exposure to these xenobiotics is *via* ingestion of contaminants in the diet but can also arise from inhalation of airborne materials or exposure to substances on their surfaces (Ames, 1989). Humans are no exception to these sources, but are additionally subjected to xenobiotics taken voluntarily in the form of drugs. Since many environmental chemicals and drugs are relatively hydrophobic (lipophilic), they often cannot be excreted unchanged and must be modified to facilitate elimination from the body (Murray, 1997). Hence, species evolved systems capable of converting xenobiotics to more polar (hydrophilic) products that can then be removed. This process is termed xenobiotic metabolism and plays an essential protective role in detoxifying and eliminating foreign substances (deBethizy and Hayes, 1994; De Groot and Vermeulen, 1997). However, not all metabolic reactions involving xenobiotics result in detoxication. Some xenobiotics require processing through several metabolic steps before being eliminated and some of the intermediates are more toxic than the parent compound (Guengerich, 1992; Pirmohamed *et al.*, 1994; Park *et al.*, 1995; Vermeulen, 1996). In these seemingly paradoxical cases, xenobiotics undergo what is termed metabolic activation (bioactivation). In fact, most cytotoxic agents (Pirmohamed *et al.*, 1994; Vermeulen, 1996) and nearly all carcinogens (Guengerich, 1992) require metabolic activation to exert their deleterious effects.

Metabolism of xenobiotics is mediated by a large range of enzymes with broad substrate specificities that catalyze the metabolism of thousands of chemicals, both natural and synthetic, to which organisms are exposed (Park *et al.*, 1995; Rendic and Di Carlo, 1997). It would appear that these enzymes evolved with the purpose of protecting

against a vast array of toxic xenobiotics. Their broad specificities toward different xenobiotics are unlike many enzymes of normal cellular metabolism. However, many of the enzymes under consideration can be shown to exhibit activity towards endogenous compounds of normal metabolism (Coon and Koop, 1983; Nebert, 1991; Hanukoglu, 1992; Kagawa and Waterman, 1995). This suggests that endogenous functions may have had some bearing on the evolution of these enzyme systems. In any case, they also represent an important mechanism for the metabolism of xenobiotics.

Xenobiotic metabolizing enzymes have been broadly classified into two groups called phase 1 and phase 2 enzymes; the reactions catalyzed by these enzymes are correspondingly termed phase 1 and phase 2 reactions (deBithizy and Hayes, 1994). The phase 1 enzymes generally metabolize chemicals to more polar products, thus increasing their potential for excretion. Some products of phase 1 metabolism are not excreted but undergo further metabolism catalyzed by phase 2 enzymes. Phase 2 reactions are typically biochemical conjugations employing endogenous molecules such as glucuronic acid (Tephly and Burchell, 1990) and glutathione (GSH) (Mitchell, 1973; Grover, 1982), that further enhance the polarity of the phase 1 products. Some conjugates arising from phase 2 reactions are also recognized by specific transport systems facilitating their excretion (Tephly and Burchell, 1990). The substrates for phase 2 enzymes are not only phase 1 metabolites but can also be parent xenobiotics (deBethizy and Hayes, 1994). In those cases where metabolism represents activation to toxic intermediates, the vast majority are phase 1 reactions (Guengerich, 1992), and a proper balance of phase 2 metabolism is necessary to inactivate these intermediates. Although few in comparison to the phase 1 enzymes, there are instances where phase 2 conjugations can produce

reactive intermediates (Coles and Ketterer, 1990; Vamvakas and Anders, 1990) that play a role in toxicity.

The predominant phase 1 enzymes are the cytochrome P450 monooxygenases (Rendic and Di Carlo, 1997). These hemoproteins are the products of a gene superfamily which evolved from a single ancestral gene: they are more than 3.5 billion years old, and were discovered over 40 years ago (Estabrook, 1998). Many laboratories have contributed to the discovery of the structure and function of these enzymes. With the advent of high-speed centrifuges in the late 1940s, separation of subcellular fractions from tissue homogenates made possible the intracellular localization of many enzyme systems. It was determined that the oxidation of many drugs was associated with the microsomal fraction of the liver (La Du *et al.* 1953, 1955; Cooper and Brodie, 1954; Axelrod, 1954, 1955). At around the same period, it was discovered that liver microsomes produced electrophilic compounds from a variety of carcinogens (Muller and Miller, 1953; Conney *et al.*, 1956). In the late 1950s, the existence of a CO-binding pigment was identified in liver microsomes of pigs and rats; this pigment was reducible by either NADPH or sodium dithionite and displayed an absorption maximum of the reduced CO-bound complex at 450 nm (Garfinkel, 1958; Klingenberg, 1958). It was determined in 1964 that this pigment was a hemoprotein located in hepatic microsomes, and was identified as a b-type hemocytochrome with an atypical absorption maximum at 450 nm (Omura and Sato, 1964). It was hence denoted cytochrome P450. Guengerich (1990) points out that the name 'cytochrome' is somewhat erroneous since electrons are not transferred to another acceptor; however, this term remains firmly established. The characteristic absorbance fingerprint that identifies a P450 is due to the presence of a

presence of a thiolate group that represents the fifth ligand to the heme iron (hemin mercaptide complex) of the hemoprotein, and is provided by a cysteinyl residue of the apoprotein moeity (Omura and Sato, 1964). This absorption property of the reduced CO bound complex is currently used for measurement of P450 content.

Cytochrome P450 enzymes are monooxygenases that catalyze the following general reaction: $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$, where RH represents the substrate for the enzyme and ROH is the oxygenated metabolite (deBethizy and Hayes, 1994). This general stoichiometry is manifested in a diverse range of chemical reactions including hydroxylation, N-, O,- and S-dealkylation, sulfoxidation, epoxidation, and deamination to name but a few. Substrates for cytochrome P450s include many drugs and xenobiotics including carcinogens (Guengerich, 1992) and cytotoxicants (Gonzalez and Gelboin, 1994) as well as endogenous compounds. Endogenous reactions include many important hydroxylations involved in steroid biosynthesis (Hanukoglu, 1992; Kagawa and Waterman, 1995) and other lipid biotransformation reactions (Coon and Coop, 1983).

Although individual cytochrome P450 isozymes possess broad substrate specificities, it is obvious that the diversity of substrates and types of chemical reactions could not be achieved by only a few isoforms. To date, there are more than 500 different isoforms (Nelson *et al.*, 1993). There are 481 described in living organisms that are divided into 74 gene families, 14 of which exist in mammals. These are further sub-divided into 26 subfamilies and 20 of these are in the human genome. The present nomenclature uses CYP to identify the P450 as a hemoprotein. The first arabic number refers to the gene family, the following letter is the subfamily, and the second number the

individual enzyme, e.g. CYP2B1 for cytochrome P4502B1. A P450 gene family is defined as having ≤ 40% sequence homology with a P450 isoform from another family. Members of the same subfamily are at least 55% identical.

The cytochromes P450 monooxygenases are dependent upon an external reducing agent for electron donation required for catalytic activity. There are 2 main classes of cytochromes P450 in terms of their electron-donating system. These are the microsomal type and the mitochondrial/bacterial type. Microsomal P450s are bound to the endoplasmic reticulum and accept electrons from another membrane-bound enzyme called NADPH-cytochrome P450 reductase which is a flavoprotein containing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Lu *et al.*, 1969; Lu *et al.*, 1970). Electrons are passed from NADPH to the flavoprotein and subsequently to P450 (Bernhardt, 1998). All of the P450s involved in metabolism of drugs and xenobiotics belong to this class (Rendic and Di Carlo, 1997).

The common catalytic cycle shared by the different isoforms of P450 has been rigorously studied, the details of which are beyond the scope of this thesis. Briefly, catalysis begins with the binding of the substrate with P450 (Rein and Jung, 1993). This orients the substrate near the active site where oxygen binds but also induces a structural change in P450 that enhances the capacity for its one-electron reduction from the ferric (Fe^{3+}) form of the hemoprotein to the ferrous (Fe^{2+}) form by cytochrome P450 reductase (Schwarz, 1991). This allows binding of molecular oxygen to P450. A second one-electron reduction provided by either P450 reductase or another enzyme called cytochrome b5, which uses NADH as the electron donor occurs; this reaction causes the insertion of 1 atom of molecular oxygen into the substrate and reduction of the other

atom to water (Schenkman, 1993). This regenerates the oxidized ferric cytochrome P450 that can initiate the catalytic cycle again.

As mentioned previously, phase 1 metabolism by the cytochrome P450 monooxygenases is not always associated with detoxification, but in some instances, these enzymes catalyze the formation of reactive metabolites. These are typically electrophilic intermediates that have the potential to cause various forms of toxic responses. Covalent binding of a metabolite to nucleic acids can cause the genotoxic process of carcinogenicity (Guengerich, 1992). Covalent adduction to nucleophilic sites on proteins can interfere with the normal physiological functions of those proteins, leading in some instances to necrosis (Bolsterli, 1993). Alternatively, the reactive intermediate or its protein adduct can act as an antigen and initiate an immune reaction (Park *et al.*, 1987; Pohl *et al.*, 1988). Metabolism of xenobiotics by embryonic or fetal cytochrome P450 can initiate teratogenicity and the mechanisms appear to involve covalent binding of electrophilic intermediates to proteins, DNA, or lipids (Wells and Winn, 1996; Wells *et al.*, 1997).

The highest overall levels of cytochrome P450 are found in the liver (Mckinnon and Mcmanus, 1996), and this tissue is therefore a major target site for numerous toxicants and carcinogens (Guengerich, 1992). Substantial levels of P450 enzymes are also found in several extrahepatic tissues, and they have been shown to mediate xenobiotic toxicity in a number of organs including the lung (Yost, 1996; Gram, 1998), kidney (Bruckner *et al.*, 1989), and gastrointestinal tract (Sugimura *et al.*, 1988). Considerable heterogeneity exists in the expression of P450 isoforms between different tissues, cell types, and individuals. This diversity can play a role in the susceptibility of a

particular organ or tissue to the toxic effects of xenobiotics due to varying rates of bioactivation of the parent chemical to reactive intermediates (Mckinnon and McManus, 1996).

Another important factor in determining the potential of a particular cell for toxic injury is its capacity for phase 2 metabolism, which depends upon availability of phase 2 enzymes and appropriate co-substrates for conjugation. The major phase 2 enzymes are the uridine diphospho (UDP) glucuronosyltransferases that catalyze the conjugation of xenobiotics or monooxygenase products with glucuronic acid. The resulting glucuronides are highly water soluble and are easily excreted in the urine or bile. Other important phase 2 enzymes catalyze detoxification reactions including sulphation, methylation, hydrolysis, and conjugation with the tripeptide glutathione (GSH) (deBethizy and Hayes, 1994).

The tripeptide GSH is γ-glutamyl cysteinyl glycine and hence contains a free sulfhydryl group. The nucleophilic thiolate ion (GS^-) is capable of reacting with relatively hydrophobic molecules bearing an electrophilic atom. This reaction serves to inactivate a vast number of potentially harmful electrophiles formed from P450-mediated metabolism of xenobiotics. Depending on the particular electrophile, conjugation with GSH can either be spontaneous or be catalyzed by a family of cytosolic enzymes called the glutathione *S*-transferases (De Groot and Vermeulen, 1997). The resulting GSH conjugates are more water soluble, and are most likely excreted in the urine, although some GSH conjugates are secreted into the bile, and excreted in the feces (deBethizy and Hayes, 1994). Glutathione conjugates that enter the systemic circulation are transported to the kidneys for further metabolism prior to excretion. The GSH conjugates first

localize in the brush border of the proximal tubules where 3 enzymatic reactions occur (Wendel *et al.*, 1977). First, an enzyme termed γ-glutamyl transpeptidase cleaves glutamic acid from cysteine. Second, the glycine group is removed by aminopeptidase M, producing the *S*-substituted cysteine conjugate. The free amino group of the cysteine is then acetylated, forming the mercapturic acid that gets excreted in the urine. Hence, GSH conjugation of an electrophilic intermediate combined with metabolism to mercapturates in the proximal tubules represents a major detoxification and elimination route for toxic agents. Therefore, availability of sufficient levels of GSH near the site of formation of potentially harmful electrophiles is often an important determinant of the extent of toxicity in response to metabolic activation (Grover, 1982). If the rate of biotransformation to electrophilic intermediates exceeds the potential for GSH conjugation, or if conjugation depletes GSH before it can be replenished, covalent binding to macromolecules and toxicity often can occur (Mitchell, 1973; Moussa and Forkert, 1992).

The members of 4 families of P450 are responsible for the majority of xenobiotic metabolism and are hence the most toxicologically significant. These include CYP1, CYP2, CYP3, and CYP4 (Rendic and Di Carlo, 1997). One isoform that has received particular interest due to its inducibility with ethanol exposure and its capacity to produce reactive intermediates that promote cytotoxicity and/or carcinogenicity is CYP2E1 (Raucy *et al*, 1993; Raucy, 1995). This isozyme is expressed constitutively in the liver where it is concentrated mainly in the centrilobular region (Ingelman-Sundberg *et al.*, 1988; Tsutsumi *et al.*, 1989). CYP2E1 is also abundant in the kidney where it is predominantly found in the proximal tubules (Ronis *et al.*, 1991). Lower levels of

CYP2E1 are found in numerous other tissues including the lung (Yang *et al.*, 1991), where it is preferentially concentrated in the Clara cells of the bronchiolar epithelium (Forkert, 1995).

CYP2E1 metabolizes a diverse set of chemicals. In general, the requirement of a molecule in being a CYP2E1 substrate is that it is relatively small and possesses hydrophobic character (Guengerich *et al.*, 1991). Many substrates display a high turnover rate from metabolism by CYP2E1 and display high affinity for this enzyme; it is not unusual for substrates to display Km values in the nM range. Some CYP2E1 substrates are metabolized exclusively by CYP2E1 at low concentrations but act as substrates for other P450s at much higher concentrations (Guengerich *et al.*, 1991; Nakajima *et al.*, 1990). The high affinity of CYP2E1 for small molecular weight substances may, in part, reflect its exclusion of larger molecules that are perhaps not compatible with the active site of CYP2E1 (Guengerich *et al.*, 1991). The finding that some CYP2E1 substrates are metabolized by other P450 isozymes, albeit with much lower affinity, supports this view. Xenobiotic substrates include alcohols, (Koop *et al.*, 1982), acetaldehyde (Richards *et al.*, 1993), acetone (Koop and Cassazza, 1985; Johansson *et al.*, 1986), aromatic hydrocarbons including benzene (Johansson and Ingelman-Sundberg, 1990), ethers including diethylether (Brady *et al.*, 1987), the halogenated anaesthetics enflurane (Richards *et al.*, 1993) and halothane (Olson *et al.*, 1991), many halogenated hydrocarbons including vinyl chloride and trichloroethylene (Guengerich *et al.*, 1991), carbon tetrachloride (Johansson and Ingelman-Sundberg, 1985), nitrosamines (Hong and Yang, 1985; Yang *et al.*, 1990; Sohn *et al.*, 1991), and drugs including acetaminophen (Morgan *et al.*, 1983; Patten *et al.*, 1989) and caffeine

(Gu *et al.*, 1992). Endogenous substrates include acetone (Koop and Cassazza, 1985; Johansson *et al.*, 1986), lipid peroxidation products including pentane (Terelius and Ingelman-Sundberg, 1986) and fatty acids such as arachidonic acid and linoleic acid (Laethem *et al.*, 1993).

A number of the CYP2E1 substrates mentioned above are metabolically activated by this enzyme to cytotoxic or carcinogenic metabolites (Raucy *et al.*, 1993; Raucy, 1995). Similar to most toxicants in general, these CYP2E1 activated xenobiotics affect mainly the liver (Zimmerman, 1978). For example, the hepatotoxicant carbon tetrachloride (Johansson and Ingelman-Sundberg, 1985) or the hepatocarinogen vinyl chloride (Guengerich *et al.*, 1991) are activated by CYP2E1. However, some substrates of CYP2E1 can also be metabolized to toxic intermediates in other tissues including the kidney (Rush *et al.*, 1984) and lung (Yost *et al.*, 1993), and hence can cause deleterious effects in these tissues. The levels of hepatic CYP2E1, and to some extent extrahepatic CYP2E1 can be modulated by nutritional factors (Hong *et al.*, 1987) and by agents such as ethanol (Lieber and DeCarli, 1968; Lieber and DeCarli, 1970; Koop *et al.*, 1982). Since hepatic concentrations of CYP2E1 can vary, so can the levels of toxic metabolites and degree of susceptibility (Raucy, 1995).

As mentioned, CYP2E1 is inducible and the extent of this induction can be quite dramatic. Induction studies in the rat have demonstrated a maximum of a 20-fold increase in hepatic CYP2E1 (Ronis *et al.*, 1993). Many of the chemicals known to cause CYP2E1 induction are also substrates for this enzyme, and hence regulate their own metabolism. Examples include ethanol (Koop *et al.*, 1982), acetone (Johansson *et al.*, 1988), pyrazole (Clejan and Cederbaum, 1990) and isoniazid (Ryan *et al.*, 1985). The

major mechanism of CYP2E1 induction for these agents appears to be post-translational stabilization of this enzyme (Ryan *et al*, 1985; Eliasson *et al.*, 1988; Winters and Cederbaum, 1992). Other mechanisms have been reported depending on the particular xenobiotic and induction protocol including increased translational efficiency (Park *et al.*, 1993) and transcription (Badger *et al.*, 1993). In humans, it is estimated that CYP2E1 can vary at least 50-fold among different individuals (Ekstrom, *et al.*, 1989; Wrighton *et al.*, 1986; Lucas *et al.*, 1993). Some of this variation may reflect the existence of genetic polymorphisms that affect the level of expression of CYP2E1 (Hayashi *et al.*, 1991). However, environmental factors including nutritional status and exposure to xenobiotics, particularly ethanol, are probably the major contributing factors to variations of CYP2E1 levels in humans.

Dietary factors can also regulate hepatic CYP2E1 levels. Starvation causes CYP2E1 induction by increasing transcription rates (Johansson *et al.*, 1990). Ethanol induction of CYP2E1 is more pronounced in conjunction with a low carbohydrate diet (Lieber and DeCarli, 1968; Lieber and DeCarli, 1970) or a diet with a high fat to carbohydrate ratio (Yoo *et al.*, 1991; Ronis *et al.*, 1991). It appears that induction by a high fat diet may be mainly due to unsaturated fatty acids since ethanol-dependent induction of CYP2E1 is more highly potentiated with unsaturated than with saturated fat (Nanji and French, 1989). A physiological condition known to cause CYP2E1 induction is insulin-dependent diabetes (Song *et al.*, 1987). These situations all cause an increase in blood acetone levels, suggesting that acetone metabolism may be an important physiological function of CYP2E1 (Song *et al.*, 1987; Badger *et al.*, 1991; Ronis *et al.*, 1991; Yoo *et al.*, 1991; Takahashi *et al.*, 1992; Pernecky *et al.*, 1994). Acetone

metabolism ultimately leads to formation of pyruvate in the gluconeogenetic pathway that occurs with a high fat supply, and is important for maintaining glucose levels during fasting. Whether acetone is a physiological inducer in these cases is unknown; however, the levels of circulating acetone during these states (100-200 μM) would not likely cause the post-translational modifications of CYP2E1 seen when animals are given high doses of acetone (Johansson *et al.*, 1988), since CYP2E1 displays a low affinity for acetone (Johansson *et al*, 1986). Instead, the evidence suggests that enhanced transcription (Johansson *et al.*, 1990) or stabilization of mRNA (Song *et al.*, 1987) are the mechanisms of induction in these cases.

The large capacity for CYP2E1 induction by many different xenobiotics suggests that exposure to one chemical can effect the metabolism and toxicity of another. Indeed, there are examples of this phenomenon. The CYP2E1 inducers acetone and pyrazole caused a 15 to 30-fold increase in the metabolic activation and hepatotoxicity associated with carbon tetrachloride in rats (Sipes *et al.*, 1973; Johansson *et al.*, 1988; Lindros *et al.*, 1990; Persson *et al.*, 1990), as assessed by the degree of lipid peroxidation and covalent binding of metabolites to proteins (Sipes *et al.*, 1973; Johansson *et al.*, 1988; Lindros *et al.*, 1990; Persson *et al.*, 1990). This potentiation was associated with a parallel increase in immunoreactive CYP2E1 levels in hepatic microsomes (Johansson *et al.*, 1988). Also, DNA methylation and hepatotoxicty associated with *N*-nitrosodimethylamine, a CYP2E1 substrate, was enhanced by ethanol and acetone (Yang *et al.*, 1990). The analgesic agent acetaminophen, when taken in high doses is metabolized to *N*-acetyl-*p*-benzoquinone imine. This electrophilic intermediate depletes cellular GSH and subsequently forms protein adducts that lead to hepatic necrosis (Morgan *et al.*, 1983; Patten *et al.*, 1989).

Much lower doses are hepatotoxic in alcoholics (Black and Raucy, 1986), and this increased susceptibility may be attributed to higher levels of CYP2E1 in alcoholics. However, it should be emphasized that increased levels of CYP2E1 may not be the only cause of synergism between ethanol and other toxicants *in vivo* since ethanol itself is a hepatotoxicant. Nevertheless, there are numerous examples of increased susceptibility to xenobiotics after CYP2E1 induction (Koop and Tierney, 1990).

While CYP2E1 induction can enhance toxic responses to xenobiotics, it is also a useful tool in the laboratory for identifying a chemical as a CYP2E1 substrate. The induction of functionally active CYP2E1 is usually measured by catalytic assays employing substrates that are specific to CYP2E1. Finding CYP2E1-specific substrates can be difficult since different P450s display overlapping specificities. However, the assays commonly used to measure CYP2E1 activities are *p*-nitrophenol (PNP) hydroxylation (Tassaneeyakul *et al.*, 1993), *N*-nitrosodimethylamine *N*-demethylation at low substrate concentrations (Yang *et al*, 1990), and chlorzoxazone 6-hydroxylation (Peter *et al.*, 1990). In addition to induction, CYP2E1-specific inhibition is also useful for determining the role of CYP2E1 in metabolizing xenobiotics. There are a number of mechanism-based inhibitors of CYP2E1. These inhibitors are also substrates for CYP2E1, and inactivate CYP2E1 irreversibly through covalent interactions between a reactive metabolite and the apoprotein or heme of CYP2E1 (Murray, 1997). Hence, catalysis is necessary for inhibition with this class of inhibitors. Mechanism-based CYP2E1 inhibitors include dihydrocapsaisin (Gannet *et al.*, 1990), and the garlic constituent diallyl sulphone ($DASO_2$) (Brady *et al.*, 1991) among others. There are also competitive inhibitors of CYP2E1 including the garlic constituents, diallyl sulphide, and

its metabolite diallyl sulphoxide (Brady *et al.*, 1991). The use of these competitive and non-competitive CYP2E1 inhibitors are very useful in determining the role of this enzyme in xenobiotic metabolism. In addition to these, monoclonal inhibitory antibodies to CYP2E1 such as the anti-rat CYP2E1 inhibitory antibody (Ko *et al*, 1987) have been useful for CYP2E1-specific inhibition.

Among the many small molecular weight chemicals thought to undergo CYP2E1-mediated metabolism and toxicity (Guengerich *et al.*, 1991) are some of the halogenated alkenes. Trichloroethylene is used as an organic solvent in industry and is both hepatocarcinogenic and hepatotoxic in rodents (Bruckner *et al.*, 1989). Several reactive metabolites have been detected, including an epoxide (trichloroethylene oxide), that are likely responsible for covalent binding to liver proteins and DNA (Bolt *et al.*, 1982; Henschler, 1985). The rates of trichloroethylene metabolism and the extent of toxicty were enhanced in rats pretreated with ethanol. Vinyl chloride is a potent hepatocarcinogen that is metabolized by CYP2E1 to several reactive intermediates including the suspected ultimate toxicant, 2-chloroethylene oxide, a reactive epoxide (Raucy *et al.*, 1993). Hence, for the chloroethylenes trichloroethylene and vinyl chloride, a role for CYP2E1 has been proposed in mediating their hepatotoxic effects by metabolizing these chemicals to reactive intermediates. Another chemical belonging to the chloroethylene class is 1,1-dichloroethylene (DCE) or vinylidene chloride. This volatile substance (Hardie, 1964) is man-made and is mass produced in industrialized nations for use in the plastics industry. It is mainly used as a monomeric intermediate (copolymer) for production of plastics including flexible films for food packaging (Birkel *et al.*, 1977). Exposure occurs *via* inhalation in the workplace, as a contaminant of

drinking water, or ingestion of unreacted (unpolymerized) monomers in food packaging (Jaeger *et al.*, 1975). Acute high exposure to DCE in the workplace has been reported to cause central nervous system depression and narcosis, while chronic exposure to low concentrations have resulted in hepatic and renal dysfunction. Contact with the skin causes irritation, and with the eyes results in conjunctivitis and mild corneal injury (Irish, 1963).

Experiments with rats demonstrated that DCE is nephrotoxic, producing acute necrosis of the proximal tubules (Jenkins and Anderson, 1978), and hepatotoxic with damage occurring mainly in the centrilobular hepatocytes. Ultrastructurally, the hepatocytes displayed mitochondrial and membrane damage with sparing of the endoplasmic reticulum (Reynolds *et al.*, 1978). It was also demonstrated that some hepatocytes had characteristics of apoptosis while others appeared necrotic (Reynolds *et al.*, 1980, 1984). DCE has also been shown to display carcinogenic properties in these tissues, and to cause both renal and hepatic tumors in mice with chronic low dose exposure (Maltoni *et al.*, 1977).

Interestingly, the most susceptible tissue to acute toxicity from DCE in animal studies is the lung, regardless of the route of administration (Forkert and Reynolds, 1982; Krijgsheld *et al.*, 1983; Forkert *et al.*, 1986). An oral dose of either 100 mg/kg or 200 mg/kg given to mice caused selective injury to the Clara cells of the bronchiolar epithelium (Forkert and Reynolds, 1982). The Clara cells showed marked dilatation of the cisternae and degeneration of the endoplasmic reticulum at a dose of 100 mg/kg. At 200 mg/kg, the Clara and ciliated cells were necrotic and the epithelium was exfoliated at 6 h after treatment. Recovery of the bronchiolar epithelium was apparent within 48 hr

after the low dose, and within 7 days after treatment with 200 mg/kg. Intraperitoneal injection with 125 mg/kg displayed similar Clara cell-selective damage (Krijgsheld *et al.*, 1983; Forkert *et al.*, 1986), with the proportion of affected cells within an airway progressing with time (Forkert *et al.*, 1986). There were no morphological changes seen in liver or kidney with this DCE dose, and only Clara cells were affected (Krijgsheld *et al.*, 1983). The finding that the Clara cells and centrilobular hepatocytes contain high levels of cytochrome P450 (Boyd, 1977; Ingelman-Sundberg *et al.*, 1985) coupled with the susceptibility of these tissues to DCE-induced toxicity, suggested that metabolism of DCE by P450 may mediate the toxic effects of this chemical.

The Clara cell necrosis occurring with a DCE dose of 125 mg/kg (Krijgsheld *et al.*, 1983; Forkert *et al.*, 1986) was associated with a 50% reduction of lung P450 activity (Krijgsheld *et al.*, 1983). In addition, covalent binding of [^{14}C]-DCE to Clara cell macromolecules was observed, and temporal relationships between the magnitude of this binding, the degree of injury, and reduction of P450 were observed (Forkert *et al.*, 1986). It was also demonstrated that covalent binding and Clara cell injury coincided with a decline in lung GSH (Forkert and Moussa, 1991; Moussa and Forkert, 1992). These studies suggested that reactive intermediates formed from P450-dependent metabolism of DCE mediated the Clara cell necrosis by binding covalently to critical macromolecules. The parallel decline in GSH strongly suggested that it is involved in DCE metabolism and may be important for detoxification of reactive metabolites of DCE. Similar findings as seen in the lung were found for DCE-induced hepatotoxicity. Covalent binding to liver proteins and hepatotoxicity were dependent upon metabolic activation by P450 (Okine and Gram, 1986; Forkert *et al.*, 1987), and were exacerbated by procedures that

lower GSH levels, such as pretreatment with diethylmaleate, fasting, or treatment with DCE when GSH was at its nadir (Jaeger *et* 1973, 1974; Mckenna *et al.*, 1978; Anderson *et al.*, 1980). Also similar to findings in the lung, the magnitude of covalent binding was associated with a corresponding decline in GSH levels (Forkert and Moussa, 1991).

Studies with rat liver microsomes showed that the primary metabolites formed from DCE were the DCE-epoxide, 2,2-dichloroacetaldehyde, and 2-chloroacetyl chloride (Liebler and Guengerich, 1983; Costa and Ivanetich, 1984; Liebler *et al.*, 1985, 1988). These are all electrophilic species making them potential candidates for depletion of GSH and covalent binding to macromolecules (Forkert and Moussa, 1991; Moussa and Forkert, 1992). However, the relative electrophilicities of these metabolites are not identical. They were shown to span a wide range of reactivity ($> 10^5$) toward the model thiols GSH and thiophenol (Liebler *et al.*, 1985). These differences could influence the role that these species play in the toxicity of DCE. In previous studies, rat liver microsomal incubations were supplemented with GSH to trap the electrophilic metabolites as stable conjugates (Liebler *et al.*, 1985; Liebler *et al.*, 1988). Three GSH adducts were formed and identified as *S*-(2,2-dichloro-1-hydroxy)ethyl glutathione [A], 2-(*S*-glutathionyl)acetyl glutathione [B], and 2-*S*-glutathionyl acetate [C] (Fig 2.1). These studies suggested that conjugate [A] arose from reaction of GSH with 2,2-dichloroacetaldehyde, while conjugates [B] and [C] formed from conjugation with the DCE-epoxide, chloroacetyl chloride or its secondary product chloroacetic acid. There was no indication as to the relative contributions of the primary metabolites in forming conjugates [B] and [C], the major products of incubations supplemented with GSH. These are important data since they represent indirect measures of the relative levels of

primary metabolites formed, and may indicate their potential contribution to GSH depletion and/or covalent binding to macromolecules.

A study using rat liver microsomes suggested that the DCE-epoxide was only a minor metabolite of DCE (Liebler and Guengerich, 1983). In this study, the DCE-epoxide was trapped with 4-(*p*-nitrobenzyl)pyridine (Nelis and Sinsheimer, 1981) and P450 was turned over by iodosobenzene rather than $NADP^+$. Since metabolic profiles from iodosobenzene-supported oxidation can differ from those using NADPH (Ortiz de Montellano), it is difficult to conclude from this study that the DCE-epoxide was only a minor metabolite contributing to the formation of conjugates [B] and [C] (Liebler *et al.*, 1985). A careful examination of the reactivities of the primary metabolites and secondary products with GSH, under conditions similar to microsomal incubations, was needed to elucidate what the likely precursors of the GSH adducts [B] and [C] are. In addition, whether these products are also formed in lung microsomal incubations is an important question for elucidation of the mechanisms mediating Clara cell necrosis associated with DCE.

Previous studies suggested a role for CYP2E1 in the metabolism and toxicity of DCE (Kainz *et al.*, 1993; Lee and Forkert, 1994). DCE evoked a dose-dependent decline in PNP hydroxylase activity in microsomal incubations of both mouse liver (Lee and Forkert, 1994) and mouse lung (Lee and Forkert, 1995). NADPH was a requirement for the inhibition of CYP2E1, suggesting the formation of reactive intermediates derived from P450-dependent metabolism of DCE. Hepatocytes isolated from mice pretreated with the CYP2E1-inducing agents ethanol or acetone sustained more severe toxic responses than those from untreated mice (Kainz *et al.*, 1993). Also, inhibition of

CYP2E1 with N, N-dimethylformamide (Mraz *et al.*, 1993) and diethyldithiocarbamate (Guengerich *et al.*, 1991) protected the hepatocytes from the cytotoxic effects of DCE (Kainz *et al.*, 1993). In addition, CYP2E1 in lung tissue has been shown to be localized predominantly in the Clara cells of the bronchiolar epithelium, a target of DCE-induced toxicity (Forkert, 1995). This finding suggests that concentration of CYP2E1-mediated metabolism of DCE in a single cell type may be responsible for the high susceptibility of the Clara cells to DCE-mediated toxicity. Further studies are required to establish definitively the role of CYP2E1 in the metabolism of DCE in liver and lung tissues of mice.

OBJECTIVES AND HYPOTHESIS

The primary objective of this investigation is to obtain further knowledge into the mechanisms mediating the hepatotoxic and pneumotoxic effects of DCE. We hypothesize that DCE is metabolized to electrophilic intermediates by a cytochrome P450-dependent mechanism in murine and human lung and liver tissues, and that CYP2E1 plays an important role in this activation. Here, we have undertaken to identify and characterize the reactive species formed in microsomes from lung and liver tissue.

Specific objectives of this investigation are:

1. To investigate the reaction of GSH with the primary metabolites of DCE and their secondary products in order to gain insight into the ability of these metabolites to capture cellular thiols.

2. To determine whether DCE is metabolized by a P450-dependent mechanism in murine lung microsomal incubations, and to compare the metabolic profile with that in murine liver. The role of CYP2E1 in catalyzing these reactions in lung and liver was also investigated.
3. To investigate formation of metabolites of DCE in human lung and liver microsomal incubations. A comparison in the rates and profile of DCE metabolism in these tissues with those in mice will be performed in order to assess the potential risk to humans exposed to DCE. The contribution of human CYP2E1 in catalyzing these reactions will also be examined in both human liver and lung microsomes.

CHAPTER 2

REACTION OF GLUTATHIONE WITH THE ELECTROPHILIC METABOLITES OF 1,1-DICHLOROETHYLENE

ABSTRACT

1,1-Dichloroethylene (DCE) requires cytochrome P450-catalyzed bioactivation to electrophilic metabolites (1,1-dichloroethylene oxide, 2-chloroacetyl chloride, and 2,2-dichloroacetaldehyde) to exert its cytotoxic effects. In this investigation, we examined the reactions of these metabolites with glutathione by spectroscopic and chromatographic techniques. In view of the extreme reactivity of 2-chloroacetyl chloride, primary reactions are likely to include alkylation of cytochrome P450, conjugation with GSH to give *S*-(2-chloroacetyl)glutathione, or hydrolysis to give 2-chloroacetic acid. Our results showed conjugation of GSH with 1,1-dichloroethylene oxide, through formation of the mono- and di-glutathione adducts, 2-S-glutathionyl acetate and 2-(*S*-glutathionyl)acetyl glutathione respectively. The observed equilibrium constant between the hydrate of 2,2-dichloroacetaldehyde and *S*-(2,2-dichloro-1-hydroxy)ethylglutathione was estimated from ^{1}H-NMR experiments to be $14 \pm 2\ M^{-1}$. Thus, 2,2-dichloroacetaldehyde is unlikely to make a significant contribution to GSH depletion as GSH concentrations above normal physiological levels would be necessary to form significant amounts of *S*-(2,2-dichloro-1-hydroxy)ethylglutathione. We also compared the formation of the glutathione conjugates in rat and mouse liver microsomes using ^{14}C-DCE. The results demonstrated a species difference; the total metabolite production was 6-fold higher in microsomes from mice, compared with samples from rat. Production of DCE metabolites in hepatic microsomes from acetone-pretreated mice was 3-fold higher than those from untreated mice suggesting a role for P450 2E1 in DCE bioactivation. These results indicate that the epoxide is the major metabolite of DCE that is responsible for GSH depletion, suggesting that it may be involved in the hepatotoxicity evoked by DCE. Furthermore, this

metabolite is formed to a greater extent in mouse than in rat liver microsomes and this difference may underlie the enhanced susceptibility found in the former species.

INTRODUCTION

1,1-Dichloroethylene (DCE) is widely used as a monomeric intermediate in the production of flexible films for food packaging. DCE has been implicated in animal studies as a potential mutagen (Bartsch *et al.*, 1975; Jones and Hathway, 1978) and carcinogen (Maltoni *et al.*, 1977) as well as having cytotoxic effects in liver, kidney, and lungs of rodents (Jaeger *et al.*, 1977; Forkert and Reynolds, 1982; Forkert and Moussa, 1991). As has been found for numerous toxic chemicals, DCE requires cytochrome P450-catalyzed bioactivation to exert its biological effects (Jones and Hathway, 1978; Costa and Ivanetich, 1982; Liebler *et al.*, 1985). Evidence that links cellular damage to the formation of DCE-metabolites includes: extensive covalent binding to protein from murine liver (Forkert and Moussa, 1991) and lung (Moussa and Forkert, 1992), and agents that inhibit DCE metabolism or protein binding diminish toxicity. Also, hepatic and pulmonary GSH levels were significantly decreased after DCE administration to mice. In rats, fasting or pretreatment with the GSH-depleting agent diethylmaleate exacerbated DCE-induced hepatotoxicity (Jaeger *et al.*, 1974; Anderson *et al.*, 1980). These findings have given rise to the hypothesis that the detoxification of DCE is primarily dependent upon availability of GSH for conjugation. This proposed mechanism is supported by results from previous studies that showed correlations between hepatocellular damage and magnitudes of both covalent binding and GSH depletion (Forkert and Moussa, 1991). It is of interest in this regard that species and tissue differences in susceptibility to DCE-induced cytotoxicity have been identified in rats and mice (Maltoni *et al.*, 1984). Furthermore, the LD_{50} value for an oral dose of

DCE is 7-fold lower in mice compared with rats (Jones and Hathway, 1978) implying a species difference in metabolic activation and/or detoxification of DCE.

Previous studies have indicated that the primary metabolites formed from DCE are DCE-epoxide, 2-chloroacetyl chloride, and 2,2-dichloroacetaldehyde (Jones and Hathway, 1978; Costa and Ivanetich, 1982; Liebler and Guengerich, 1983; Costa and Ivanetich, 1984; Liebler *et al.*, 1985;; Liebler *et al.*, 1988*)* (Fig. 2.1). All are electrophilic compounds of variable reactivity, which could be expected to influence their target selectivity and efficacy as toxicants (Liebler *et al.*, 1985; Liebler *et al.*, 1988). Secondary metabolites are produced as the result of further oxidation, hydrolysis and conjugation reactions to give the compounds shown in Fig. 2.1. In previous work, the primary reactive metabolites of DCE were trapped as their glutathione conjugates and analyzed by HPLC (Liebler *et al.*, 1985; Liebler *et al.*, 1988). Rat liver microsomes were shown to catalyze the formation of three glutathione adducts identified as S-(2,2-dichloro-1-hydroxy) ethylglutathione [A], 2-(S-glutathionyl) acetyl glutathione [B], and 2-S-glutathionyl acetate [C], (Fig. 2.1). The studies suggested that although [A] is formed by addition of GSH to 2,2-dichloroacetaldehyde, [B] and [C] could result from GSH conjugation with either the DCE-epoxide, 2-chloroacetyl chloride, or the secondary metabolite 2-chloroacetic acid (Liebler *et al.*, 1985). Interestingly, a fourth GSH conjugate S-(2-chloroacetyl)glutathione [D] which is an obligatory intermediate in the formation of [B] from 2-chloroacetyl chloride was not observed. In the present investigation, we examined the reaction of GSH with the primary metabolites by spectroscopic and chromatographic techniques. The results provide insight into the

relative ability of the metabolites to scavenge cellular thiols and/or alkylate biopolymers that may contribute to the mechanism of DCE-mediated toxicity.

Recent studies in mice have shown that P450 2E1 is involved in the biotransformation as well as toxicity of DCE (Kainz *et al.*, 1993; Lee and Forkert, 1994). The greater susceptibility to DCE-induced toxicity in mice compared with rats (McKenna *et al.*, 1977; Jones and Hathway, 1978) suggests that relative levels of P450 2E1 enzyme may differ in the two species. Here we have undertaken to assess this species difference in the metabolism of DCE by comparing the relative amounts of GSH conjugates produced in hepatic microsomes from rats and mice. We have also examined bioactivation of DCE in microsomes from mice pretreated with ethanol, acetone or pyridine. These solvents have been shown to be inducers of P450 2E1, a P450 isozyme that has been implicated in the oxidation of numerous low molecular weight cancer suspects, including trichloroethylene (TCE) and DCE (Kainz *et al.*, 1993; Guengerich *et al.*, 1991; Hewitt and Plaa, 1983). Our results implicate the metabolites of DCE and in particular the DCE-epoxide, which are formed to a greater extent in mouse than in rat liver, as mediating the toxic effects of DCE.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Aldrich (Milwaukee, WI), BDH (Toronto, Ontario) or Sigma (St. Louis, MO) unless stated otherwise. ^{14}C-DCE (97% pure by GLC) was obtained from Amersham Canada Ltd., (Oakville, Ontario) as a 30 μCi/ml solution in corn oil diluted to a specific activity of 0.15 nCi/nmol. Benzphetamine was a generous gift from Health Canada (formerly the Department of National Health and Welfare, Canada). 2,2-Dichloro-1-methoxyethanol, the hemiacetal of 2,2-dichloroacetaldehyde was synthesized according to the following procedure. Briefly, 5.6 g (57.4 mmol) of N,O-dimethylhydroxylamine hydrochloride was dissolved in ethanol free chloroform. 2,2-Dichloroacetyl chloride (7.7 g, 52.2 mmol, Kodak, Rochester, NY) and pyridine (9 ml) were added at 0°C. The solution was stirred at room temperature for 2 hours. The solvent was removed and the product (dichloroacetyl-N,O-dimethylhydroxylamide) was isolated by ether extraction and purified by flash chromatography on silica gel with 3:2 ethyl acetate:hexane as eluant. Dichloroacetyl-N,O-dimethylhydroxylamide was reduced to 2,2-dichloro-1-methoxyethanol with DIBAL (3 ml) in tetrahydrofuran at - 78 °C under an atmosphere of nitrogen. The solution was stirred for 5 min, the reaction quenched with methanol, acidified with citric acid, and the product extracted into ether. 2,2-Dichloro-1-methoxyethanol was characterized by ^{1}H-NMR ($CDCl_3$): δ 1.58 (s, 1H) -OH; 3.52 (s, 3H) OCH_3; 4.71 (s, 1H) Cl_2CH-C<u>H</u>; 5.66 (d, 1H) Cl_2C<u>H</u>-CH. ^{13}C-NMR ($CDCl_3$): 56.06 -OCH_3, 73.06 Cl_2CH-, 98.11 - CH(OMe)OH. In water 2,2-dichloro-1-methoxyethanol exchanges with water to give the hydrate, $Cl_2CHCH(OH)_2$ and methanol; exchange is complete within ten min. ^{1}H-NMR

(D_2O): δ 3.38 (s, 3 H, CH_3OH), 5.30 (d, 1 H, J = 2.6 Hz, $-CH(OD)_2$), 5.92 (d, 1 H, J = 2.6 Hz, Cl_2CH). The spectral data were consistent with published values (Kainz *et al.*, 1993).

The DCE-epoxide was synthesized by oxidation of DCE with MCPBA in CH_3CN or $CHCl_3$ according to (Liebler *et al.*, 1985) with minor modifications. Briefly 2.00 g (11.6 mmol) of MCPBA and 620 µl (7.76 mmol) of DCE were dissolved in 10 ml of dry acetonitrile or chloroform and the solution stirred at 60 °C for 60 min. The solution was distilled in vacuo (20 mmHg) into a flask immersed in a dry ice/acetone bath. The DCE-epoxide was identified by the characteristic absorbance at 560 nm of the 4-(*p*-nitrobenzyl)pyridine derivative (Guengerich *et al.*, 1979) and by ^{1}H-NMR spectra of small scale oxidations in $CDCl_3$ which showed a singlet at 3.2 ppm as reported previously for the methylene protons of the DCE-epoxide (Liebler and Guengerich, 1983).

Synthesis and Characterization of DCE GSH Conjugates. Conjugate [C] was synthesized as described previously (Liebler *et al.*, 1985). The spectroscopic data confirmed the identity of the product as 2-S-glutathionyl acetate. ^{1}H NMR (D_2O) δ2.21 (q, 2 H, *J* = 7.4 Hz, Glu-β), 2.58 (m, 2 H, Glu-γ), 3.07 (m, 2 H, Cys-β), 3.38 (s, 2 H, $-CH_2COOD$), 3.87 (t, 3 H, J = 6.3 Hz, Glu-α), 4.01 (s, 2 H, Gly), 4.65 (dd, 1 H, *J* = 4.9, 8.6 Hz, Cys-α); Electrospray-MS (positive ion , 1% aq.CH_3COOH, pH 2.4), m/z 366 (M+1) (11 %).

Conjugate [D] was synthesized according to (Liebler *et al.*, 1988). ^{1}H NMR (D_2O) δ2.11 (m, 1 H, Glu-β^1), 2.32 (m, 1 H, Glu-β^2), 2.54 (m, 2 H, Glu-γ), 2.97 (m, 2

H, Cys-β), 4.06 (s, 2 H, -CH_2COOD), 4.06 (s, 2 H, Gly-α), 4.23 (s, 2 H, $ClCH_2$-),4.49 (dd, 1 H, *J* = 5, 10 Hz, Glu-α), 4.61 (t, 1 H, *J* = 6 Hz, Cys-α); Electrospray-MS (positive ion , 1% aq. CH_3COOH, pH 2.4), m/z 384 (MH^+) (10 %), 386 (MH^+ + 2) (3 %). The spectral data (^1H NMR, electrospray-MS) were consistent with reported values (Liebler *et al.*, 1988).

Conjugate [B] was synthesized by reacting the DCE-epoxide with GSH as follows. The DCE-epoxide in acetonitrile was added to a 25 ml solution of 50 mM potassium phosphate buffer (pH 7.4) containing 140 mg (0.45 mmol) GSH. The solution was stirred for 20 min at 25 °C. The acetonitrile was removed en vacuo and the resulting aqueous solution was washed with ether. The water was concentrated in vacuo and subjected to semipreparative HPLC with an Ultrasphere ODS column (5 µm, 10 x 250 mm, Beckman) with a flow rate of 5.0 ml/min, and 0.2% H_3PO_4 in H_2O at pH 3.15 as the mobile-phase composition. The eluants obtained from HPLC were all kept frozen until evaporated to minimize hydrolysis. Two major GSH conjugates were produced identified as 2-S-glutathionylacetate [C] and 2-(*S*-glutathionyl)acetylglutathione [B]. The spectral data of [B] and [C] were consistent with reported values (Liebler *et al.*, 1988). The data for [B] are presented here. ^1H NMR (D_2O) δ1.75 - 2.0 (m, 4 H, Glu-β), 2.14 - 2.28 (m, 4H, Glu-γ), 2.86 - 3.13 (m, 4 H, Cys-β), 3.64 (s, 4 H, Glu-α), 4.18 (s, 2 H, -CH_2CO-), Glu-α and Cys-α peaks obscured by solvent suppression of HOD peak; positive ion Electrospray-MS (1% aq. CH_3COOH, pH 2.4), m/z 657 (MH^+) (9 %).

Conjugate [A] was synthesized by combining 6.68 µl (59.9 µmol) of 2,2-dichloro-1-methoxyethanol with 18.4 mg (1.0 equivalents) of GSH in 600 µl of D_2O (pH 7.4). ^1H

NMR (D_2O) δ 2.21 (q, 2 H, J = 7.1 Hz, Glu-β), 2.59 (m, 2 H Glu-γ), 3.22 (m, 2 H, Cys-β), 3.87 (t, 1 H, J = 6.4 Hz, Glu-α), 4.02 (s, 2 H, Gly-α), 4.70 (m, 1 H, Cys-α), 5.34 (dd, 1 H, J = 2.7, 8.0 Hz, Cl_2CH-), 6.22 (dd, 1 H, J = 2.6, 8.4 Hz, -CH(OD)SG). The D_2O was removed and the solid redissolved in H_2O to exchange the acidic protons. The H_2O was removed and the solid was analyzed by FAB-MS (positive ion, glycerol), m/z 420 (0.2 %, MH^+), 307 (91 %, GSH). Electrospray mass spectral analysis (1% aq. CH_3COOH, pH 2.4, positive ion) of a solution of 0.025 M 2,2-dichloro-1-methoxyethanol and 0.25 M GSH (pH 7.4) showed m/z 420 (MH^+), 422 ($MH^+ + 2$), and 424 ($MH^+ + 4$).

Analysis of GSH Reactions with DCE Metabolites. The equilibrium constant (K_{obs}) for the formation of [A] from 2,2-dichloroacetaldehyde was obtained from the integration of the -CH(OD)SG peak and the $-CH(OH)_2$ peak from the ^1H NMR spectra according to the following derivation.

$$K_{eq} = \frac{[Cl_2CHCH(OH)SG]\,[H_2O]}{[Cl_2CHCH(OH)_2]\,[GSH]}$$

$$[GSH]_T = [Cl_2CHCH(OH)SG] + [GSH] = 0.1\ M$$

$$[Cl_2CHCH(OH)_2]_T = [Cl_2CHCH(OH)SG] + [Cl_2CHCH(OH)_2] = 0.1\ M$$

$$[H_2O] = 55.5\ M$$

$$R = \frac{[Cl_2CHCH(OH)SG]}{[Cl_2CHCH(OH)_2]}$$ (obtained from ^{1}H-NMR integration data, mean ± S.D. of 4 determinations)

Therefore,

$$K_{obs}D_2O = \frac{K_{eq}}{[H_2O]} = \frac{R}{[GSH]_T - R\,[Cl_2CHCH(OH)_2]_T \over 1 + R}$$

The equilibrium constant in H_2O was estimated using the solvent deuterium isotope effect K_{H_2O}/K_{D_2O} of 0.44 determined for the addition of a series of thiols to acetaldehyde (Leinhard and Jencks, 1966).

The reaction of 2-chloroacetyl chloride and conjugate [D] with excess GSH was analyzed by combining 2-chloroacetyl chloride with GSH in phosphate buffer (pH 7.4) for final concentrations of 0.5 mM and 5.0 mM respectively. The ability of glutathione S-transferase to catalyze the reaction was checked by including the enzyme (0.3 mg/ml, Sigma) in the incubation. Similar incubations were also carried out with 2-chloroacetic acid and conjugate [C]. Due to difficulties in accurately determining the concentration of the synthesized DCE-epoxide, 25 µl of the $CHCl_3$ solution was added (1.0 mM for 100 % yield of DCE-epoxide) to a wide range of GSH concentrations (0.8 - 10 mM). All incubations were conducted for 30 min at 25 °C in potassium phosphate buffer (pH 7.4) and terminated by chilling in an ice bath followed by the addition of perchloric acid (50 µl/ml). The incubations were analyzed by two different HPLC methods as described below (Adduct Identification).

Animals and Treatments. The procedures for the handling and treatment of animals were in accordance with the appropriate ethical standards of Queen's University. Male Sprague-Dawley rats (150-225 g) and male CD-1 mice (25-30 g) were obtained from Charles River Canada (St. Constant, Quebec, Canada). The animals were maintained on a 12 h light-dark cycle and were fed Purina Rodent Chow and water *ad libitum*. Treatment of mice was carried out usin

g the following protocols: (i) acute acetone (5 ml/kg of 50% acetone in 0.9% saline) was administered by gavage on day 1 and mice were sacrificed 24 h later, (ii) chronic acetone given as 1% acetone in drinking water for 8 days (Forkert *et al.*, 1994), and (iii) pyridine was given intraperitoneally at a dose of 200 mg/kg body weight on day 1 and mice were sacrificed 24 h later (*27*). Microsomes were prepared and protein and P450 content were determined by standard procedures (Thompson et al., 1987). The P450 concentrations for liver microsomes in nmol/mg protein were 0.78 (rat), 0.63 (mouse), 0.13 (acute acetone mouse), 0.21 (chronic acetone mouse), 1.8 (pyridine mouse).

Incubations. Microsomal incubations were performed at 25 °C for 30 min in 50 mM phosphate buffer (pH 7.4) with 1.5 nmol/ml P450, ^{14}C-DCE (S.A. 0.15 nCi/nmole) in corn oil (10 mM) and GSH (5.0 mM). An NADPH generating system consisting of 0.4 mM $NADP^+$, 7.5 mM glucose-6-phosphate, and 1 unit/ml of glucose-6-phosphate dehydrogenase were used together with 5.0 mM $MgCl_2$. For control incubations, $NADP^+$ was omitted. The reactions were initiated by the addition of $NADP^+$ and the vials were sealed with stoppers due to the volatility of DCE. The incubations were terminated by chilling in an ice bath followed by the addition of perchloric acid (50 µl/ml). Due to the low specific activity of ^{14}C-DCE, relatively large volumes of the

vehicle (50 µl/ml) were necessary to observe the metabolites. This led to difficulties in accurately determining the exact radioactivity due to inadequate mixing of the corn oil and buffer. Also, incubations could not be performed at physiological temperature since the boiling point of DCE is 32 ^{0}C and experiments at 37 ^{0}C gave irreproducible results. To determine the effect of 50 µl/ml corn oil on P450 activity, we measured the formation of formaldehyde from N-dealkylation of benzphetamine in untreated mouse liver microsomes by the spectrophotometric method described previously (Nash, 1953). In addition, P450 2E1 activity was assessed by hydroxylation of *p*-nitrophenol as described (Reinke and Moyer, 1985). In both cases, P450 activity did not differ in incubations performed in the presence or absence of corn oil.

Adduct Identification. The incubates were centrifuged at 13,000 rpm for 6 min to precipitate microsomal protein. All incubates were stored at - 70 °C until analysis. Samples (100 µl) were analyzed with a reverse phase C-18 column (5 µm, 4.6 x 250 mm, Microsorb-MV) at 200 nm with a total flow rate of 1.0 ml/min. The isocratic mobile phase was 0.2% H_3PO_4 (pH 3.15). For identification of GSH conjugates, 0.3 ml aliquots of the column effluent were collected during each run and radioactivity was measured with a Beckman model LS 5801 liquid scintillation counter. Concentrations of the GSH conjugates were estimated by summing the radioactivity associated with each peak and converting the data to nanomolar amounts using the specific activity of the ^{14}C-DCE. Alternatively, the samples were derivatized as described previously (Reed *et al.*, 1980). Briefly, the pH was adjusted upon addition of sodium bicarbonate and 250 µl aliquots of the supernatant were derivatized with 250 µl of 0.12 M ethanolic 2,4-dinitrofluorobenzene. The pH was adjusted to 6 by addition of 2.5 µl 6 M HCl. Aliquots

(100 µl) were analyzed by HPLC with a aminopropyl silica column, (5 µm, 4.0 x 250 mm, SGE NH_2-8/5) at 360 nm with a total flow rate of 0.75 ml/min. The mobile phase consisted of 95% solvent A (80% methanol/20% water) (v/v) and 5% solvent B (8M ammonium acetate, 3.4 M acetic acid in 80% methanol/20% water, pH 5.9) at 0.75 ml/min for 10 min, increased to 40% B over 15 min, isocratic for 10 min, and increased to 99% B over the last 15 min. For identification of GSH conjugates, 0.225 ml aliquots of the column effluent were collected during each run and concentrations of the GSH conjugates were estimated as described above.

Instrumentation. HPLC experiments were performed on a Shimadzu LC-10A gradient HPLC with an SPD-10AV UV detector and SIL-10A auto injector. Peaks were integrated with Shimadzu Ezchrom software and a 486-33 computer. UV spectra were measured with a Hewlett Packard Model 8452 diode array UV spectrophotometer, and ^1H NMR spectra were obtained with a Bruker AC-F 200 or AM 400 spectrometer at 200 MHz or 400 MHz respectively. FAB, electrospray, and EI mass spectra were obtained with a VG Quattro instrument. The electrospray conditions were 4 µl/min of 1% aq. CH_3COOH (positive ion) using a Phoenix HPLC system 20.

Statistical Analysis. Statistical analysis was performed using the unpaired Student "t" test. Significance was determined at $P < 0.05$.

RESULTS

HPLC Analyses of DCE Incubations. The oxidation of DCE to electrophilic metabolites by hepatic microsomes was determined by trapping these reactive species with GSH (Liebler *et al.*, 1985). Incubation of DCE with mouse liver microsomes, derivatization of the post incubate with 2,4-dinitrofluorobenzene and separation by HPLC gave the radiochromatogram shown in Fig. 2.2. The three GSH conjugates (Peaks 1 - 3) were previously identified as [A], [B], and [C] in order of increasing elution time (Liebler *et al.*, 1985). Our results agree with the asignment of [B] and [C] to peaks 2 and 3 however, we believe peak 1 is conjugate [D] resulting from reaction of the 2-chloroacetyl choride with GSH (see below). Separation of the microsomal products by reverse phase HPLC (no derivatization) also gave 2 major peaks (Fig. 2.3A) and a minor product. These peaks completely disappeared in the absence of GSH (Fig. 2.3B) and in incubations without $NADP^+$ (data not shown). An increase in radioactivity eluting from the column between 2 and 4 min in the incubation without GSH was also observed (Fig. 2.3B). Glycolic acid and formaldehyde, produced from decomposition of the DCE-epoxide (Liebler and Guengerich, 1983), elute in this region; however, we cannot at the present time confirm their identity. In all cases studied, analysis of the conjugates without derivatization produced peaks eluting with 3 - 4 times the radioactivity compared to the derivatization method. This increased sensitivity cannot be fully explained by dilutions required for the derivatization and may be the result of instability of the conjugates to the derivatization process which may cause 25 - 50 % destruction.

Reaction of GSH with DCE-metabolites. In order to investigate the source of the GSH conjugates produced in the microsomal incubations, we examined the chemical reaction of GSH with the primary metabolites of DCE; 2-chloroacetyl chloride, 2,2-dichloracetaldehyde, and DCE-epoxide.

2-Chloroacetyl chloride. Under the reaction conditions used in the present study, the only GSH conjugate produced from addition of GSH to 2-chloroacetyl chloride was [D]. ^{1}H-NMR experiments using 0.2 M GSH and 0.1 M 2-chloroacetyl chloride in D_2O (pH 7.4) revealed the formation of [D] as well as hydrolysis of the 2-chloroacetyl chloride to 2-chloroacetic acid. HPLC analyses of incubations performed using a 10:1 ratio of GSH to 2-chloroacetyl chloride only showed the formation of [D] (Fig. 2.4). Conjugates [B] and [C] were not observed with either HPLC method. The pseudo first-order rate constant for reaction of [D] with GSH in water has been determined to be 0.0058 min^{-1} (Liebler *et al.*, 1988), which suggests that little if any formation of [B] during the 30 min reaction period would be observed. Derivatization of purified [D] with 2,4-dinitrofluorobenzene and HPLC analysis gave a peak with similar chromatographic properties to that of peak #1 produced in microsomal incubations (Fig. 2.2). This peak had previously been identified as conjugate [A] (Liebler *et al.*, 1985).

Formation of conjugate [C] in microsomal incubations could arise from conjugation of 2-chloroacetic acid with glutathione, or alternatively by hydrolysis of 2-S-glutathionylacetyl chloride which is the initial product of attack of GSH on the DCE-epoxide (Fig. 2.1). In order to determine the plausibility of the former hypothesis, 2-chloroacetic acid was combined with GSH with and without rat liver glutathione S-transferase. The solutions were monitored by ^{1}H-NMR for 1 hour, during which time no

peaks corresponding to [C] were observed in either sample (data not shown). As the non-enzymatic first-order rate constant of reaction of GSH with $ClCH_2COOH$ is very slow [k_{obs} = 0.006 min^{-1} (Liebler *et al.*, 1985)], we believe that it is unlikely that conjugate [C] is derived from 2-chloroacetic acid under the conditions of our microsomal incubations.

2,2-Dichloroacetaldehyde. ^1H NMR analysis of the reaction of the hydrate of 2,2-dichloroacetaldehyde with GSH (Fig. 2.5) revealed that the equilibrium between [A] ($Cl_2CHCH(OH)SG$) (Fig. 2.1) and the hydrate of 2,2 dichloroacetaldehyde heavily favors the hydrate at physiological concentrations of GSH. The observed equilibrium constant was estimated as described in the methods section to be $K_{obs}^{H_2O} = 14 \pm 2\ M^{-1}$. The equilibrium constant for thiohemiacetal formation (K_S) and the hydration equilibrium constant (K_h) are related to the Taft polar substituent constant σ^* according to the following relationships.

$$\log K_S = 1.65\ \sigma^* + 1.41 \quad \text{(Kanchuger and Byers, 1979)}$$

$$\log K_h = 1.68\ \sigma^* - 0.033 \text{ (Kanchuger and Byers, 1979)}$$

As the σ^* value for Cl_2CH is 1.94 (Perrin *et al.*, 1981), K_S for formation of [A] can estimated at 41,000 M^{-1}. Similarly, the estimated K_h for the hydration of 2,2-dichloroacetaledehyde is 1,700 M^{-1}. The observed association constant (K_{obs}) is a function of these equilibria as follows.

$$K_{obs} = \frac{K_S}{(1 + K_a/[H^+])\,(1 + K_h)} \quad \text{(Kanchuger and Byers, 1979)}$$

Thus, with the knowledge of K_a for the thiol group of GSH (pK_a 9.1) and the pH of the solution, K_{obs} was calculated to be 23 M^{-1} in the pH range of 3 to 8. This calculated equilibrium constant is in the same range as the experimental value of 14 M^{-1}. The ratio of hydrate to [A] would hence be 14:1 in microsomal incubations containing 5 mM GSH. All attempts to analyze [A] either directly or as its 2,4-dinitrobenzene derivative by HPLC failed presumably because the derivatization process and/or chromatographic separation drives the equilibrium toward the free hydrate. The synthesized hydrate standard had a retention time of 6 min using the reverse phase HPLC conditions and radioactivity eluting in this region can be observed both in the presence and absence of GSH (Fig. 2.3).

DCE-Epoxide. The reaction of the synthesized DCE-epoxide with GSH gave an electrospray mass spectrum consistent with the formation of both [B] (MH^+, 657) and [C] (MH^+, 366) (Fig. 2.6). Excess GSH (MH^+, 308) and GSSG (MH^+, 613) formed from MCPBA-catalyzed oxidation of GSH were also observed. Analysis of the same sample 24 hours later showed an increase in intensity of the 366 ion ([C]), whereas the 657 ion ([B]) had completely disappeared (data not shown). Conjugate [B] is known to hydrolyze to [C] in aqueous solution [$t_{1/2} \sim 3$ h, (Liebler *et al.*, 1985)]. Ion exchange HPLC analysis of the 2,4-dinitrobenzene derivatives (Fig. 2.7A) gave peaks with identical retention times to those of conjugates [B] and [C] in the radiochromatograms from microsomal incubations. The chromatographic properties of derivatized [B] and [C] were consistent with published chromatograms (Liebler *et al.*, 1985). Reverse phase HPLC analysis of the underivatized reaction using 2.0 mM GSH only showed the formation of [B] (Fig. 2.7B) although [C] could be observed in samples that were

analyzed at later time periods or in reactions where lower concentrations of GSH were used. Conjugate [D] was not detected in any of the experiments indicating that during the synthesis of the DCE-epoxide, rearrangement to 2-chloroacetyl chloride did not occur. These data suggest that [B] and [C] arise from the DCE-epoxide. Moreover, we believe that conjugate [B] is formed from non-enzymatic reaction of 2 molecules of GSH with the DCE-epoxide, and that conjugate [C] is formed from hydrolysis of either 2-*S*-glutathionylacetyl chloride or [B] (Fig. 2.1). Glutathione S-transferase-mediated catalysis of the reaction between the synthesized DCE-epoxide and GSH was not examined however, and it is possible that formation of the conjugates is enzyme catalyzed in vivo.

Taken together, the above data suggest that conjugates [B] and [C], which are the major GSH conjugates produced in DCE microsomal incubations, arise from the reaction of GSH with the DCE-epoxide. Addition of GSH to the 2-chloroacetyl chloride gives [D] which corresponds to peak 1 in the ion exchange HPLC analysis of microsomal incubations. The results also indicate that conjugate [A] is unlikely to be observed under the experimental conditions used in the present studies as physiological concentrations of GSH heavily favor the free hydrate.

Cytochrome P450-Dependent Oxidation of DCE. Incubations of rat and mouse liver microsomes with DCE produced the DCE-epoxide GSH conjugates ([B] and [C]); however, the amounts of [D] produced were close to the limit of detection (0.5 nmol/mg protein/min) and could not be precisely quantified. Also, considerable variation was observed with [D] between the two HPLC separation methods, a problem not encountered with [B] and [C]. The total amounts of conjugates formed ([B] + [C]) in

mouse liver (6.3 $\pm$ 0.9 nmol/nmol P450/min) were significantly higher than in rat liver (1.1 $\pm$ 0.3 nmol/nmol P450/min).

The effects of exposure to acetone and pyridine on the conversion of DCE to [B] and [C] were measured with hepatic microsomes from treated and untreated mice. As shown in Fig. 2.8, chronic acetone treatment enhanced the formation of the DCE-epoxide GSH conjugates 3-fold compared to microsomes from untreated mice. In contrast, acute treatments with acetone or pyridine did not significantly increase DCE metabolism.

DISCUSSION

There is substantial evidence in mice linking DCE-induced hepatotoxicity to electrophilic metabolite formation and subsequent binding to cellular nucleophiles. This species-related effect has been ascribed to enhanced metabolism of DCE by murine liver compared to rat liver in vivo (Jones and Hathway, 1978). In the present *in vitro* investigation, we trapped these reactive intermediates with GSH to determine if murine liver microsomes have a greater overall ability to metabolize DCE than rat microsomes. The results showed significant increases (6-fold) in the levels of GSH conjugates produced in microsomal incubations with DCE, compared with levels produced in analogous experiments with rat liver microsomes. The greater *in vivo* susceptibility of mice to DCE-mediated toxicity may therefore be linked to more efficient metabolism of DCE by a specific P450 enzyme present at higher levels in the mouse.

Previous studies have attempted to indirectly determine isozyme-selective metabolism and toxicity of DCE by using P450 inducers. These studies have produced equivocal results; pretreatment of rats (Carlson and Fuller, 1972) or mice (Forkert *et al.*, 1986; Kainz *et al.*, 1993) with the prototypic P450 2B and P450 1A inducing agents phenobarbital and 3-methylcholathrene, respectively, did not potentiate the hepatotoxic effects of DCE. Pretreatment of mice with these inducers also failed to alter *in vivo* covalent binding of DCE (Forkert *et al.*, 1986). In contrast, other studies have shown increased covalent binding in the livers of mice pretreated with the same inducing agents (Okine *et al.*, 1985); however, the effect was slight and may not reflect isozyme-selective metabolism of DCE. Of significance is the finding that pretreatment with ethanol or acetone augmented DCE-mediated hepatotoxicity (Kainz *et al.*, 1993; Hewitt and Plaa,

1983). Here we have shown that hepatic microsomes from mice receiving a chronic acetone treatment regimen were the most effective in metabolizing DCE to GSH conjugates (Fig. 2.8); a 3-fold enhancement in metabolite production was observed compared to levels in microsomes from control mice. No significant differences were observed between incubations using microsomes from untreated mice or mice treated acutely with acetone or pyridine; however, the rank order for total conjugate production was chronic acetone > acute acetone > acute pyridine > untreated. These results are consistent with P450 2E1-selective metabolism of DCE, and the extent of conjugate formation correlated with the level of induction of the P450 2E1 enzyme observed for these treatment regimens (Forkert *et al.*, 1994). Recent studies in mice have provided evidence to demonstrate selective bioactivation of DCE by P450 2E1 and not by P450 2B, a P450 enzyme also induced by acetone treatment (Lee and Forkert, 1994).

Previous studies examined the reactivity of the DCE metabolites with GSH to determine their role in GSH depletion and alkylation of other cellular components (Liebler *et al.*, 1985; Liebler *et al.*, 1988). Three major GSH conjugates labeled [A], [B], and [C] (Fig 2.1) were produced in rat liver microsomal incubations. Conjugates [B] and [C] were believed to result from reaction of GSH with either the DCE-epoxide, 2-chloroacetyl chloride, or the hydrolysis product 2-chloroacetic acid (Fig 2.1). Our results agreed with the above mentioned data in that we also determined conjugates [B] and [C] to be the major GSH adducts formed. However, our study indicated that the DCE-epoxide is the exclusive precursor for both conjugates [B] and [C]. Reaction of the synthesized DCE-epoxide with GSH produced both conjugates [B] and [C] as determined by HPLC and electrospray mass spectrometry. Previous studies have suggested that the

DCE-epoxide is only a minor metabolite produced from the P450-catalyzed oxidation of DCE (Liebler and Guengerich, 1983). The discrepancies between our study and earlier work may be ascribed to the detection methods used for measuring DCE-epoxide formation. One assay which was used routinely involved reacting the DCE-epoxide with 4-(*p*-nitrobenzyl)pyridine and measuring the absorbance of the colored product at 560 nm. The 4-(*p*-nitrobenzyl)pyridine method has great sensitivity but with the disadvantage of instability of its final color (Nelis and Sinsheimer, 1981). We have measured the half life of the DCE-epoxide 4-(*p*-nitrobenzyl)pyridine derivative and estimated it to be 60 s at 25 ^{0}C. The method used to quantify DCE-epoxide formation from DCE using purified P450 enzymes was the fluorimetric procedure based on alkylation of nicotinamide (Nelis and Sinsheimer, 1981). $NADP^{+}$ interfered with the fluorescent product and thus DCE-epoxide formation was studied using iodosobenzene-supported oxidation of DCE by P450 (Liebler and Guengerich, 1983). The catalytic turnover of P450 by iodosobenzene can result in a very different metabolic profile compared to NADPH (Ortiz de Montellano, 1986). Also, the pyridine nitrogen of nicotinamide is a relatively weak nucleophile and competing hydrolysis and alkylation reactions may also lower the amount of DCE-epoxide trapped. Our results with GSH as the trapping agent suggests that much more of the DCE-epoxide is formed as judged by the amount trapped by the more reactive thiol nucleophile.

We did not observe conjugates [B] or [C] in similar experiments with the 2-chloroacetyl chloride and GSH; only [D] and 2-chloroacetic acid were obtained using a 2:1 molar ratio of GSH to 2-chloroacetyl chloride. Under pseudo-first order conditions when GSH concentration is 10-fold higher than 2-chloroacetyl chloride, only [D] was

observed. To the best of our knowledge, the hydrolysis rate constant for 2-chloroacetyl chloride has not been determined, although it should be similar to that of acetyl chloride [k_{obs} = 1100 s^{-1} (Palling and Jencks, 1984)]. Reaction with the highly nucleophilic thiol moiety of GSH should be orders of magnitude faster than hydrolysis, suggesting that all the 2-chloroacetyl chloride formed, that escapes from P450, should react with GSH giving [D] observed in microsomal incubations. Once the pool of GSH is depleted, hydrolysis to 2-chloroacetic acid will predominate. The high reactivity of 2-chloroacetyl chloride also implicates it in the reported destruction of P450 2E1 through alkylation of nucleophilic groups on the apoprotein (Lee and Forkert, 1994).

Formation of conjugate [C] from reaction of GSH with the 2-chloroacetic acid or conjugate [B] from a similar reaction with [D] in microsomal incubations is dubious due to the relatively slow rate of reaction of these compounds with GSH in aqueous solution [$t_{1/2}$ ~ 2 h (Liebler *et al.*, 1985, 1988)]. Since incubations were terminated at 30 min, it is unlikely that any production of conjugate [B] or [C] from these metabolites would occur. It is possible these conjugates are produced from these precursors *in vivo* either by a non-enzymatic process or through glutathione S-transferase mediated metabolism.

Conjugate [A], formed from attack of GSH on the 2,2-dichloroacetaldehyde was previously reported to be the first GSH conjugate eluting in the HPLC analysis of microsomal incubations (Liebler *et al.*, 1985). Our results indicate that it is unlikely that conjugate [A] will be observed under the conditions of the present study or *in vivo* unless the cellular levels of GSH were relatively high (> 10 mM). Using the experimental constant, the calculated ratio of the hydrate of 2,2-dichloroacetaldhyde to conjugate [A] in our derivatized microsomal experiment would be approximately 28:1. This indicates

that the 2,2-dichloroacetaldehyde is unlikely to be responsible for significant GSH depletion. In addition, the 2,2-dichloroacetaldehyde was less toxic than DCE to isolated mouse hepatocytes (Kainz *et al.*, 1993), arguing against its role as an intermediate on the bioactivation pathway. However, the *in vivo* formation of the 2,2-dichloroacetaldehyde may contribute to alkylation of cellular proteins as reported for the 2-chloroacetaldehyde metabolite formed from P450-catalyzed oxidation of vinyl chloride (Guengerich *et al.*, 1979, 1981). Experiments in isolated rat hepatocytes showed that 2-chloroacetaldehyde formed irreversible GSH conjugates presumably through displacement of the α-chlorine in a S_N2 reaction (Sood and O'Brien, 1993). A similar S_N2 displacement reaction would be more difficult for 2,2-dichloroacetaldehyde (or the hydrate) due to steric crowding caused by the additional chlorine atom. In contrast, the same study showed that protein thiols formed reversible adducts with 2-chloroacetaldehyde which suggests that reaction occurred at the carbonyl moiety. In the hydrophobic environment of a protein, an analogous reaction could also occur for 2,2-dichloroacetaldehyde.

The relative electrophilicities of the metabolites of DCE spans a wide range [$> 10^5$ (Liebler *et al.*, 1985)]. The results from the present study have provided additional insight into the fate of DCE-metabolites in cellular systems (Fig. 2.1). The extreme reactivity of the 2-chloroacetyl chloride suggests that reaction within the active site of P450, alkylation of GSH in the immediate environment of P450 to give [D], or hydrolysis to give 2-chloroacetic acid are likely the primary reactions of this metabolite. Furthermore, alkylation of cellular proteins or nucleic acids remote from its site of formation are unlikely events. The DCE-epoxide is the most plausible candidate for GSH depletion in forming conjugates [B] and [C]. Its intermediate reactivity also implicates it

in alkylation of cellular biopolymers. It is unlikely that the 2,2-dichloroacetaldehyde will significantly alter intracellular GSH concentrations, although alkylation of nucleophilic residues on proteins or DNA may contribute to damage caused by DCE. Finally, our results suggest that the hepatotoxic effects of DCE are the result of P450 2E1-dependent bioactivation to reactive intermediates of which the DCE-epoxide may be the ultimate toxic species.

FIG. 2.1. Proposed pathways for DCE metabolism in the presence of GSH.

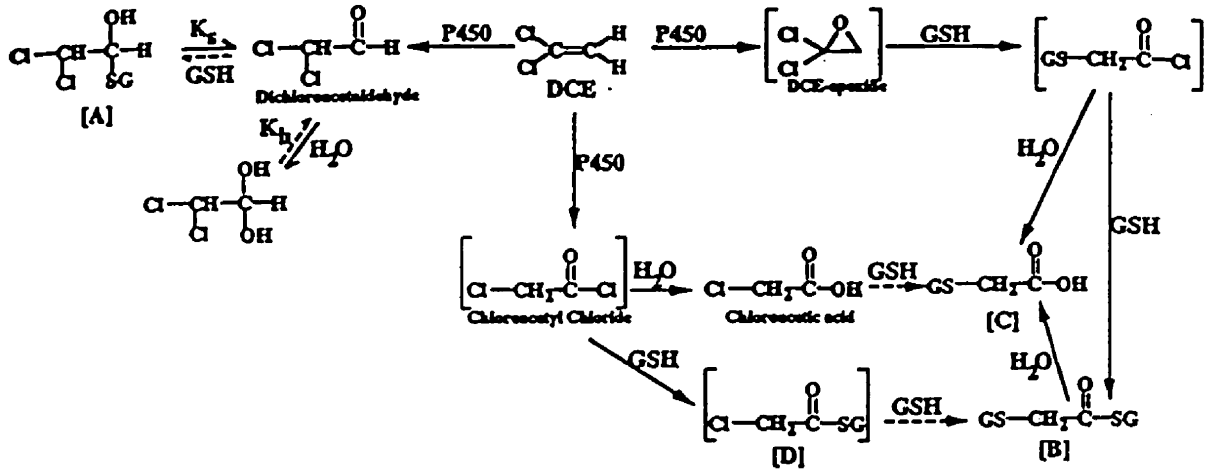

[A]
GSH
P450
DCE
P450
GSH
P450
GSH
GSH
[C]
GSH
[D]
GSH
[B]

FIG. 2.2. HPLC analysis of derivatized glutathione adducts produced from 10 mM 14C-DCE by mouse liver microsomes in the presence of an NADPH-generating system and 5.0 mM GSH after 30 min incubation at 25°C. The sample was derivatized with 2,4-dinitrofluorobenzene as described in Materials and Methods. Radioactivity eluting from the HPLC column was measured in fractions collected at 18s intervals. The figure represents data from which the radioactivity eluting in the NADPH sample has been subtracted.

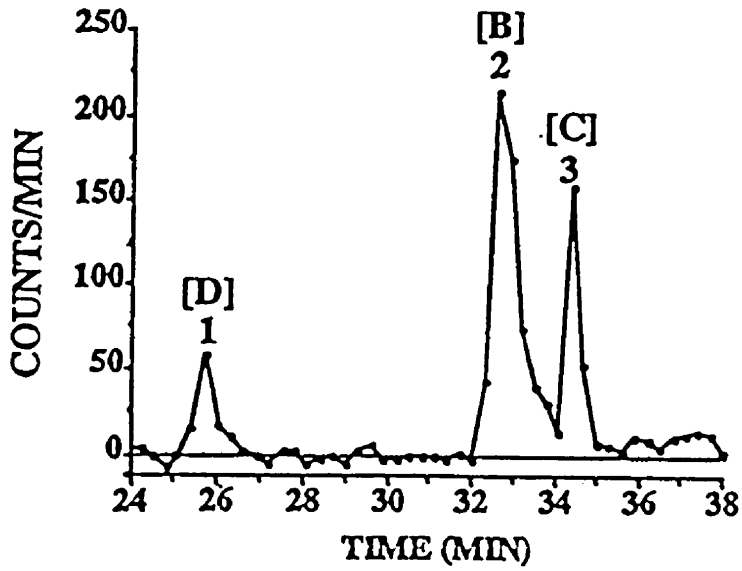

250
200
150
100
50
0
COUNTS/MIN
[D]
1
[B]
2
[C]
3
24
26
28
30
32
34
36
38
TIME (MIN)

FIG. 2.3. HPLC analysis of microsomal incubate in the presence (A) or absence (B) of 5.0 mM GSH. Incubation conditions were 10mM ^{14}C-DCE, mouse liver microsomes (1.5nmol/ml P450), NADPH-generating system, 25°C, pH 7.4, 30 min. incubation time. The samples were analysed on C-18 reverse phase column as described in Materials and Methods. Radioactivity eluting from the HPLC column was measured in fractions collected at 18s intervals. The figure represents data from which the radioactivity eluting in the NADPH sample has been subtracted.

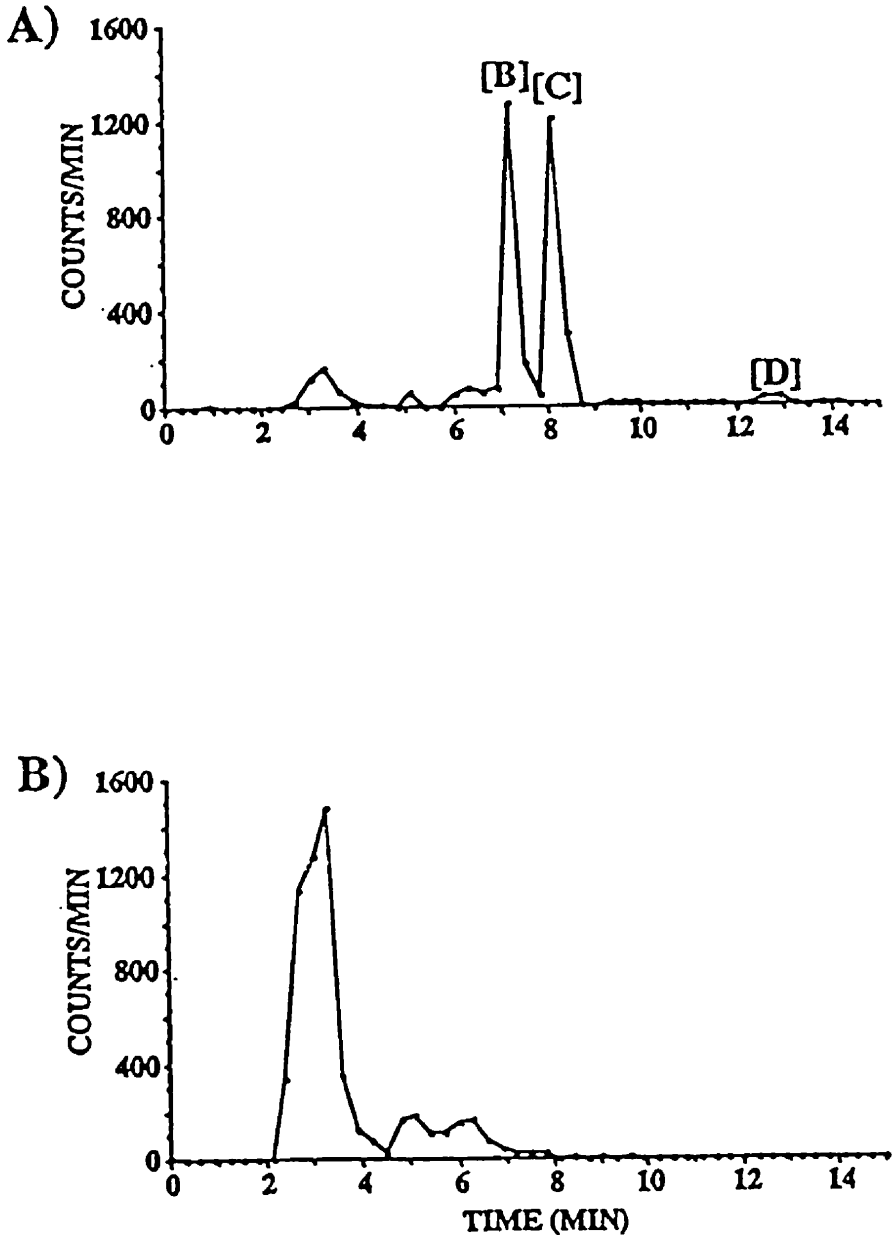

A)
1600
1200
800
400
0
COUNTS/MIN
[B]
[C]
[D]
0
2
4
6
8
10
12
14
B)
1600
1200
800
400
0
COUNTS/MIN
0
2
4
6
8
10
12
14
TIME (MIN)

FIG. 2.4. Reverse-phase HPLC analysis of the reaction between 2-chloroacetyl chloride (0.5 mM) and GSH (5.0 mM) after 30 min. incubation at 25°C.

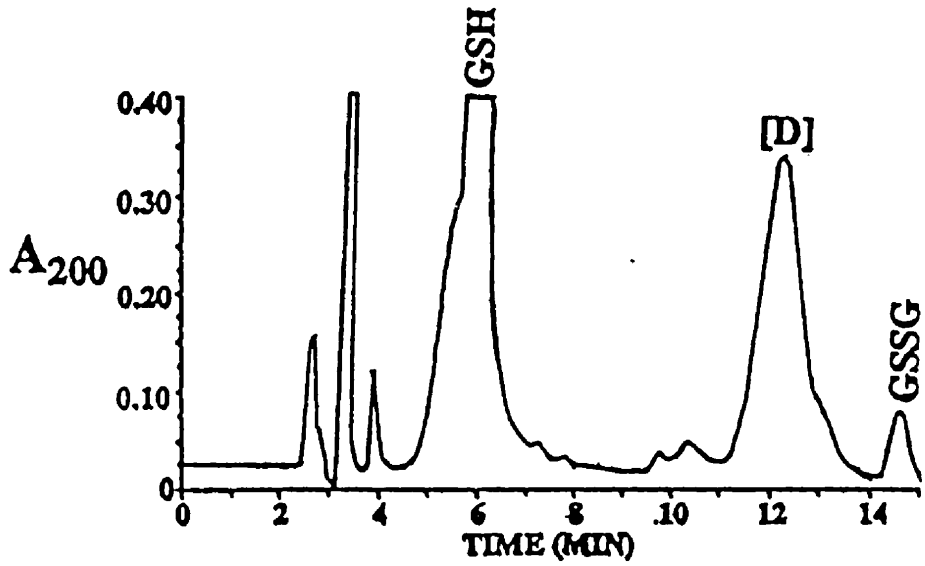
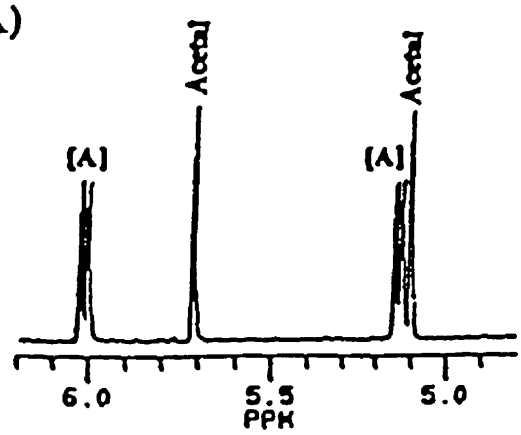

GSH
[D]
GSSG
0.40
0.30
0.20
0.10
0
A_{200}
0
2
4
6
8
10
12
14
TIME (MIN)

FIG. 2.5. ^{1}H-NMR monitoring of reaction of acetal (100 mM) and GSH (100 mM) pD 5.0. In the pH range of 3-8, the equilibrium is pH independent (Kanchuger and Byers, 1979). Peaks shown at (A) were taken at the equilibrium of the reaction while addition of excess GSH (200 mM final concentration) shifted the reaction towards conjugate [A] (B). Peaks due to glutathione are not shown (<5 ppm).

A)



B)

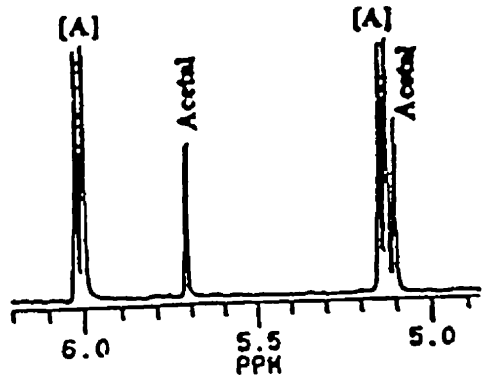


FIG. 2.6. Electrospray mass spectrum (positive ion) of the products obtained from reaction of the DCE epoxide with GSH.

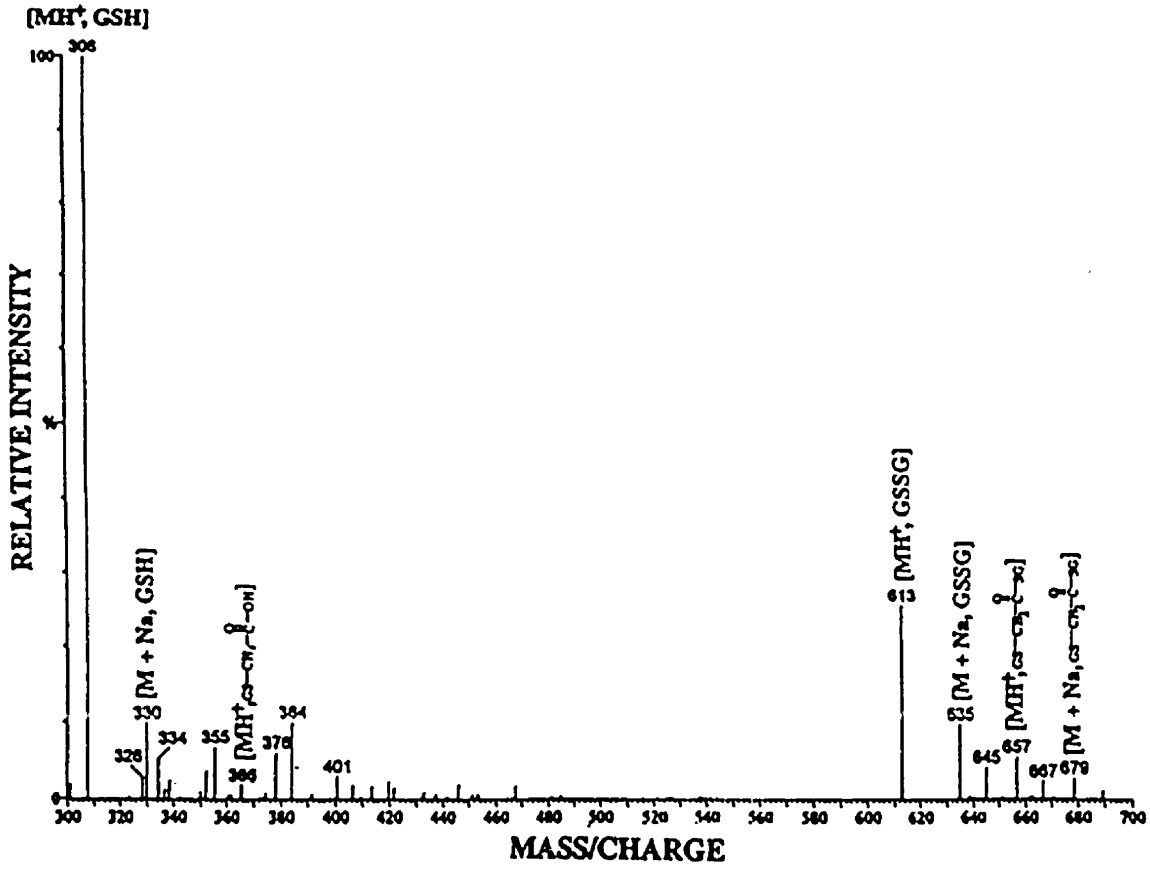
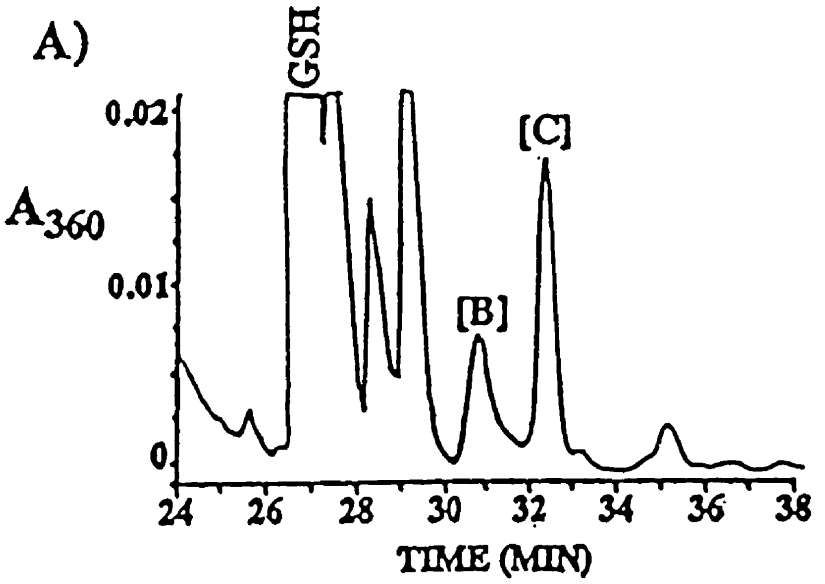

[MH+, GSH]
RELATIVE INTENSITY
MASS/CHARGE
[M + Na, GSH]
[MH+, GSSG]
[M + Na, GSSG]

FIG. 2.7. Analysis of the sample of a mixture of synthesized DCE-epoxide (approximate concentration 0.4 mM) added to 1.6 mM GSH, pH 7.4 by ion exchange HPLC after derivatization (A) and by reverse phase HPLC without derivatization (B).


A)
GSH
0.02
A_{360}
0.01
0
[B]
[C]
24
26
28
30
32
34
36
38
TIME (MIN)

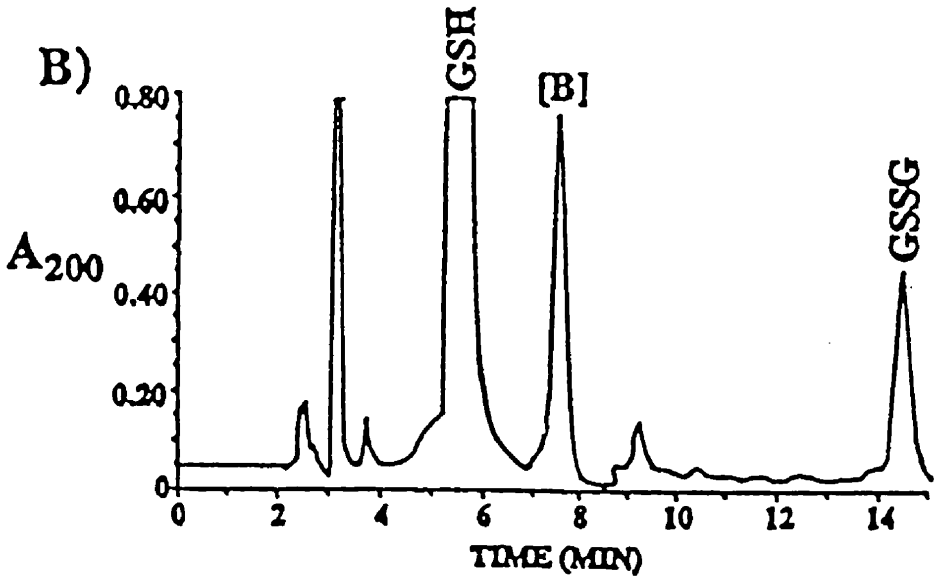
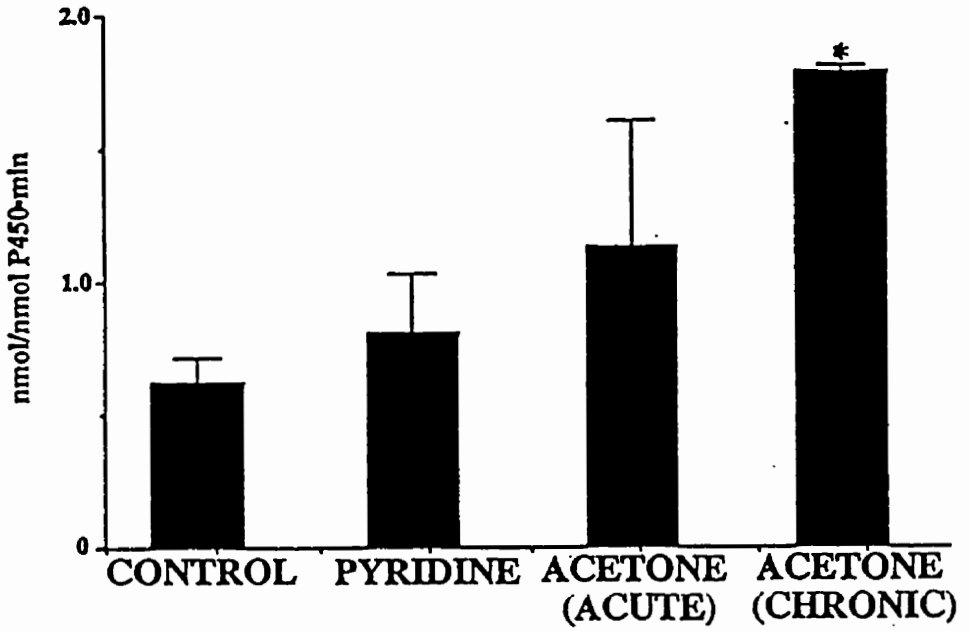

B)
GSH
[B]
GSSG
0.80
0.60
A_{200}
0.40
0.20
0
0
2
4
6
8
10
12
14
TIME (MIN)

FIG. 2.8. Quantities of DCE-epoxide trapped as the GSH conjugates [B] and [C] in incubations with 10 mM ^{14}C-DCE by various mouse hepatic microsomes in the presence of an NADPH-generating system and 5.0 mM GSH after 30 min. at 25°C. Microsomes were prepared from untreated, pyridine-treated, acute acetone-treated and chronic acetone-treated mice. Results are the mean ± S.D. of three determinations. $^{*}P < 0.05$, significantly different from untreated mouse liver microsomes. These values are lower limits based on the amount of ^{14}C-DCE added as described in Materials and Methods.


2.0
1.0
0
nmol/nmol P450-min
*
CONTROL
PYRIDINE
ACETONE (ACUTE)
ACETONE (CHRONIC)

CHAPTER 3

CYP2E1-DEPENDENT BIOACTIVATION OF 1,1-DICHLOROETHYLENE IN MURINE LUNG: FORMATION OF REACTIVE INTERMEDIATES AND GLUTATHIONE CONJUGATES

ABSTRACT

We investigated the cytochrome P450-dependent metabolism of 1,1-dichloroethylene (DCE) in murine lung microsomal incubations. The metabolites were identified as their glutathione conjugates or hydrolyzed products, analyzed by HPLC and quantified with [^{14}C]-DCE. We determined the relative quantities of DCE-metabolites formed in lung microsomal incubations, and compared them to those produced in liver. Furthermore, we used antibody inhibition experiments to investigate the CYP2E1-dependent metabolism of DCE in lung. Our results demonstrated that reactive intermediates were generated from DCE in the lung microsomal incubations. The DCE-epoxide (12.6 ± 1.4 pmol/mg protein/min) was the major metabolite formed and was identified as two glutathione conjugates, 2-(*S*-glutathionyl) acetyl glutathione [B] and 2-*S*-glutathionyl acetate [C]. Lower levels of the acetal of 2,2-dichloroacetaldehyde (3.6 ± 0.25 pmol/mg protein/min) were detected. The ratio of acetal to DCE epoxide was higher in lung (0.30 ± 0.04) than in liver (0.12 ± 0.02). Preincubation of microsomes with a CYP2E1-inhibitory monoclonal antibody resulted in a maximal inhibition of 50 % of the formation of both the acetal and the glutathione conjugates derived from the DCE-epoxide. These data demonstrated that lung CYP2E1 metabolizes DCE to reactive intermediates of which the DCE-epoxide is both the major metabolite formed and is an efficient scavenger of glutathione, implicating it as an important toxic species mediating DCE-induced lung cytotoxicity.

INTRODUCTION

Pulmonary and hepatocellular cytotoxicities are elicited after administration of 1,1-dichloroethylene (DCE), a chemical used in the plastics manufacturing industry and identified as a widespread water contaminant (Forkert and Reynolds, 1982; Forkert *et al.*, 1986; Coleman *et al.*, 1976). Previous studies have established that the cytotoxic responses in both tissues are associated with cytochrome P450-catalyzed metabolic activation of DCE to reactive intermediates that bind covalently to cellular macromolecules (Okine and Gram, 1986; Forkert *et al.* 1987). The extent of binding is inversely related to loss of cellular glutathione (GSH) without any apparent threshold, so that severities of cellular damage paralleled the decline in GSH levels (Moussa and Forkert, 1992; Forkert and Moussa, 1991). Furthermore, covalent binding is significantly inhibited in lung and liver microsomal incubations performed in the presence of GSH (Okine and Gram, 1986). In addition, hepatotoxicity is exacerbated in experimental manoeuvres that diminish GSH levels, such as pretreatment with diethylmaleate, fasting or treatment with DCE during the diurnal cycle when GSH is at its nadir (McKenna *et al.*, 1978; Andersen *et al.*, 1980; Jaeger *et al.*, 1973, 1974). These findings strongly implicated conjugation of DCE-metabolites with GSH as an important metabolic event associated with mechanisms modulating the cytotoxic response.

Previous studies in rats have identified the primary metabolites of DCE formed in hepatic microsomal incubations as DCE-epoxide, 2,2-dichloroacetaldehyde, and 2-chloroacetyl chloride (Liebler *et al.*, 1985; 1988; Liebler and Guengerich, 1983; Costa and Ivanetich, 1984). All are electrophilic metabolites which undergo secondary reactions including further oxidation, conjugation with GSH and/or hydrolysis. We have

recently investigated the formation of the three primary DCE- metabolites in liver microsomal incubations by identifying these species as their GSH conjugates and/or their hydrolyzed products (Dowsley *et al.*, 1995). The major products formed were the GSH conjugates, 2-(*S*-glutathionyl) acetyl glutathione [B] and 2-*S*-glutathionyl acetate [C], which are believed to be derived from the DCE-epoxide (Fig. 3.1). *S*-(2,2-dichloro-1-hydroxy) ethyl glutathione [A], the GSH conjugate formed from reaction of GSH with 2,2-dichloroacetaldehyde (Liebler *et al.*, 1985), was not observed in our experiments (Dowsley *et al.*, 1995). The acetal, together with chloroacetic acid and S-(2-chloroacetyl)-glutathione [D], the hydrolysis and GSH-conjugated products of 2-chloroacetyl chloride, respectively, were detected, but the levels were minimal, compared to those obtained for the DCE-epoxide-derived conjugates [B] and [C]. These results suggested that the DCE-epoxide is the major metabolite formed in liver microsomal incubations and appears to be an efficient scavenger of GSH, resulting in the GSH depletion reported in murine liver after DCE exposure (Forkert and Moussa, 1991; 1993).

Of relevance in the context of regulatory mechanisms mediating DCE-induced cytotoxicities is the cytochrome P450 isozyme-selective metabolic activation of the chloroethylene to reactive intermediates. Our recent studies have detected a 3-fold higher production of the DCE-epoxide GSH conjugates in incubations with liver microsomes of mice treated chronically with acetone (Dowsley *et al.*, 1995). This treatment regimen induces members of both the CYP2E and CYP2B subfamilies (Johansson *et al.*,1988) and implicates these P450 isoforms in DCE metabolism. However, our previous studies have provided findings to support bioactivation of DCE in liver microsomal incubations by the CYP2E1 but not the CYP2B enzyme (Lee and Forkert, 1994), suggesting that formation

of the DCE-epoxide is catalyzed by CYP2E1; this proposed pathway in the lung remains to be established definitively.

As has been found in the liver, diminution of GSH is closely linked to covalent binding and DCE-induced pneumotoxicity (Moussa and Forkert, 1992). Our recent studies have suggested that CYP2E1 is the primary enzyme catalyzing the biotransformation of DCE in lung microsomal incubations (Lee and Forkert, 1995). However, the specific metabolites generated by metabolic activation of DCE in the lung have not been delineated and the major species formed through conjugation with GSH and/or hydrolysis have not been identifed. As a result, it is not known whether the metabolic pathway for DCE is the same in lung and liver tissues. In addition, the role that CYP2E1 plays in the formation of the DCE-metabolites in lung or liver has not been examined directly. In the present studies, we have investigated the cytochrome P450-catalyzed metabolism of DCE to reactive metabolites in lung microsomes from mice. We have determined the relative proportions of the individual metabolites and also the extent to which these metabolites formed GSH conjugates. Finally, we have used antibody inhibition experiments to investigate the role of pulmonary CYP2E1 in the production of DCE-metabolites.

MATERIALS AND METHODS

Chemicals and reagents. Chemicals were purchased from suppliers as follows: Aldrich Chemical Co. (Montreal, Québec, Canada): 1,1-dichloroethylene (> 99% purity), glutathione, phosphoric acid (85%); BDH Chemical Co. (Toronto, Ontario, Canada): methanol (HPLC grade); Sigma Chemical Co.: (St. Louis, MO): glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$. All other chemicals and reagents were purchased from standard suppliers. ^{14}C-DCE (99% pure by GLC) was obtained from Amersham Corp. (Arlington Hights, IL) as a 485 μCi/ml solution diluted to a specific activity of 11.3 nCi/nmol. 2,2-Dichloro-1-methoxyethanol, the hemiacetal of 2,2-dichloroacetaldehyde was synthesized as described previously (Dowsley *et al.*, 1995; Kainz *et al.*, 1993). 2,2-Dichloro-1-methoxyethanol rapidly undergoes hydrolysis when dissolved in an aqueous solution and forms the hydrate of 2,2-dichloroacetaldehyde (acetal). The glutathione conjugates of the DCE metabolites, Conjugates [A], [B], [C], and [D] were synthesized and characterized as described in our previous studies (Dowsley *et al.*, 1995). These compounds were used as standards for metabolite identification.

Animal treatment. Female CD-1 mice (20-25 g body weight) were purchased from Charles River Canada (St. Constant, Québec, Canada). Mice were maintained on a 12-h light/dark cycle and were freely provided with food (Purina Rodent Chow) and water. The mice were acclimatized to laboratory conditions for at least 7 days and were then killed by cervical dislocation.

Preparation of Microsomes. Lungs from 25 mice were pooled and homogenized in 4 volumes of ice-cold phosphate buffered KCl (1.15% KCl, 100 mM K_2HPO_4, 1.5 mM

EDTA, pH 7.4). Lung and liver microsomes were prepared according to procedures described previously (Forkert *et al.*, 1987; Matsubara *et al.*, 1974), with minor modifications. Microsomal pellets were resuspended in 100 mM phosphate buffered KCl at a volume of 0.2 ml/g of the original weight of lung tissues. Aliquots (200 µl) were dispensed into Eppendorf tubes, layered over with argon, frozen in liquid nitrogen and stored at -70°C. Protein concentrations of the microsomal samples were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Microsomal incubations. Microsomal incubations were perfomed as described previously (Dowsley *et al.*, 1995). Briefly, incubations were conducted at 25°C for 30 min in a total volume of 0.5 ml 50 mM phosphate buffer containing 1.5 mM detapac (pH 7.4), 5.0 mg/ml protein, [^{14}C]-DCE (specific activity, 5.21 nCi/nmol) in mineral oil (2mM), 10.0 mM $MgCl_2$, 15 mM GSH and an NADPH-generating system (15.0 mM glucose-6-phosphate, 2 units/ml of glucose-6-phosphate dehydrogenase and 0.8 mM $NADP^+$). The incubation time and protein concentration used for the lung and liver experiments were within the linear range for the production of the DCE-metabolites. The reactions were terminated by chilling in an ice bath and the subsequent addition of perchloric acid (50 µl/ml). Control samples were performed simultaneously and consisted of reactions carried out under conditions in which $NADP^+$ was omitted.

Antibody inhibition studies. To determine the involvement of CYP2E1 in the metabolism of DCE, a CYP2E1-specific monoclonal antibody (Mab 1-91-3; Ko *et al.*, 1987) was used for immunoinhibition of the CYP2E1 isozyme. A monoclonal antibody specific for egg white lysozyme, Mab HyHel 9, (Smith-Gill *et al.*, 1982) was used as a control for nonspecific reactions. Microsomes were preincubated with the inhibitory

antibodies at 25°C for 15 min, after which GSH, [^{14}C]-DCE and components of the NADPH-generating system were dispensed into the incubation mixtures; the reaction was allowed to proceed for 30 min at 25°C. Antibody protein: microsomal protein ratios of 0.25, 0.5, and 1.0 were used for the CYP2E1 antibody, while a ratio of 0.5 was used for Mab HyHel 9. Control and experimental samples were performed simultaneously.

Metabolite identification. The incubates were centrifuged at 13,000 rpm for 6 min to pellet the microsomal proteins. Samples (100 µl) were analyzed with a reverse phase C-18 column (5 µm, 4.6 x 250 mm, Microsorb-MV). The column effluent was monitored at at 200 nm. The isocratic mobile phase was 0.2 % H_3PO_4 (pH 3.15) and the flow rate was 1.0 ml/min. For detection of metabolites, 0.3 ml aliquots of the column effluent were collected for each sample and levels of radioactivity were determined with a Beckman Model LS 7000 liquid scintillation counter. Concentrations of the metabolites were estimated by summing the radioactivity associated with each peak and converting the data to picomolar amounts, using the specific activity of the [^{14}C]-DCE. To confirm the identity of metabolites, co-elution of standards of the GSH conjugates [B] and [C] with the radioactive peaks was achieved under a second set of HPLC conditions as described previously (Liebler *et al.*, 1985; Dowsley *et al.*, 1995). Briefly, the pH of samples was adjusted by adding sodium bicarbonate, and 250 µl aliquots of the supernatant were derivatized with 250 µl of 0.12 M ethanolic 2,4-dinitroflourobenzene. The pH was adjusted to 6.0 by adding 2.5 µl of 6 M HCl. Aliquots of 100 µl were analyzed by HPLC with an aminopropyl silica column, (5 µm, 4.0 x 250 mm, SGE NH_2-8/5) at 360 nm with a flow rate of 0.75 ml/min. The mobile phase was 95 % solvent A (80 % methanol/20 % water) (v/v) and 5 % solvent B (8 M ammonium acetate, 3.4 M acetic acid in 80 %

methanol/20 % water, pH 5.9) at 0.75 ml/min for 10 min, increased to 40 % B over 15 min, isocratic for 10 min, and increased to 99 % B over the last 15 min. Detection of radiolabeled conjugates [B] and [C] was achieved by collecting the column effluent and counting the radioactivity as described above.

Instrumentation. HPLC experiments were performed on a Shimadzu LC-10A gradient HPLC with an SPD-10AV UV detector and SIL-10A auto injector. Peaks were integrated with Shimadzu Ezchrom software and a 486-33 computer. UV spectra were determined with a Hewlett Packard Model 8452 diode array UV spectrophotometer.

Statistical analysis. Data are expressed as mean ± S.D. Statistical analysis was performed with the unpaired Student's *t* test or one-way analysis of variance followed by the Student -Newman-Keuls test to identify significant differences between experimental groups. The level of significance was set at $P < 0.05$.

RESULTS

Oxidation of DCE by pulmonary cytochrome P450. HPLC analysis of lung microsomal incubations revealed three major DCE-metabolites. The peaks were identified by co-elution of authentic standards under two different HPLC conditions (Dowsley *et al.*, 1995) as the acetal of 2,2-dichloroacetaldehyde, conjugates [B] and [C] in order of increasing elution time (Fig. 3.2, panel A). These peaks were not observed when $NADP^+$ was omitted from the incubation mixtures. In the absence of GSH (Fig. 3.2, panel B), the GSH conjugates [B] and [C] disappeared with corresponding appearance of a new peak at 3 min. Glycolic acid and formaldehyde, which are decomposition products of the DCE-epoxide elute in this region. The amount of acetal metabolite detected was unaffected by the presence of GSH, confirming our previous work, which showed that little, if any, of the acetal reacts with GSH to form conjugate [A] (Fig. 3.2, panels A and B). The rate of formation of the DCE-epoxide GSH conjugates (conjugates [B] + [C]) was 12.6 ± 1.4 pmol/mg protein/min, while acetal production was 3.6 ± 0.25 pmol/mg protein/min. We observed only negligible quantities of either conjugate [D], or chloroacetic acid, the GSH conjugate and hydrolysis product of 2-chloroacetyl chloride, respectively. Hence, the major metabolites produced in lung microsomal incubations supplemented with GSH are the DCE-epoxide GSH conjugates and the acetal of 2,2-dichloroacetaldehyde.

Tissue-specific metabolism of DCE. Previous studies have shown that the major metabolites of DCE formed in murine liver microsomal incubations were conjugates [B] and [C] (Dowsley *et al.*, 1995). Negligible amounts of the acetal were detected and this result may be due to the low specific activity of [^{14}C]-DCE added to the microsomal incubations or the low concentrations of liver microsomal protein used, compared to the

levels used in the lung microsomal incubations described herein. To determine whether the relatively higher levels of the acetal metabolite observed in lung *versus* liver microsomal incubations were related to protein concentrations and/or specific activity of [^{14}C]-DCE, incubations of liver and lung microsomes were performed simultaneously with identical amounts of [^{14}C]-DCE and protein content. Liver microsomal incubations produced significantly higher amounts of all three metabolites than those with lung microsomes (Fig. 3.2, panel C). However, in comparison to levels obtained in lung microsomes, incubations of liver microsomes yielded a significantly lower ratio of acetal metabolite, compared to the sum of DCE-epoxide GSH conjugates (Fig. 3.2, panel A). The mean ratio of the acetal/DCE-epoxide conjugates in the hepatic experiments was 0.12 ± 0.02. This was significantly lower than observed for the pulmonary incubations which was 0.30 ± 0.04. microsomes. These data indicate that the proportions of specific metabolites formed from DCE in lung microsomes differ from those formed in liver microsomes.

Antibody inhibition studies. Previous studies have shown that the amount of conjugates [B] and [C] could be enhanced by using microsomes from animals pretreated with inducers of CYP2E1 (Dowsley *et al.*, 1995). In the present studies, we have used a CYP2E1-specific inhibitory Mab to investigate more definitively the participation of CYP2E1 in the bioactivation of DCE by lung microsomes. As shown in Table 3.1, the CYP2E1 Mab caused significant inhibition in the production of both DCE-epoxide GSH adducts at the three antibody concentrations used, whereas the acetal metabolite was inhibited only at the higher antibody concentrations. The inhibition of the DCE-epoxide conjugates was significantly greater than that for the acetal at the 0.25 ratio but was not

different at ratios of either 0.50 or 1.0. Maximal inhibition of both metabolites (~ 50 %) was achieved at the 0.50 ratio; increasing the ratio to 1.0 did not increase the magnitude of the inhibitory effect. Incubations were also performed with microsomes preincubated with the nonspecific Mab, HyHel 9, as a control to assess for nonspecific effects on metabolite production. No significant difference was observed in reactions with HyHel 9, compared with those found in control incubations in which no antibodies were used.

DISCUSSION

Considerable evidence has accumulated to support the premise that the pneumotoxic effects of DCE are the result of its metabolism by pulmonary cytochrome P450 to reactive intermediates (Okine and Gram, 1986; Forkert *et al.*, 1987; Krijgsheld *et al.*, 1983). However, the metabolites formed from DCE in the lung have not been identified. Identification of the specific metabolites generated from DCE is relevant to elucidation of mechanisms mediating lung cytotoxicity and may provide findings to explain the high susceptibility of this tissue to DCE exposure. In contrast to lack of data in the lung, the reactive intermediates produced from hepatic bioactivation of DCE have been delineated and identified as 2,2-dichloroacetaldehyde, DCE-epoxide and 2-chloroacetyl chloride in rat liver microsomal incubations (Liebler *et al.*, 1985, 1988; Liebler and Guengerich, 1983; Jones and Hathway, 1978). We have recently reported that similar DCE-metabolites, as has been identified in rat liver microsomal incubations, were also formed in murine liver microsomal incubations (Dowsley *et al.*, 1995). The DCE-epoxide was the major metabolite produced and was identified as reaction products with GSH, termed conjugates [B] and [C] (Fig. 3.1). Other metabolites formed were 2-chloroacetyl chloride and 2,2-dichloroacetaldehyde. The 2-chloroacetyl chloride was trapped as its GSH conjugate [D], whereas 2,2-dichloroacetaldehyde was detected as its hydrolyzed product, acetal (Fig. 3.1). However, relative to the DCE-epoxide, minimal amounts of these two metabolites were detected in the liver microsomal incubations. Conjugation of 2,2-dichloroacetaldehyde with GSH to yield conjugate [A] has also been demonstrated but the levels produced were negligible. These findings in mice suggested that the DCE-epoxide is likely the species responsible for diminution of hepatic GSH *in*

vivo and, following its depletion, covalent binding of DCE to cellular macromolecules ensue. Thus, the DCE-epoxide may be the ultimate toxic species that mediates DCE-induced hepatocellular necrosis. It is unknown whether similar metabolites are generated by metabolism of DCE in lung, a tissue that is highly susceptible to DCE-induced toxicity but containing considerably less cytochrome P450 and GSH than the liver (Krijgsheld *et al.*, 1983; Forkert *et al.*, 1986; Forkert and Moussa, 1993; Moussa and Forkert, 1992).

Here we have undertaken to investigate the metabolism of DCE to reactive intermediates by murine lung microsomes. The DCE-epoxide was the predominant metabolite detected, as estimated from the amounts of GSH conjugates [B] and [C] formed. We believe the sum of conjugates [B] and [C] to be an accurate estimate of the quantity of DCE-epoxide that escaped the active site of P450. The known breakdown products of the DCE-epoxide in aqueous solutions are formaldehyde and glycolic acid. Both of these were detectable by our HPLC method but were negligible in incubations containing GSH. In addition, production of conjugates [B] and [C] in liver microsomal incubations was unaffected by the presence of purified glutathione-*S*-transferase (Liebler *et al.*, 1985), indicating that the DCE-epoxide is highly reactive towards GSH non-enzymatically. It is possible that some of the DCE-epoxide formed reacted within the active site of P450 and hence was not detected by our methods. We observed only negligible quantities of either 2-chloroacetic acid or the GSH conjugate [D] in the lung microsomal incubations; these metabolites are believed to arise from 2-chloroacetyl chloride. However, the acetal of 2,2-dichloroacetaldehyde was also an important metabolite produced in lung incubations and was approximately 30% of the levels of the

DCE-epoxide. Conjugate [A], the reaction product of 2,2-dichloroacetaldehyde with GSH, was not found in the lung microsomal incubations. This finding is consistent with the results of our previous work which showed that the equilibrium between the acetal of 2,2-dichloroacetaldehyde and its GSH conjugate heavily favors the acetal (Dowsley *et al.*, 1995). Thus, it is unlikely that 2-chloroacetyl chloride or 2,2-dichloroacetaldehyde contributes significantly to the depletion of GSH evoked by DCE (Moussa and Forkert, 1992; Forkert and Moussa, 1991, 1993). These data suggested that the DCE-epoxide is likely the species responsible for the depletion of GSH *in vivo* reported previously in the lung (Forkert and Moussa, 1993; Moussa and Forkert, 1992).

We observed relatively substantial production of the acetal of 2,2-dichloroacetaldehyde in this investigation (Fig. 3.2), a result that contrasts with that of our previous work with murine liver microsomes in which low amounts of the acetal were detected (Dowsley *et al.*, 1995). A variable that may account for this difference is that our lung incubations were performed at higher microsomal protein concentrations (5-7 fold higher) than in incubations with liver microsomes. Because the primary metabolites of DCE span a wide range of reactivities (Liebler *et al.*, 1985), microsomal protein concentrations may affect the relative proportions of the metabolites that become bound to macromolecules and thereby escape detection. To investigate this proposed mechanism, we performed hepatic and lung microsomal reactions in simultaneous incubations with the same protein and substrate concentrations. Our results demonstrated that, in comparison with levels detected in lung microsomes, reactions with liver microsomes yielded, in absolute amounts, higher production of both the GSH conjugates and the acetal metabolite (Fig. 3.2). This result is not surprising and may be ascribed to

the markedly higher levels of cytochrome P450 present in liver *versus* lung microsomes. However, the ratio of acetal to DCE-epoxide was significantly lower in liver, compared with that obtained in the lung (Fig. 3.2). These findings demonstrated that, in comparison with that in liver, metabolism of DCE in the lung favours to a greater extent the formation of the acetal of 2,2-dichloroacetaldehyde, suggesting that metabolic mechanisms may differ between the two tissues. Whether this difference has any toxicological significance is at present unclear and remains to be investigated.

Data are available to implicate bioactivation of DCE by the cytochrome P450 isozyme, CYP2E1. Previous studies have reported that hepatocytes isolated from mice treated with ethanol or acetone and incubated in the presence of DCE sustained more severe cytotoxic effects than hepatocytes from untreated mice (Kainz *et al.*, 1993); ethanol and acetone are agents known to induce the CYP2E1 enzyme in murine liver (Forkert *et al.*, 1991). Incubation of hepatocytes with the CYP2E1 inhibitors, N,N-dimethylformamide (Mraz *et al.*, 1993) and diethyldithiocarbamate (Guengerich *et al.*, 1991), decreased the severity of the cytotoxic reactions caused by DCE (Kainz *et al.*, 1993). Our recent studies have examined P450-selective enzyme inactivation by DCE and have provided data to support metabolic activation of DCE by CYP2E1 in lung and liver microsomal incubations (Lee and Forkert, 1994, 1995). We have also reported an increase in the formation of the DCE-epoxide GSH conjugates [B] and [C] in incubations with hepatic microsomes from mice treated chronically with acetone, compared to microsomes from untreated mice (Dowsley *et al.*, 1995). The present studies are an extension of this previous work and we have investigated herein the specific metabolites produced from the pulmonary oxidation of DCE by CYP2E1. Our results revealed that

antibody inhibition of CYP2E1 resulted in significant reduction in the levels of both the conjugates, [B] and [C], as well as the acetal metabolite. Maximal inhibition observed was ~50 % for both metabolites, suggesting that CYP2E1 is responsible for at least 50 % of the metabolism of DCE in lung microsomes. These results suggest the involvement of other P450 isoforms in DCE bioactivation and/or incomplete inhibition of the CYP2E1 enzyme. The lack of complete inhibition achieved by high concentrations of the CYP2E1 antibody in our experiments is consistent with findings reported in previous studies with liver microsomes (Thomas *et al.*, 1987; Nakajima *et al.*, 1992). Interestingly, the use of purified CYP2E1 in a reconstituted system produced complete inhibition of CYP2E1-dependent *N*-nitrosodimethylamine demethylation (Thomas *et al.*, 1987). These findings suggested that it may not be possible to completely inhibit CYP2E1 catalytic activity in a microsomal system, and has been postulated to be due to interference by NADPH-cytochrome P450 reductase or inaccessibility of a portion of CYP2E1 to the antibody (Thomas *et al.*, 1987).

In summary, we report that DCE is bioactivated in murine lung microsomes to electrophilic intermediates by a cytochrome P450-dependent metabolism. Our data supported an important role for CYP2E1 in this metabolic activation. The DCE-epoxide is the major metabolite produced, as assessed by formation of the two GSH conjugates [B] and [C]; the acetal of 2,2-dichloroacetaldehyde was formed at lower levels. A difference in the metabolism of DCE was observed between lung and liver microsomes, with the production of relatively higher levels of the acetal of 2,2-dichloroacetaldehyde in the former. Taken together, our findings suggested that DCE-induced Clara cell damage

is mediated by CYP2E1-dependent metabolic activation to electrophilic metabolites of which the DCE-epoxide may be the most important reactive species.

TABLE 3.1. Inhibition of DCE-metabolites by an anti-CYP2E1 Antibody in lung microsomes

Experiment	DCE Metabolites (pmol/mg protein/min)	
	2,2-Dichloroacetaldehyde	DCE-epoxide
	% of control (-Mab)	
Anti-CYP2E1 Mab[b]		
0.25	90.2 ± 6.7	59.9 ± 5.4[d,f]
0.50	54.8 ± 7.1[d,e]	50.1 ± 5.4[d]
1.00	55.3 ± 5.2[d,e]	53.7 ± 5.8[d]
Mab Hyhel 9[c]		
0.50	123.3 ± 15.0[g]	90.8 ± 6.0[g]

[a]Reaction mixtures contained 5 mg/ml of microsomal protein and incubations were performed at 25^{0}C for 30 min.
[b]Anti-CYP2E1 Mab protein:microsomal protein concentration ratios of 0.25-1.00 were employed.
[c]An irrelevant Mab Hyhel 9 was used to control for non-specific reactions.
[d]Significantly different from control (-Mab), $P < 0.01$
[e]Significantly different from a Mab protein:microsomal protein concentration ratio of 0.25, $P < 0.01$.
[f]Significantly different from levels of 2,2-dichloroacetaldehyde, $P < 0.01$.
[g]Significantly different from reactions with an anti-CYP2E1 Mab, $P < 0.05$.

FIG. 3.1. Metabolic pathway for DCE.

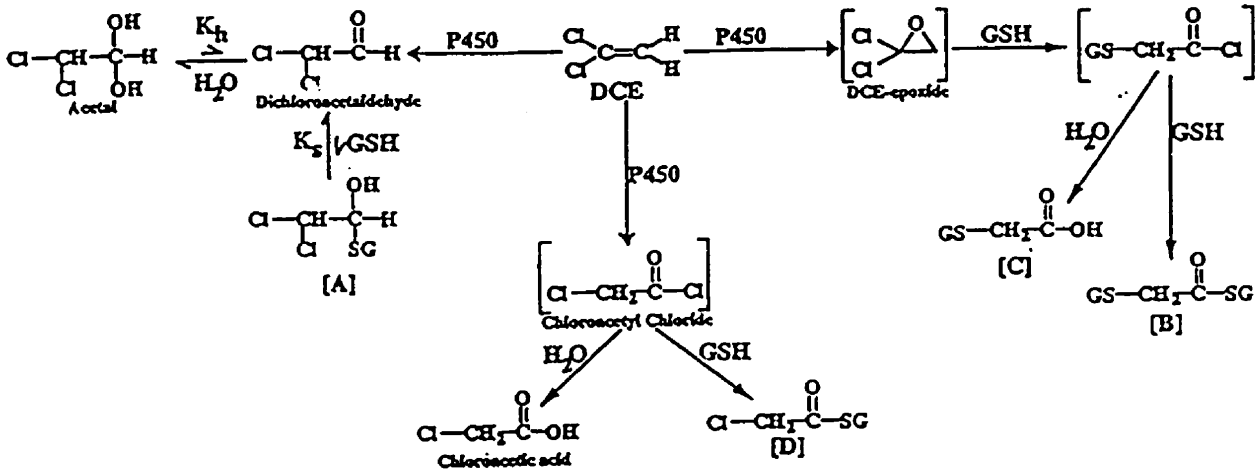
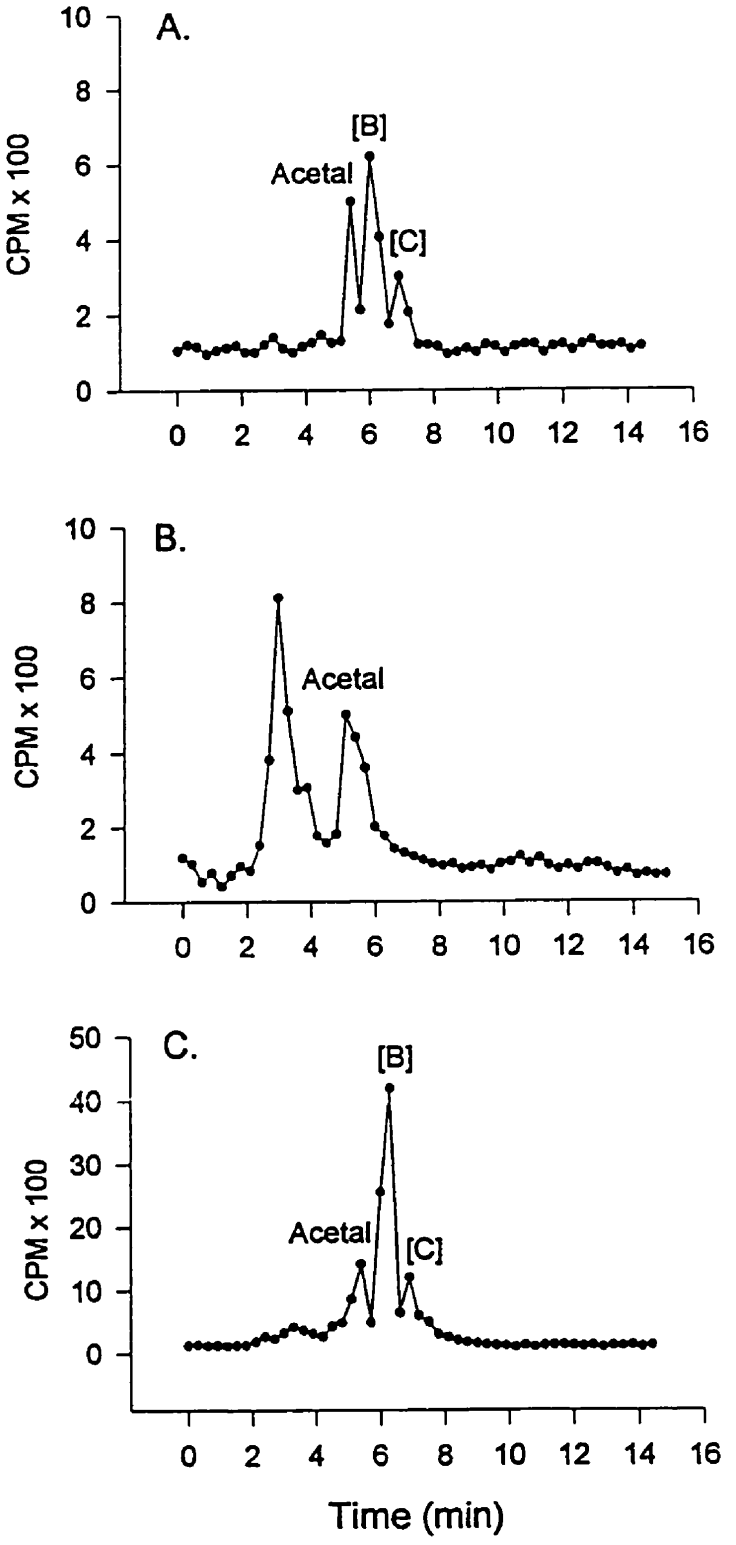

Acetal
Dichloroacetaldehyde
P450
DCE
P450
DCE-epoxide
GSH
[A]
P450
Chloroacetyl Chloride
H2O
GSH
Chloroacetic acid
[D]
H2O
GSH
[C]
[B]

FIG. 3.2. HPLC analysis of GSH conjugates [B] and [C] and the acetal of 2,2-dichloroacetaldehyde in lung (A and B) and liver (C) microsomal incubations performed in the presence (A and C) or absence (B) of GSH. Reactions were performed in a volume of 0.5 ml 50 mM phosphate buffer, pH 7.4, containing 5.0 mg per ml of microsomal protein, 2 mM [^{14}C]-DCE and 15.0 mM GSH, and were carried out in the presence of an NADPH-generating system for 30 min at 25°C. Details of components used in the incubations are detailed in "Materials and Methods". Radioactivity eluting from the HPLC column was measured in fractions collected at 0.3 min intervals and expressed as counts per min (CPM).


A.
[B]
Acetal
[C]
CPM x 100
10
8
6
4
2
0
0 2 4 6 8 10 12 14 16
B.
Acetal
CPM x 100
10
8
6
4
2
0
0 2 4 6 8 10 12 14 16
C.
[B]
Acetal
[C]
CPM x 100
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16
Time (min)

CHAPTER 4

CYTOCHROME P450-DEPENDENT BIOACTIVATION OF 1,1-DICHLOROETHYLENE TO A REACTIVE EPOXIDE IN HUMAN LUNG AND LIVER MICROSOMES

ABSTRACT

We investigated the cytochrome P450-dependent metabolism of 1,1-dichloroethylene (DCE) by human lung and liver microsomes, and compared the results in analogous experiments in mice. Metabolites were identified by HPLC analysis of their glutathione conjugates and/or hydrolyzed products, and were detected by using [^{14}C]DCE. The role of human CYP2E1 in the metabolic reactions was examined by comparing *p*-nitrophenol hydroxylase activities with levels of metabolites formed, and by employing the CYP2E1-selective inhibitor diallyl sulfone ($DASO_2$). The major products formed in microsomal incubations containing NADPH were the DCE-epoxide-derived glutathione conjugates 2-(*S*-glutathionyl) acetyl glutathione [B] and 2-*S*-glutathionyl acetate [C]. Lower levels of the acetal of 2,2-dichloroacetaldehyde were also detected. In lung samples from 8 patients, the amounts of DCE-epoxide formed ranged from 15.6 ± 4.23 to 34.9 ± 12.75 pmol/mg protein/min. The levels in murine lung were higher at 40.0 ± 3.8 pmol/mg protein /min. In liver samples from 5 patients, the levels of DCE-epoxide ranged from 46.5 ± 8.3 to 240.0 ± 10.5 pmol/mg protein/min, while levels in murine liver were 83.0 ± 6.2 pmol/mg protein/min. The rank order of levels of the DCE-epoxide formed in the different groups corresponded nearly identically with relative levels of *p*-nitrophenol hydroxylase activity present. $DASO_2$ inhibited the formation of the DCE-epoxide in liver samples from all 4 patients (20-65 % inhibition), whereas only 1 of 5 human lung samples exhibited this inhibition (27 %). Hence, the DCE-epoxide is the major metabolite formed from DCE in human lung and liver microsomes and a strong role for CYP2E1 was implicated in catalyzing this bioactivation in human liver.

INTRODUCTION

1,1-Dichloroethylene (DCE), a chemical used in the manufacture of plastics and a widespread water contaminant, causes pulmonary and hepatocellular injury in experimental animals (Coleman *et al.*, 1976; Forkert and Reynolds, 1982; Forkert *et al.*, 1986). Previous studies have determined that the mechanism of DCE-induced injury involves cytochrome P450-catalyzed metabolism to reactive intermediates (Okine and Gram, 1986; Forkert *et al.*, 1987). Exposure to DCE causes dose-dependent increases in covalent binding and concomitant decreases in cellular glutathione (GSH) in both lung and liver (Forkert and Moussa, 1991; Moussa and Forkert, 1992). The severity of tissue injury correlates with the extent of binding and parallels the decline in GSH, suggesting that binding of reactive intermediates to critical macromolecules mediates the cytotoxicity. Conjugation of DCE metabolites with GSH represents a detoxication reaction. The hepatotoxic effects of DCE were exacerbated by procedures that lower GSH levels (Jaeger *et al.*, 1973, 1974; McKenna *et al.*, 1978 Anderson *et al.*, 1980).

Previous studies in rat liver have identified the primary metabolites formed in microsomal incubations from DCE as 2,2-dichloroacetaldehyde, the DCE-epoxide, and 2-chloroacetyl chloride (Lieber and Guengerich, 1983; Costa and Ivanetich, 1984; Liebler *et al.*, 1985, 1988). In our recent studies in murine lung and liver, the major metabolic products formed in microsomal incubations supplemented with GSH were the conjugates, 2-*S*-glutathionyl acetyl glutathione [B] and 2-*S*-glutathionyl acetate [C] (Dowsley *et al.*, 1995, 1996). These products are believed to arise from conjugation of GSH with the reactive intermediate DCE-epoxide (Dowsley *et al.*, 1995; Fig. 4.1). The acetal of 2,2-dichloroacetaldehyde is detectable in lung microsomal incubations. However, conjugate

[A], the product of GSH conjugation with 2,2-dichloroacetaldehyde, was not detected in our experiments, suggesting that this reaction is unlikely to contribute to GSH depletion or to be responsible for DCE-induced cytotoxicity (Dowsley *et al.*, 1995). This assumption is consistent with results from previous studies that showed that isolated hepatocytes incubated with 2,2-dichloroacetaldehyde evoked less toxic effects than incubation with the parent compound (Kainz *et al.*, 1993). Conjugate [D] and chloroacetic acid, the GSH-conjugated and hydrolysis products of 2-chloroacetyl chloride, respectively, were formed at minimal levels in both liver and lung microsomal incubations (Dowsley *et al.*, 1995, 1996). Hence, the DCE-epoxide is the major metabolite formed from DCE *in vitro*, and is an efficient scavenger of GSH, suggesting that it is the most plausible candidate for mediating the toxic effects of DCE. This is supported by results from several studies that show a striking correlation between susceptibility to lung or liver injury in rodents, and the rate of production of the DCE-epoxide (Dowsley *et al.*, 1995; Forkert *et al.*, 1996a, 1996b). For example, mice are more susceptible than rats to DCE-induced injury. The LD_{50} for an oral dose of DCE is 7-fold lower in mice than in rats (Jones and Hathway, 1978). Our studies have also shown that the DCE-epoxide is produced in microsomal incubations at a level that is 6-fold higher in mice than in rats (Dowsley *et al.*, 1995). These data supported the proposal that the DCE-epoxide may be responsible for DCE-induced toxicity by depleting GSH and binding to cellular macromolecules.

We have identified CYP2E1 as a major P450 isozyme involved in the metabolism of DCE in murine lung and liver (Dowsley *et al.*, 1995, 1996; Forkert *et al.*, 1996a, 1996b). Moreover, treatment of mice with diallyl sulfone ($DASO_2$) inhibited CYP2E1 and

protected against the Clara cell damage induced by DCE (Forkert *et al.*, 1996b). Our previous studies in mice indicated that the degree of DCE-induced Clara cell cytotoxicity is linked to magnitudes of CYP2E1-dependent bioactivation of DCE to the epoxide (Forkert *et al.*, 1996a). The expression of CYP2E1 in human lung and liver (Wrighton *et al.*, 1986; Ekström *et al.*, 1989; Wheeler *et al.*, 1991; Lucas *et al.*, 1993) has raised the possibility that exposure to DCE results in generation of the DCE-epoxide in these tissues. It is of importance to determine the capacity of human liver and lung CYP2E1 to metabolize DCE to reactive intermediates, including the DCE-epoxide, so as to obtain data to better assess the potential risk to humans exposed to DCE and other low molecular weight chemicals that are substrates for CYP2E1. The objectives of the present study are to investigate the P450-mediated metabolism of DCE in human lung and liver microsomal incubations. We have also compared the metabolic profiles with those in murine lung and liver to evaluate relevance of the murine model for future studies in this area. The role that human CYP2E1 plays in DCE bioactivation is also examined by preincubation of human microsomes with $DASO_2$, a CYP2E1-specific inhibitor.

MATERIALS AND METHODS

Chemicals and Reagents. Chemicals were obtained from suppliers as follows: 1,1-dichloroethylene (> 99 % purity), phosphoric acid (85 % v/v) and GSH (Aldrich Chemical Co., Montréal, Québec, Canada); glucose-6-phosphate and glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, MO); $NADP^+$ (BDH Chemical Co., Toronto, Ontario, Canada); $DASO_2$ (Parish Chemical Co., Orem, UT). [^{14}C]-DCE (99 % pure by GLC, specific activity 11.3 nCi/nmol) was obtained from Amersham Corp., Arlington Heights, IL, and was diluted to 300 μCi/ml for our experiments. The DCE-epoxide-derived GSH conjugates [B] and [C] were synthesized as described previously (Dowsley *et al.*, 1995,1996), and were used as standards for metabolite identification. Other chemicals and reagents were purchased from standard suppliers.

Animal Treatment. The procedures for the handling and treatment of animals was in accordance with the ethical standards of Queen's University. Female CD-1 mice, weighing 20 to 25 g, were obtained from Charles River Canada (St. Constant, Québec, Canada). The mice were kept in an animal facility maintained on a 12-hr light/dark cycle, and were freely provided with food (Purina Rodent Chow) and water. The mice were housed for 7 days following arrival to acclimatize to laboratory conditions, and were then sacrificed by cervical dislocation.

Preparation of Microsomes. Human lung tissue (10-50 g) was obtained from Kingston General Hospital, Kingston, Ontario, Canada, from consenting patients undergoing surgical lobectomies. Tissues distant from the primary lesions were surgically excised, placed on ice, and were immediately transferred to a biohazard facility. Human liver

tissue samples were rapidly frozen in liquid nitrogen, and were shipped from the Department of Surgery, University of Arizona, Tuscon, Arizona. The liver tissue was thawed in a laminar flow hood before homogenizing.

For preparation of murine microsomes, lungs from 25 mice or livers from 10 mice were pooled. The human tissues were not pooled but were retained as individual samples. The tissues were homogenized in 4 volumes of cold phosphate buffered KCl (100 mM K_2HpO_4, 1.15 % KCl, 1.15 mM EDTA, pH 7.4), and microsomes were prepared as described previously (Forkert *et al.*, 1987; Matsubara *et al.*, 1987). Microsomal pellets were resuspended in phosphate-buffered KCL in a volume of 0.2 ml/g tissue weight (mouse) or 0.1 ml/g tissue weight (human). Aliquots of the microsomal suspension were dispensed into Eppendorf tubes, frozen in liquid nitrogen and stored at −70°C. Protein concentrations of the microsomal samples were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Microsomal Incubations. Microsomal incubations were performed as described in our previous studies (Dowsley *et al.*, 1995, 1996). Reactions were performed at 25°C for 30 min in a total volume of 0.5 ml phosphate buffer containing 1.5 mM EDTA. Incubation mixtures contained 5.0 mg per ml of microsomal protein, [^{14}C]-DCE (2 mM, specific activity 7.5 nCi/nmol), 10.0 mM $MgCl_2$, 15 mM GSH, and an NADPH-generating system (15.0 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, and 0.8 mM $NADP^+$). The protein concentrations and the incubation time used were both within the linear range for formation of metabolites. The reactions were initiated with DCE, and were terminated by chilling the samples in an ice bath. The microsomal proteins were precipitated with perchloric acid (50 µl/ml) and centrifugation.

Preincubation of Microsomes with $DASO_2$. The involvement of CYP2E1 in the formation of DCE-metabolites in human lung and liver incubations was examined by preincubation of the microsomes with the CYP2E1-selective inhibitor, $DASO_2$ (Forkert *et al.*, 1996; Brady *et al.*, 1991). Previous studies have shown that $DASO_2$ inhibits the CYP2E1 enzyme in both lung (Forkert *et al.*, 1996) and liver (Brady *et al.*, 1991). Microsomes were preincubated with 20 mM $DASO_2$ in the presence of an NADPH-generating system for 30 min at 37°C. The samples were then centrifuged at 105,000 x g for 30 min to recover the microsomal proteins. The microsomal pellet was homogenized and resuspended in 100 mM phosphate buffered KCl, pH 7.4. The conditions for DCE incubation were the same as those described for the microsomal incubations with DCE. Control incubations consisted of incubations performed without $DASO_2$.

p-Nitrophenol Hydroxylase Activity. Microsomes from human and murine liver and lung were resuspended in 100 mM K_2HPO_4 buffer, pH 6.8, using 3.0 mg per ml of microsomal protein. *p*-Nitrophenol (PNP) hydroxylase activity was used as a selective enzyme marker for CYP2E1, and was determined by the method of Koop (1986). Incubations were performed in a total volume of 2 ml and contained 3.0 mg/ml protein and an NADPH-generating system (7.5 mm glucose-6-phosphate, 5.0 mM $MgCl_2$, 2 U of glucose-6-phosphate dehydrogenase, and 0.4 mM $NADP^+$). The reaction mixtures were preincubated for 3 min at 37°C and subsequently PNP in dimethyl sulfoxide (DMSO) (4 µl, 200 µM) was added and allowed to react for a further 10 min. The reaction was terminated by cooling the samples in an ice bath. The microsomal proteins were precipitated by addition of perchloric acid (70 %, 50 µl) and centrifugation. The supernatant was obtained, NaOH (100 µl, 0.9 M) was added to a 1.0 ml aliquot, and the

formation of 4-nitrocatechol was determined spectrophotometrically at 546 nm. Levels of 4-nitrocatechol formed were determined by relating absorbances to a standard calibration curve of known amounts of 4-nitrocatechol. The assay was carried out under linear conditions of time and protein concentrations.

Metabolite Identification. Synthesized standards of DCE metabolites were characterized by reverse phase HPLC analysis using a C-18 column (5 μm, 4.6 x 250 mm, Microsorb-MV, Rainin Instruments Co., Inc., Woburn, MA). The mobile phase consisted of 0.2 % H_3PO_4 (pH 2.0), and was run isocratically at a flow rate of 1.0 ml/min. The column effluent was monitored at 200 nm. For analysis of microsomal incubations, 100 μl aliquots of the supernatant from each microsomal incubation were injected onto the HPLC. Fractions (0.25 ml) of the column effluent were collected and levels of radioactivity were determined by liquid scintillation spectroscopy (Beckman Model LS 7000 liquid scintillation counter). Identification of the metabolites was achieved by radiochemical detection of the fractions eluting from the column with retention times corresponding to synthesized standards. Concentrations of the metabolites were estimated by summing the radioactivity associated with each peak and converting the data to picomolar amounts using the specific activity of the [^{14}C]-DCE.

Instrumentation. HPLC experiments were conducted on a Beckman System Gold Programmable Solvent Module 126 HPLC with a Beckman System Gold Module 168 UV detector. UV spectra for all other assays were determined with a Hewlett Packard Model 8452 diode array UV spectrophotometer.

Statistical Analysis. Data are expressed as mean ± S. D. Statistical analysis was performed with one-way analysis of variance followed by the Tukey test to identify significant differences between experimental groups ($p < 0.05$).

RESULTS

Metabolism of DCE by Human Lung and Liver Microsomes. Three major peaks were observed in the radiochromatograms obtained in the human lung incubations (Fig 4.2A). These were identified by co-elution with authentic standards as the acetal of 2,2-dichloroacetaldehyde, and conjugates [B] and [C] in order of increasing elution time. These peaks were not observed when $NADP^+$ was omitted from the incubation mixtures. In the absence of GSH, conjugates [B] and [C] were also not present, while two peaks eluting at 3.0 and 4.0 min appeared (data not shown). These peaks corresponded to the retention times of formaldehyde and glycolic acid, respectively, and are the hydrolysis products of the DCE-epoxide (Liebler *et al.*, 1985). However, these were negligible in comparison with conjugates [B] and [C]. Human lung microsomes from 8 subjects (HL-1 to HL-8) were used to assess the metabolism of DCE in this tissue. The rate of formation of the DCE-epoxide, as estimated from the sum of [B] and [C], ranged from 15.6 ± 4.2 (HL-8) to 34.9 ± 12.7 (HL-5) pmol/mg protein/min (Table 4.1). Levels of the acetal of 2,2-dichloroacetaldehyde were lower and were less variable between microsomes from the different patients, with a mean rate of formation of 5.2 ± 0.9 pmol/mg protein/min. HPLC analysis of human liver microsomal incubations from 5 patients (HLv-1 to HLv-5) yielded similar metabolites as those detected in the human lung microsomal incubations, but at higher levels (Fig. 4.2B). There was also considerable variability in the extents to which the DCE-epoxide-derived GSH conjugates were formed in the liver microsomes from the 5 patients (Table 4.2). The sum of [B] and [C] ranged from 46.5 ± 8.3 (HLv-1) to 240.0 ± 10.5 (HLv-2) pmol/mg protein/min; the highest level of the DCE-epoxide produced was about 5-fold of that at

the lowest. The mean rate of formation of the acetal in the human liver samples (8.0 ± 1.5 pmol/mg protein/min) was only slightly higher than those detected in the human lung samples, and was less variable between the patients. Compared with the formation of the DCE-epoxide, the amounts of acetal produced in the liver were relatively negligible (Fig 4.2B, Table 4.2).

Species Comparison in DCE Metabolism and CYP2E1 Activity. We compared the rates of formation of the DCE-epoxide and the acetal of 2,2-dichloroacetaldehyde in incubations with murine lung and liver microsomes with the amounts formed in those with human lung and liver microsomes. The specific activity of the [^{14}C]-DCE used was the same in all the microsomal incubations. The mean rate of formation of the DCE-epoxide in murine lung microsomal incubations was 1.7-fold higher than the level in the human lung samples from the 8 patients investigated (Tables 4.1 and 4.3). However, lung samples from patients HL-1, HL-5 and HL-6 formed the epoxide at rates that were 74%, 70% and 87% of the levels found in murine lung, respectively. The mean level of DCE-epoxide formed by liver microsomes from the 5 patients was 1.8-fold higher than those formed by murine liver microsomes (Tables 4.2 and 4.3). Levels in murine liver were higher than in samples from livers of patients HLv-1 and HLv-4, but lower than HLv-2, HLv-3, and HLv-5. Therefore, in the microsomal samples examined here, murine liver microsomes formed levels of the DCE-epoxide that were in the mid-range of the rates detected in the human liver incubations. The acetal was formed in both murine lung and liver microsomal incubations, but the levels were low, compared with the amounts of epoxide detected (Table 4.3). However, acetal levels were slightly higher in microsomes from mice than from humans (Tables 4.1,4.2,4.3).

Our previous studies established an important role for CYP2E1 in the metabolism of DCE to the epoxide in murine liver and lung microsomes (Dowsley *et al.*, 1995, 1996). In the present investigation, we measured PNP hydroxylase activities of microsomal samples from the lungs of 4 patients (HL-1 to HL-4) and the livers of 3 patients (HLv-1 to HLv-3), and compared the levels with those obtained in lungs and livers of mice. The results are summarized in Figs.4.3 and 4.4. Activities of PNP hydroxylase from the 4 patients had a mean rate of 0.10 ± 0.03 nmol/mg protein/min. The mean rate in murine lung microsomes (0.70 ± 0.08 nmol/mg/min) were about 7-fold higher than those of human lung (Fig 4.3). The 3 human liver samples showed a large range in the amounts of hydroxylase activity. Patient HLv-1 had the lowest level (1.33 ± 0.11 nmol/mg protein/min), while the quantities in HLv-2 (2.59 ± 0.07 nmol/mg protein/min) and HLv-3 (2.87 ± 0.07 nmol/mg protein/min) were higher and were similar to each other. In mice, the hydroxylase activity in liver microsomes was significantly higher than in lung microsomes (Figs 4.3 and 4.4) but was significantly lower than detected in liver microsomes from patients HLv-2 and HLv-3 (Fig. 4.4). The rank order of the levels of catalytic activities in human and murine lung and liver microsomes was nearly identical to the levels of production of the DCE-epoxide (Figs 4.3 and 4.4). There was a linear correlation between the formation of the DCE-epoxide and PNP hydroxylase activity for the 4 human lung samples (HL-1 to HL-4) (R^2 = 0.80, Fig 4.5, top panel) and for the human liver samples (HLv-1 to HLv-3) (R^2 = 0.99, Fig 4.5, bottom panel).

Effects Of $DASO_2$ on DCE-Epoxide Formation in Human Lung and Liver Microsomes. The rank order for production of the DCE-epoxide corresponded to that for PNP hydroxylase activity in the human and murine lung and liver incubations (Figs. 4.3

and 4.4). These findings prompted us to examine the role of CYP2E1 in catalyzing the formation of the DCE-epoxide in human liver and lung incubations. We measured the effect of $DASO_2$, a potent CYP2E1-specific inhibitor, on the production of the DCE-epoxide in human lung and liver microsomes. Five human lung samples and 4 human liver samples were examined. In the human lung experiments, samples from only 1 of 5 patients, (HL-1), exhibited inhibition (27%) of DCE-epoxide formation in the microsomal incubations (Table 4.4). In contrast to the lung, $DASO_2$ caused significant inhibition of DCE-epoxide formation in all the human liver microsomal samples (Table 4.4).

DISCUSSION

Considerable data have accumulated to implicate the DCE-epoxide as the ultimate toxic species involved in the hepatotoxic and pneumotoxic effects of DCE in mice. (Dowsley *et al.*, 1995, 1996; Forkert *et al.*, 1996a, 1996b). The DCE-epoxide-derived GSH conjugates [B] and [C] are the major products formed in murine liver and lung microsomal incubations (Dowsley *et al.*, 1995, 1996). Previous studies have identified species-, sex- and age-dependent differences in formation of the epoxide, as assessed by the levels of [B] and [C] formed (Dowsley *et al.*, 1995, Jones and Hathway, 1978; Forkert *et al.*, 1996a, 1996b). The findings indicated a strong correlation between the rate of production of the DCE-epoxide and susceptibility to DCE-induced injury. Here we have extended the findings from this previous work in experimental animals to studies in the human, and have undertaken to investigate the capacity of human lung and liver microsomes to bioactivate DCE to reactive intermediates and to determine whether the DCE-epoxide is a major product formed.

Our results demonstrated that human liver microsomes possess a strong capacity to metabolize DCE to the DCE-epoxide, as estimated from the sum of [B] and [C] produced (Fig. 4.2, Table 4.2). The major products formed were [B] and [C], whereas the acetal of 2,2-dichloroacetaldehyde was detected at almost negligible levels (Table 4.1). There was considerable variation, spanning a 5-fold range, in the levels of the DCE-epoxide generated in liver microsomes from the different patients (Fig. 4.3A). Liver microsomes from three patients metabolized DCE to the epoxide at levels that were 2.5 to 3-fold higher than that in murine liver microsomes. These data suggested that humans exposed to DCE may be potentially at risk, and that some individuals may sustain

hepatotoxic effects greater than those seen in the mouse. Importantly, this risk factor in humans will also depend on individual differences in cellular GSH levels for detoxication. Glutathione *S*-transferase activity in humans will probably not play an important role as the DCE-epoxide is highly reactive towards GSH non-enzymatically (Liebler *et al.*, 1985). In a previous study, we determined that production of the DCE-epoxide in liver microsomes was 6-fold higher in mice than in rats (Dowsley *et al.*, 1995). Since epoxide formation in the human liver experiments described herein were within the range of the levels in murine liver, it appears that the mouse is a better model than the rat for assessing human risk to DCE-induced hepatotoxicity.

We also detected formation of reactive intermediates in microsomal incubations from the 8 human lung samples. As was found in the human liver experiments, [B] and [C] were the major products detected, indicating that the DCE-epoxide is the major metabolite formed in human lung microsomes (Fig. 4.2, Table 4.1). The acetal of 2,2-dichloroacetaldehyde was also detected, and amounted to about 25% of the epoxide. The relative level of acetal was lower in human liver microsomes, and was formed at about 7% of the epoxide (Table 4.2). The relatively higher levels of the acetal in human lung *vs.* human liver are consistent with findings obtained in the lungs of mice in previous studies (Dowsley *et al.*, 1996). Nevertheless, the quantities of acetal produced were low in both human lung and liver, suggesting that DCE epoxidation is the preferential route of metabolism. It remains unclear whether this has any toxicological significance, although most of the available evidence indicated that 2,2-dichloroacetaldehyde is not an important metabolite in the DCE bioactivation pathway (Dowsley *et al.*, 1995; Kainz *et al.*, 1993). The 8 human lung microsomal samples metabolized DCE to the epoxide at levels that

spanned more than a 2-fold range (Table 4.1). The mean level was about 50% of the amount formed in lung microsomes from mice. This would suggest that susceptibility to DCE-induced lung injury is likely to be less severe in humans than in mice. However, the levels of epoxide formed in the human lung incubations were variable, suggesting that individuals capable of generating higher epoxide levels may be more vulnerable to the pneumotoxic effects of DCE.

Our previous studies have established a role for CYP2E1 in the bioactivation and ensuing toxicity of DCE in murine lung and liver (Lee and Forkert, 1994, 1995; Dowsley *et al.*, 1995, 1996; Forkert *et al.*, 1996a, 1996b). Here we have evaluated the role of human CYP2E1 in DCE metabolism by comparing microsomal levels of PNP hydroxylation with the amounts of DCE-epoxide formed. The large variations in PNP hydroxylation in human liver were reflected in the relative levels of DCE-epoxide formed. A relationship between enzyme catalytic activity and DCE-epoxide levels was observed in that the rank order among murine and human lung and liver for these parameters was nearly identical (Figs. 4.3 and 4.4). However, the ratio of DCE-epoxide to PNP hydroxylase activity was much higher in human lung as compared to the other groups (Figs. 4.3 and 4.4) suggesting that CYP2E1 may not be as important in catalyzing the metabolism of DCE in human lung. In addition, preincubation with $DASO_2$, which has been shown previously to selectively inhibit CYP2E1 (Forkert *et al.*, 1996b), caused a reduction of 20-65 % in the formation of the DCE-epoxide in human liver microsomes. These findings suggested that human liver CYP2E1 catalyzes the formation of the DCE-epoxide, and that variations in human liver CYP2E1 are likely to be manifested in the rate of formation of this metabolite. Interestingly, levels of human liver CYP2E1 can

vary at least 50-fold among individuals (Wrighton *et al.*, 1986; Lucas *et al.*, 1993). This variability may reflect the existence of genetic polymorphisms (Hayashi *et al.*, 1991), and/or exposure to inducing agents such as acetone and ethanol (Johansson *et al.*, 1988, 1990; Badger *et al.*, 1993; Lucas *et al.*, 1993; Takahashi *et al.*, 1993). Alcoholics or individuals exposed to CYP2E1 inducing agents may be at greater risk to the hepatotoxic effects of DCE due to greater production of the epoxide. Conversely, exposure to CYP2E1 inhibitors, such as the garlic derivative $DASO_2$, may lower DCE activation as well as the associated risk.

Although we observed substantial cytochrome P450-mediated formation of the DCE-epoxide in our human lung microsomal incubations, it did not appear that CYP2E1 was as important in this tissue as in human liver. For example, it is unlikely that the low levels of PNP hydroxylase activity in the human lung microsomal samples (7-fold lower than murine lung) could account for the relatively substantial production of the DCE-epoxide in this tissue , which amounted to about 50 % of that in murine lung. In addition, preincubation of microsomes with high concentrations of $DASO_2$ was effective in inhibiting DCE-epoxide formation in only 1 of 5 human lung microsomal samples. It is noteworthy that the lung sample from patient HL-1 exhibited 27% inhibition in the levels of DCE-epoxide formed after preincubation with $DASO_2$, but it also had the highest levels of CYP2E1 and DCE-epoxide of the 4 samples examined (Fig. 4.3). The lack of CYP2E1 inhibition in 4 of the human lung samples may reflect the presence of low CYP2E1 levels rather than an inability of human lung CYP2E1 to metabolize DCE. Our experiments with the human microsomes supported the premise that human CYP2E1 catalyzes the metabolism of DCE to the epoxide, and suggested that this P450 will also

play a role in human lung, if present in sufficient quantities. Of relevance is identification of several genetic polymorphisms of human CYP2E1 (Hayashi *et al.*, 1991). These polymorphic variations occur in the noncoding regions of CYP2E1; polymorphisms in the 5'flanking region of human CYP2E1 have been shown to cause a 10-fold variation in the transcriptional regulation of the gene (Hayashi *et al.*, 1991). This finding suggests that certain individuals may have higher expression of lung CYP2E1 than found in our samples. These individuals could potentially bioactivate DCE to the DCE-epoxide at levels that are as high or higher than in the lungs of mice, a species with high susceptibility to DCE-induced Clara cell damage (Forkert and Reynolds, 1982; Forkert *et al.*, 1996a). Studies with additional samples having CYP2E1 activity at least as high as HL-1, are needed to determine the full capacity of CYP2E1 in metabolizing DCE in human lung tissue.

Our studies in murine lung microsomes revealed that 50% of the levels of DCE-epoxide produced was attributed to CYP2E1, as assessed by immunoinhibition studies with a CYP2E1 monoclonal antibody (Dowsley *et al.*, 1996). We postulated that the remaining 50% is catalyzed by other P450 isozymes as yet unidentified (Dowsley *et al.*, 1996). These studies are currently under investigation in our laboratory. Our results also suggested that other P450s might be involved in the formation of the DCE-epoxide in human lung. This is not surprising based on the finding that, in addition to CYP2E1, human lung has been shown to express several P450 isozymes, including CYP1A1, CYP2B6, CYP2F1, CYP3A4, CYP4B1 (Wheeler and Guenther, 1991). Further studies are required to determine the role of these isozymes in catalyzing the metabolism of DCE in human lung.

In summary, our data showed that human lung and liver microsomes bioactivated DCE to the DCE-epoxide by a cytochrome P450-dependent mechanism, as assessed by the NADPH-dependent formation of the GSH conjugates [B] and [C]. The amounts of the DCE-epoxide formed correlated generally with levels of PNP hydroxylase activity detected in the microsomal samples, and were inhibited by $DASO_2$, suggesting that CYP2E1 is involved in DCE metabolism in human lung and liver.

TABLE 4.1. Formation of DCE metabolites in human lung microsomes

Patient	DCE Metabolites (pmol/mg protein/min)	
	DCE-Epoxide	2,2-Dichloroacetaldehyde
HL-1	29.4 ± 3.0	5.8 ± 0.3[c]
HL-2	19.3 ±5.2	4.1 ± 0.5[c]
HL-3	18.5 ± 4.6[a,b]	4.7 ± 0.8[c]
HL-4	18.5 ± 3.3[a,b]	5.6 ± 0.4[c]
HL-5	34.9 ± 12.8	6.3 ± 0.7[c]
HL-6	28.1 ± 3.2	5.7 ± 1.3[c]
HL-7	23.2 ± 2.9	4.5 ± 0.7[c]
HL-8	15.6 ± 4.2[a,b]	5.0 ± 0.7[c]

Microsomal incubations were performed at 25°C for 30 min. Reaction mixtures in a total volume of 0.5 ml contained 5 mg/ml of microsomal protein, [^{14}C]-DCE (2 mM, specific activity 7.5 nCi/nmol) and an NADPH-generating system. Levels of DCE-epoxide were estimated from the sum of conjugates [B] and [C], and 2,2-dichloroacetaldehyde from amounts of the acetal. Data are presented as mean ± S.D. of triplicate determinations from microsomal preparations of individual patients.

[a]Significantly different from HL-1 ($P < 0.05$).
[b]Significantly different from HL-5 ($P < 0.05$).
[c]Significantly different from DCE-epoxide ($P < 0.05$).

TABLE 4.2. Formation of DCE metabolites in human liver microsomes

Patient	DCE Metabolites (pmol/mg protein/min)	
	DCE-epoxide	2,2-Dichloroacetaldehyde
HLv-1	46.5 ± 8.3[a]	6.7 ± 0.9[b]
HLv-2	240.0 ± 10.5	10.5 ± 1.1[b]
HLv-3	202.3 ± 25.5	8.2 ± 0.2[b]
HLv-4	54.8 ± 12.5[a]	6.5 ± 0.4[b]
HLv-5	200.8 ± 50.8	8.3 ± 0.2[b]

Microsomal incubations were performed at 25°C for 30 min. Reaction mixtures in a total volume of 0.5 ml contained 5 mg/ml of microsomal protein, [^{14}C]-DCE (2 mM, specific activity 7.5 nCi/nmol) and an NADPH-generating system. Levels of DCE-epoxide were estimated from the sum of conjugates [B] and [C], and 2,2-dichloroacetaldehyde from amounts of the acetal. Data are presented as mean ± S. D. of triplicate determinations from microsomal preparations of individual patients.

[a]Significantly different from HLv-2, HLv-3 and HLv-5 ($P < 0.05$).
[b]Significantly different from DCE-epoxide ($P < 0.05$).

TABLE 4.3. Formation of DCE metabolites in murine lung and liver microsomes

Patient	DCE Metabolites (pmol/mg protein/min)	
	DCE-epoxide	2,2-Dichloroacetaldehyde
Lung	40.3 ± 3.8	9.7 ± 2.0[a]
Liver	83.3 ± 6.2[b]	12.0 ± 2.2[a,c]

Microsomal incubations were performed at 25°C for 30 min. Reaction mixtures in a total volume of 0.5 ml contained 5 mg/ml of microsomal protein, [^{14}C]-DCE (2 mM, specific activity 7.5 nCi/nmol) and an NADPH-generating system. Levels of DCE-epoxide were estimated from the sum of conjugates [B] and [C], and 2,2-dichloroacetaldehyde from amounts of the acetal. Data are presented as mean ± S.D. of triplicate determinations from three different microsomal preparations.

[a] Significantly different from levels of DCE-epoxide ($P < 0.05$).
[b] Significantly different from levels of DCE-epoxide in lung ($P < 0.05$).
[c] Significantly different from levels of 2,2-dichloroacetaldehyde in lung ($P < 0.05$).

TABLE 4.4. Effects of $DASO_2$ on DCE-epoxide formation in human lung (HL) and liver (HLv) microsomes

Patient	-$DASO_2$ + DCE	+ $DASO_2$ + DCE
HL-1	19.1 ± 2.2	14.0 ± 1.1[a]
HL-2	20.6 ± 3.5	21.1 ± 5.8
HL-3	13.8 ± 4.2	13.5 ± 3.6
HL-4	18.8 ± 3.7	21.4 ± 7.5
HL-9	30.1 ± 2.0	27.0 ± 1.2
HLv-3	120.5 ± 8.5	76.0 ± 9.2[b]
HLv-6	95.8 ± 6.2	33.9 ± 7.2[b]
HLv-7	86.3 ± 5.5	69.5 ± 4.8[b]
HLv-8	84.7 ± 5.7	40.5 ± 2.0[b]

Reaction mixtures in a total volume of 0.5 ml contained 5 mg/ml of microsomal protein, 20 mM $DASO_2$ and an NADPH-generating system, and were preincubated for 30 min at 37°C. The microsomes were recovered by centrifugation and precipitation, and were incubated with [^{14}C]-DCE (2 mM, specific activity 7.5 nCi/nmol) and an NADPH-generating system at 25°C for 30 min. Levels of DCE-epoxide were estimated from the sum of conjugates [B] and [C], and 2,2-dichloroacetaldehyde from amounts of the acetal. Data are presented as mean ± S. D. of triplicate determinations from microsomal preparations of individual patients.

[a] Significantly different from -$DASO_2$ + DCE ($P < 0.05$).

[b] Significantly different from –$DASO_2$ + DCE ($P < 0.01$).

FIG. 4.1 Proposed pathway of DCE metabolism (taken from Dowsley *et al.*, 1996)

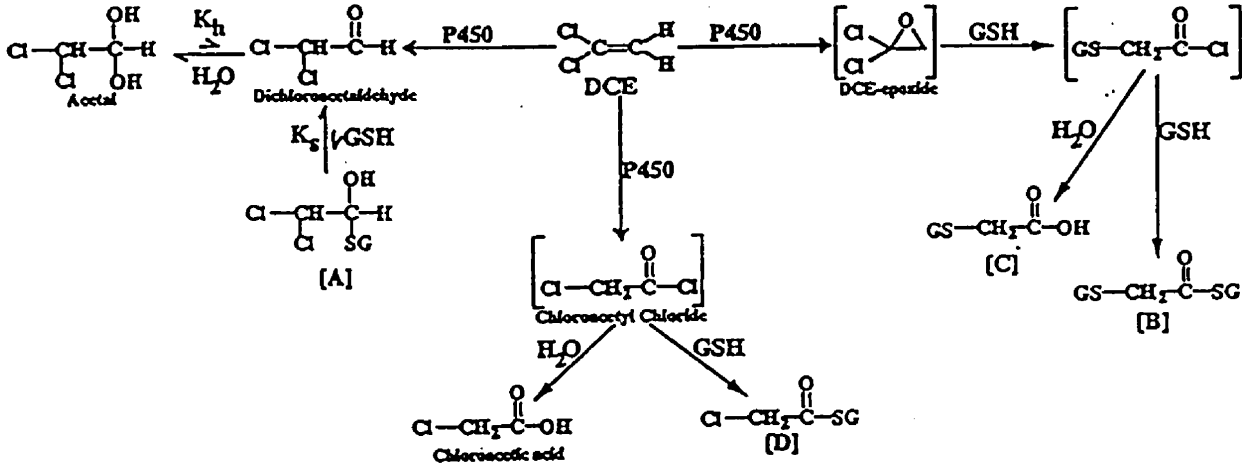

Acetal
K_h
H_2O
Dichloroacetaldehyde
P450
DCE
P450
DCE-epoxide
GSH
K_s
GSH
[A]
P450
Chloroacetyl Chloride
H_2O
GSH
Chloroacetic acid
[D]
H_2O
GSH
[C]
[B]

FIG. 4.2. HPLC analysis of human lung (A) and liver (B) microsomal incubations.

Reactions were conducted in a total volume of 0.5 ml of 50 mM phosphate buffer, pH 7.4, at 25°C 30 min. The reaction mixtures contained 5.0 mg/ml microsomal protein, 2 mM [^{14}C]DCE (specific activity 7.5 nCi/nmol) 15.0 mM GSH, and an NADPH-generating system. Microsomal proteins were precipitated and the supernatant was subjected to HPLC analysis. Radioactivity was determined in fractions (0.25 ml) eluting from the column. The peaks were identified as the GSH conjugates, 2-(*S*-glutathionyl)acetyl glutathione [B] and 2-*S*-glutathionyl acetate [C] and the acetal of 2,2-dichloroacetaldehyde.

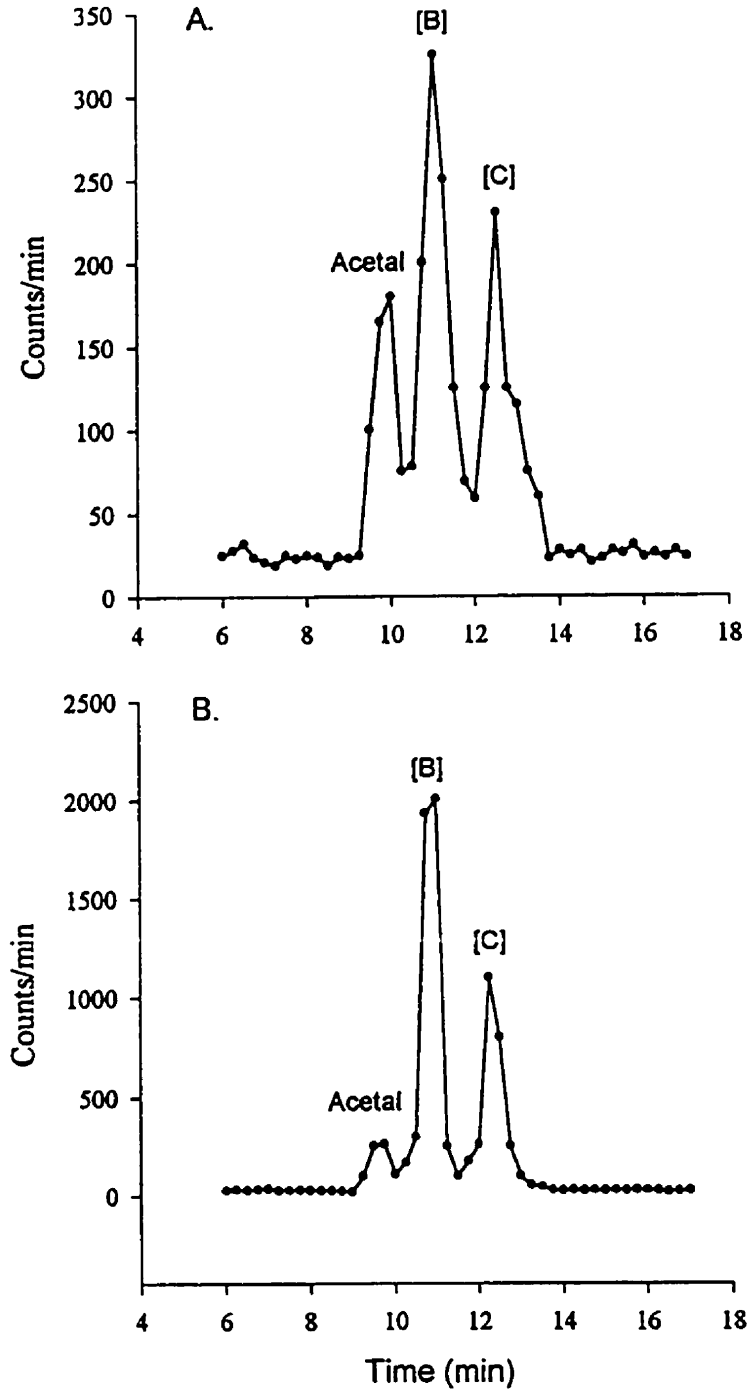

A.
[B]
[C]
Acetal
Counts/min
350
300
250
200
150
100
50
0
4
6
8
10
12
14
16
18
B.
[B]
[C]
Acetal
Counts/min
2500
2000
1500
1000
500
0
4
6
8
10
12
14
16
18
Time (min)

FIG. 4.3. Formation of DCE-epoxide and PNP hydroxylase activity in microsomes of human (HL) and murine (MLu) lung.

Levels of the epoxide were estimated from the total levels of [B] and [C]. Data are expressed as the mean ± S. D. of triplicate determinations for each human lung sample, while values for murine lung are derived from triplicate determinations from each of 3 separate microsomal preparations. Details of the microsomal incubations with DCE and the measurements for PNP hydroxylase activity are described in *Materials and Methods*.

[a]Significantly different from HL-2, HL-3, HL-4, and MLu ($P < 0.05$).

[b]Significantly different from HL-1, HL-4, and MLu ($P < 0.05$).

[c]Significantly different from HL-1, HL-2, HL-3, MLu ($P < 0.05$)

[d]Significantly different from HL-3 and HL-4 ($P < 0.05$).

[e]Significantly different from all human lung samples ($P < 0.05$).

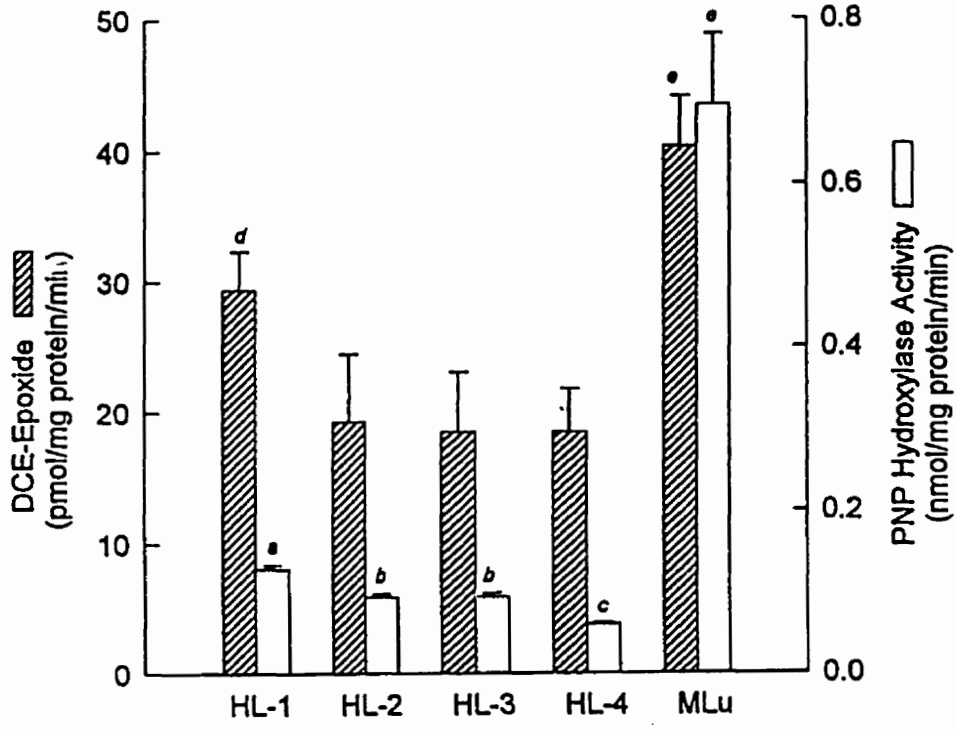

DCE-Epoxide
(pmol/mg protein/min)
PNP Hydroxylase Activity
(nmol/mg protein/min)
50
40
30
20
10
0
0.8
0.6
0.4
0.2
0.0
d
a
b
b
c
e
e
HL-1
HL-2
HL-3
HL-4
MLu

FIG. 4.4 Formation of DCE-epoxide and PNP hydroxylase activity in microsomes from human (HLv) and murine (MLv) liver.

Levels of the epoxide were estimated from the total levels of [B] and [C]. Data are expressed as the mean ± S. D. of triplicate determinations for each human lung sample, while values for murine lung are derived from triplicate determinations from each of 3 separate microsomal preparations. Details of the microsomal incubations with DCE and the measurements for PNP hydroxylase activity are described in *Materials and Methods*..

*Significantly different from values for HLv-1 and MLv, $P < 0.05$.

†Significantly different from values for HLv-1 and MLv, $P < 0.05$.

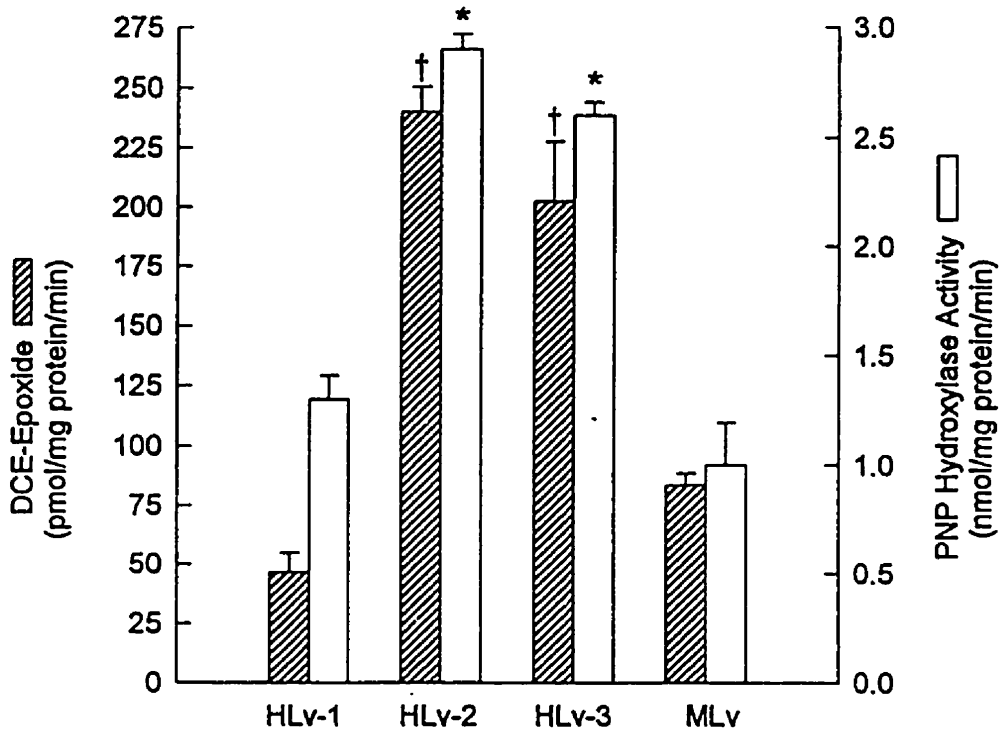
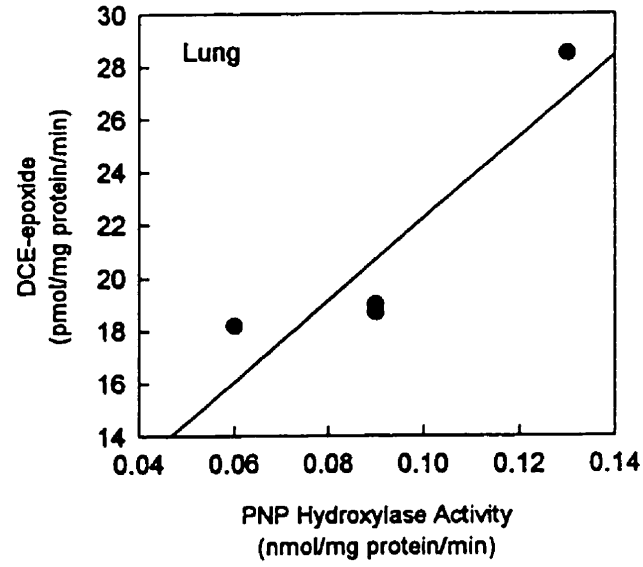
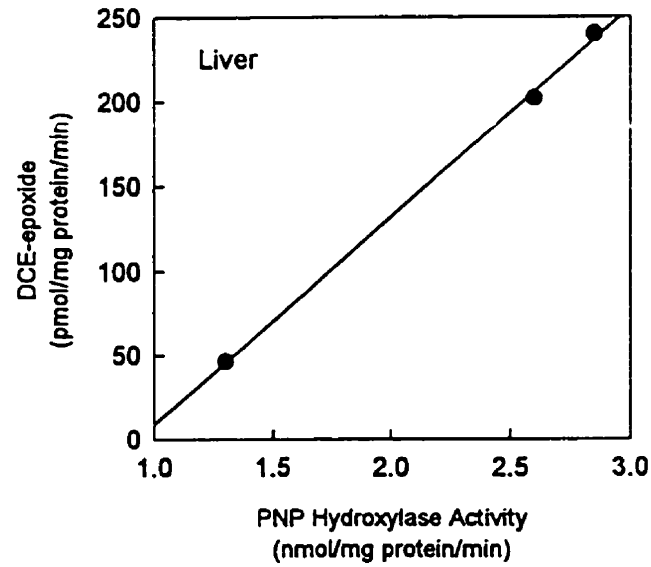

DCE-Epoxide (pmol/mg protein/min)
PNP Hydroxylase Activity (nmol/mg protein/min)
275
250
225
200
175
150
125
100
75
50
25
0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
†
*
†
*
HLv-1
HLv-2
HLv-3
MLv

FIG. 4.5. Regression analysis of the relationship between DCE-epoxide formation and levels of CYP2E1-dependent PNP hydroxylase activity in 4 human lung microsomal samples ($R^2 = 0.80$, top panel) and 3 human liver microsomal samples ($R^2 = 0.99$, bottom panel).


Lung
DCE-epoxide
(pmol/mg protein/min)
30
28
26
24
22
20
18
16
14
0.04
0.06
0.08
0.10
0.12
0.14
PNP Hydroxylase Activity
(nmol/mg protein/min)


Liver
DCE-epoxide
(pmol/mg protein/min)
250
200
150
100
50
0
1.0
1.5
2.0
2.5
3.0
PNP Hydroxylase Activity
(nmol/mg protein/min)

CHAPTER 5

GENERAL DISCUSSION

This thesis explores the cytochrome P450-dependent metabolism of 1,1-dichloroethylene (DCE), an event believed to mediate the toxicity of this chemical in lung and liver (Okine and Gram, 1986; Forkert *et al.*, 1987). The studies described herein have characterized the specific DCE metabolites formed, provided insight into their relative abilities to deplete GSH, and have examined the P450 isozyme selectivity in catalyzing the formation of these products.

Our first study described in Chapter 2 was initiated by our preliminary data that conflicted with those of published reports regarding the source of the GSH conjugates [B] and [C], the major products formed in rat hepatic microsomal incubations (Liebler *et al.*, 1985). The results of these previous studies had proposed that reaction of GSH with either the DCE-epoxide or 2-chloroacetyl chloride could produce conjugates [B] and [C] (Liebler *et al.*, 1985) (Fig 2.1). We were not able to reproduce these results that generated [B] and [C] from 2-chloroacetyl chloride or chloroacetic acid. This prompted us to undertake a detailed analysis of the reactivity of the three primary DCE metabolites with GSH under conditions similar to microsomal incubations to gain information about the relative abilities of these products to scavenge cellular GSH and/or alkylate macromolecules. By analyzing the data from these reactions in our experiments, it was determined that the DCE-epoxide is likely the exclusive precursor to the GSH conjugates [B] and [C] in hepatic microsomal incubations (Fig 2.7). We did not observe formation of conjugate [B] or [C] from reaction of 2-chloroacetyl chloride with GSH (Fig 2.4). Instead, this reaction yielded only conjugate [D], an additional conjugate formed in microsomal incubations (Figs 2.2, 2.3A, and 2.4).

Also in contrast to findings of previous studies (Liebler *et al.*, 1985), we did not observe conjugate [A], the GSH conjugate of 2,2-dichloroacetaldehyde, in our microsomal incubations. We determined that the equilibrium between conjugate [A] and the free hydrate (acetal) of 2,2-dichloroacetaldehyde heavily favored the acetal. Using this experimental equilibrium constant, it was calculated that a ratio of acetal to [A] of 14:1 would be expected in microsomal incubations employing 5 mM GSH, suggesting that conjugate [A] is a very minor product of DCE metabolism in microsomal incubations supplemented with GSH (Dowsley *et al.*, 1995).

The implications of our results are interesting. Our finding that conjugates [B] and [C] arise exclusively from the DCE-epoxide, and are the major products formed in both rat (Liebler *et al.*, 1985) and mouse (Dowsley *et al.*, 1995) hepatic microsomal incubations (Liebler *et al.*, 1985) (Fig 2.2, 2.3) suggested an important role for this metabolite in the reported GSH depletion that accompanies DCE-induced covalent binding and toxicity in liver (Forkert and Moussa, 1991). The intermediate reactivity of the DCE-epoxide (Liebler *et al.*, 1985) also implicated it in the alkylation of cellular macromolecules. Interestingly, the LD_{50} value for an oral dose of DCE is 7-fold lower in mice compared with rats (Jones and Hathway, 1978), and we observed a 6-fold higher production of the DCE-epoxide in liver microsomal incubations from the former species (Fig 2.8). Taken together, our results implicated the DCE-epoxide as an important metabolite mediating the toxic effects of DCE.

The equilibrium between the acetal and conjugate [A] heavily favored the acetal at physiological pH and GSH concentrations and suggested that 2,2-dichloroacetaldehyde will not contribute significantly to the GSH depletion observed *in vivo* (Forkert and Moussa, 1991; Moussa and Forkert, 1992). The possibility still exists that 2,2-dichloroacetaldehyde could play a role in DCE-induced covalent binding to macromolecules similar to what has been reported for chloroacetaldehyde, a metabolite of vinyl chloride (Guengerich *et al.*, 1979, 1981). However, the significance of this is probably minor in light of findings from previous studies that demonstrated significant exacerbation of DCE-induced hepatotoxicity in animals with depleted GSH levels (Jaeger *et al.*, 1974; Forkert and Moussa, 1992). This would not have been expected if 2,2-dichloroacetaldehyde was the ultimate toxic species because GSH levels should have little bearing on covalent binding of the aldehyde to macromolecules, as GSH does not react appreciably with this metabolite (Fig 2.5).

The relatively low levels of 2-chloroacetyl chloride formed in our incubations suggested that the predominant fate of this extremely reactive molecule (Liebler *et al.*, 1985) may be alkylation of P450 near its site of formation. It may be responsible for the reported mechanism-based decline in P450 content and CYP2E1-dependent catalytic activity (Lee and Forkert, 1994, 1995). Any 2-chloroacetyl chloride that escapes the active site of P450 would most likely react with GSH located nearby to form conjugate [D] or hydrolyze to chloroacetic acid, but the high reactivity of this molecule would likely prevent it from reaching a distant macromolecular target. To the best of our knowledge, the

work described in Chapter 2 represented the first to provide evidence for identifying which of the three primary DCE-metabolites mediated toxicity; our data strongly suggested a role for the DCE-epoxide.

The lung is also susceptible to DCE-induced damage, and there is evidence to suggest that the pneumotoxic effects of DCE arise from metabolism of this chemical to reactive intermediates by cytochrome P450 (Okine and Gram, 1986; Forkert *et al.*, 1987; Moussa and Forkert, 1992). Hence, we investigated the microsomal metabolism of DCE in murine lung. This undertaking represented a challenge since the lung contains considerably lower levels of cytochrome P450 compared with those in the liver (McKinnon and Mcmanus, 1996), but was important given that the specific metabolites formed in lung had not been identified. Our results demonstrated that similar to DCE metabolism in the liver, the major products formed in the lung were the DCE-epoxide derived GSH conjugates [B] and [C]. We also observed formation of the acetal of 2,2-dichloroacetaldehyde in murine lung microsomes; this metabolite was formed at higher rates relative to the DCE-epoxide in these experiments than in murine liver incubations (Fig. 3.2). However, as mentioned, it would appear that 2,2-dichloroacetaldehyde does not play a major role in DCE-induced toxicity (Dowsley *et al.*, 1995; Kainz *et al.*, 1993). Only neglible amounts of chloroacetic acid or conjugate [D], the hydrolysis product and GSH conjugate of 2-chloroacetyl chloride, respectively, were formed in murine lung (Fig. 3.2). Hence, similar to what was postulated from our studies with murine liver

microsomes, our results from the murine lung experiments suggested a role for the DCE-epoxide in mediating DCE-induced pneumotoxicity.

Of interest in the context of mechanisms mediating the toxic effects of DCE is the isozyme-selectivity in the metabolism of this chemical. Our previous studies supported a role for CYP2E1 in the metabolic activation of DCE in murine liver (Lee and Forkert, 1994) and lung (Lee and Forkert, 1995) microsomal incubations. We have therefore examined the role of this isozyme in catalyzing the formation of reactive intermediates in these tissues. Our results suggested a strong role for CYP2E1 in DCE metabolism in both the liver (Dowsley *et al.*, 1995) and lung (Dowsley *et al.*, 1996). We observed a 3-fold higher production of the DCE-epoxide conjugates [B] and [C] in murine liver microsomal incubations from mice pretreated with the CYP2E1-inducing agent, acetone (Fig. 2.8). In our murine lung experiments, we used a CYP2E1-selective inhibitory monoclonal antibody (Ko *et al.*, 1987), and inhibited the formation of the DCE-epoxide conjugates by 50% suggesting that at least 50% of the DCE-epoxide formed was catalyzed by CYP2E1. Since it appeared that only a fraction of the DCE-epoxide formed was catalyzed by CYP2E1, these data suggested that other isozymes of P450 may also catalyze the oxidation of DCE in murine lung. Alternatively, it may not be possible to inhibit 100 % of CYP2E1-dependent catalytic activity with the inhibitory antibody. A lack of complete inhibition with high concentrations of the inhibitory antibody is consistent with findings from previous studies with rat liver microsomes (Thomas *et al.*, 1987, Nakajima *et al.*, 1992). It has been suggested that interference by cytochrome P450 reductase or

inaccessibility of the entire portion of CYP2E1 to the antibody may be responsible for this phenomenon (Thomas *et al.*, 1987). Nevertheless, there was a strong role for CYP2E1 in the metabolism of DCE in liver and lung.

The capacity for CYP2E1-dependent metabolism of DCE in murine lung microsomes described above is interesting in light of the observation that the cell type most susceptible to DCE-induced toxicity in lung are the Clara cells (Forkert and Reynolds, 1982; Okine and Gram, 1986). Isolated Clara cell fractions displayed the highest levels of covalent binding as compared with fractions containing type II cells or fractions containing a mixture of lung cells (Forkert *et al.*, 1990). More recently, pulmonary CYP2E1 protein and mRNA have been shown to be localized predominantly in these cells (Forkert *et al.*, 1995). Taken together, these data suggested that the Clara cell-selective toxicity associated with DCE is due to CYP2E1-dependent bioactivation to reactive intermediates within these cells, and that the DCE-epoxide may be the most important reactive species. This concept is supported by results of recent studies in our laboratory (Forkert *et al.*, 1996a, 1996b). Lung microsomes from adult female CD-1 mice were found to have about 45% higher levels of CYP2E1-dependent PNP hydroxylase activity than lung microsomes from male adult mice (Lee and Forkert, 1995). This gender-related difference in CYP2E1 expression is reflected in the greater susceptibility of females than males to the pneumotoxic effects of DCE; the DCE dose that was required to produce a comparable level of Clara cell injury was about twice the amount in male *versus* female mice (Forkert *et al.*, 1996a). This sexual dimorphism was reflected in a 2-fold higher production of the DCE-

epoxide in microsomes from females than from males. Hence, data from this study suggested that the enhanced susceptibility of female CD-1 mice to DCE-induced pneumotoxicty is, in part, a result of higher CYP2E1 expression and epoxide formation.

The role of CYP2E1 in mediating DCE-induced toxicity was further confirmed in studies in which CYP2E1 was inhibited with the garlic derivative, $DASO_2$ (Forkert *et al.*, 1996b). The results were exciting in that protection of the bronchiolar epithelium was achieved in female CD-1 mice pretreated with $DASO_2$ prior to being given a dose of 75 mg/kg DCE, a regimen that caused bronchiolar necrosis in mice that were not pretreated with $DASO_2$ (Forkert *et al.*, 1996a, 1996b). The protective effects correlated with significantly lower levels of CYP2E1-dependent p-nitrophenol hydroxylase activity and DCE-epoxide formation (Forkert *et al.*, 1996b). These studies supported the contention that the DCE-induced Clara cell damage is mediated by CYP2E1-dependent metabolism of DCE to the DCE-epoxide, and that protection against these effects can be achieved by inhibition of CYP2E1, producing a concomitant decline in production of the DCE-epoxide.

The bronchiolar necrosis associated with DCE in animal studies is similar in many respects to what has been reported for other pulmonary toxicants including 4-ipomeanol and naphthalene (Gram *et al.*, 1998). 4-Ipomeanol, a natural contaminant of sweet potatoes, and naphthalene both cause Clara cell-selective injury to rodents at doses below those that affect the liver and kidney (Dutcher and Boyd, 1979; Mahvi *et al.*, 1977; Warren *et al.*, 1982). The

mechanisms mediating the cytotoxicity of these chemicals involve cytochrome P450-dependent metabolism to reactive intermediates that bind covalently to macromolecules (Boyd *et al.*, 1975; Boyd and Burka, 1978; Tong *et al.*, 1982; Warren *et al.*, 1982). The importance of GSH in detoxification by conjugating with reactive intermediates is also described for these pneumotoxicants. In the case of 4-ipomeanol, the GSH conjugates were formed non-enzymatically (Buckpitt and Boyd, 1980). This mechanism is also apparent for formation of the DCE-epoxide GSH conjugates [B] and [C], levels of which are not increased with the addition of glutathione-*S*-transferases in microsomal incubations (Liebler *et al.*, 1985). In contrast, conjugation of naphthalene-derived reactive intermediates with GSH is catalyzed by these enzymes (Smart and Buckpitt, 1983). Although 4-ipomeanol, naphthalene, and DCE are all metabolized by cytochrome P450 to reactive intermediates, and are toxic to Clara cells, these chemicals differ with respect to the pulmonary P450s that catalyze these reactions. For 4-ipomeamol, CYP3A4, CYP1A2, and CYP4B1 (Czerwinski *et al.*, 1991; Yost, 1996) have been implicated in its metabolism, while CYP2F2 (Chang *et al.*, 1996) has been shown to metabolize naphthalene to 2 enantiomeric epoxides. The lack of importance of CYP2E1 in metabolizing 4-ipomeanol or naphthalene is not surprising given that these are larger molecules than the majority of CYP2E1 substrates that are low molecular weight compounds (Guengerich *et al.*, 1991). The results of these studies in animals support the assumption that the high susceptibility of the Clara cells to the effects of toxicants is due to localization of cytochrome P450 monooxygenases in this cell type.

In our final study (Chapter 4), we examined the capacity of human and murine lung and liver microsomes to metabolize DCE to reactive intermediates, and compared the rates of their formation with those in the mouse in an effort to assess the potential risk to humans exposed to this chemical. Our results demonstrated that both human lung and liver microsomes metabolized DCE to reactive intermediates by a cytochrome P450-dependent mechanism. Similar to our studies in mice, the DCE-epoxide-derived conjugates [B] and [C] were the major products formed, while lower levels of the acetal of 2,2-dichloroacetaldehyde were also detected (Tables 4.1 and 4.2; Fig. 4.2). The rates of formation of the DCE-epoxide in human lung spanned a 2-fold range and had a mean level that was approximately 50% of the values in murine lung (Tables 4.1, 4.3). The variation in human liver samples was more pronounced and spanned more than a 5-fold range (Table 4.2). Importantly, some human liver microsomal samples produced the DCE-epoxide at rates that were 2.5 to 3-fold higher than those in mice. A large range of CYP2E1-dependent *p*-nitrophenol hydroxylase activity was also seen in the human liver microsomal samples (Fig. 4), and the levels corresponded with the rates of formation of the DCE-epoxide, suggesting a role for this P450 in human liver metabolism of DCE. This was further supported by experiments with the CYP2E1 inhibitor, $DASO_2$; preincubation of human liver microsomes with $DASO_2$ caused a significant decline in the formation of conjugates [B] and [C] (Table 4.4). The results of this work therefore suggest that humans exposed to DCE may be susceptible to its hepatotoxic and pneumotoxic effects due to cytochrome P450-dependent metabolism to the DCE-epoxide.

These data also strongly support a role for human CYP2E1 in this bioactivation in liver and imply that individuals with high levels of CYP2E1 expression may be particularly sensitive to DCE-induced hepatotoxicity.

It did not appear that CYP2E1 was as important for catalyzing the formation of the DCE-epoxide in our human lung microsomes as it was in human liver microsomes. $DASO_2$ inhibited the levels of the GSH conjugates by 27% in only 1 sample (Table 4.4). This sample also had the highest level of CYP2E1-dependent *p*-nitrophenol hydroxylase and yielded the highest formation of DCE-epoxide (Table 4.1, Fig. 4.3). This finding suggests that CYP2E1 may play a role in bioactivation of DCE in lung if present in sufficient quantities. This concept is plausible in view of findings showing that several genetic polymorphisms in the non-coding regions of human CYP2E1 can markedly affect the transcriptional regulation of this gene (Hayashi *et al.*, 1991). The incidence of these alleles ranges from 0.10 to 0.18 in the Caucasian population but is considerably higher in Asians at 0.18 to 0.43 (Hayashi *et al.*, 1991). Individuals with levels of CYP2E1 expression that are higher than the samples described herein may bioactivate DCE to the DCE-epoxide at enhanced rates and be more susceptible to lung injury. However, substantial levels of the DCE-epoxide were formed in our lung microsomal samples that were unaffected by CYP2E1 inhibition, suggesting that another isozyme (s) metabolizes DCE in human lung. As mentioned previously, a maximum of 50% inhibition in DCE-epoxide formation was achieved in our murine lung incubations, using high concentrations of a CYP2E1-specific inhibitory monoclonal antibody (Dowsley *et al.*, 1996). The identity of additional

P450 isozymes in metabolizing DCE remains to be elucidated. However, we have performed preliminary studies to address this proposal and have obtained promising data. We have detected conjugates [B] and [C] in incubations containing DCE and human CYP2F1 expressed in a lymphoblastoid cell line;this is a P450 enzyme present in human lung (Yost, 1996). Future studies will investigate the role of CYP2F1 and the mouse homologue CYP2F2 (Gram *et al.*, 1998) in DCE metabolism.

Our data strongly suggest that an epoxide is the ultimate toxic species causing the hepatotoxic and pneumotoxic effects of DCE. Epoxide hydrolases are enzymes that catalyze the hydrolysis of epoxides to less reactive dihydrodiols (Seidegard and Ekstrom, 1997). Considerable variation has been observed in the expression of microsomal and soluble epoxide hydrolases in humans (Mertes *et al.*, 1985). Therefore, individual differences in expression of these enzymes can play a role in the toxic responses to epoxides (Seidegard and Ekstrom, 1997). It is unknown whether the DCE-epoxide is a substrate for epoxide hydrolases. We observed negligible quantities of the dihydrodiol of the DCE-epoxide (glycolic acid) in our murine and human microsomal incubations containing excess GSH, but observe higher levels of this product in incubations devoid of GSH. It is of interest to determine if the ratio of glycolic acid to conjugates [B] and [C] increases in incubations supplemented with microsomal and/or soluble epoxide hydrolase, and containing moderate or low GSH levels. In addition, the employment of knockout mice may prove useful in assessing the potential protective role of epoxide hydrolase.

Important future studies will be to identify the critical macromolecules targeted by DCE. As the DCE-epoxide is implicated in the alkylation of proteins, an initial step may be to design a hapten consisting of synthesized DCE-epoxide covalently bound to amino acids. The successful generation of polyclonal antibodies to the DCE-epoxide bound to the sulfhydryl of cysteine, for example, could be used to identify the *in vivo* protein adducts. Developing antibodies of sufficient specificity may pose a considerable challenge considering the small molecular weight of the DCE-epoxide. In summary, the results of the work in this thesis support our hypothesis that the hepatotoxic and pneumotoxic effects of DCE are a result of cytochrome P450-dependent metabolism to reactive intermediates and that the CYP2E1 isozyme plays a strong role in catalyzing this reaction. Our analysis of the reactivity of the primary DCE metabolites with GSH combined with our characterization of the products formed in murine and human liver and lung microsomal incubations strongly support the DCE-epoxide as the major toxic species involved in depletion of GSH and alkylation of macromolecules. Furthermore our studies with human microsomes suggest that humans exposed to DCE may potentially be at risk to the deleterious effects of this chemical.

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