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HUMAN METABOLISM AND METABOLIC INTERACTIONS OF DEPLOYMENT-RELATED CHEMICALS

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It has been suggested that chemicals and, more specifically, chemical interactions, are involved as causative agents in deployment-related illnesses. Unfortunately, this hypothesis has proven difficult to test, because toxicological investigations of deployment-related chemicals are usually carried out on surrogate animals and are difficult to extrapolate to humans. Other parts of the problem, such as the definition of variation within human populations and the development of methods for designating groups or individuals at significantly greater risk, cannot be carried out on surrogate animals, and the data must be derived from humans. The relatively recent availability of human cell fractions, such as microsomes, cytosol, etc., human cells such as primary hepatocytes, recombinant human enzymes, and their isoforms and polymorphic variants has enabled a significant start to be made in developing the human data needed. These initial studies have examined the human metabolism by cytochrome P450, other phase I enzymes, and their isoforms and, in some cases, their polymorphic variants of compounds such as chlorpyrifos, carbaryl, DEET, permethrin, and pyridostigmine bromide, and, to a lesser extent, other chemicals from the same chemical and use classes, including solvents, jet fuel components, and sulfur mustard metabolites. A number of interactions at the metabolic level have been described both with respect to other xenobiotics and to endogenous metabolites. Probably the most dramatic have been seen in the ability of chlorpyrifos to inhibit not only the metabolism of other xenobiotics such as carbaryl and DEET but also to inhibit the metabolism of steroid hormones.

Key Words: Alcohol dehydrogenase; Aldehyde dehydrogenase; Carbamates; Chemical warfare agents; Cytochrome P450; Deployment-related chemicals; Jet fuel components; Metabolic interactions; Metabolism; Organophosphorus compounds; Pyrethroids; Repellents; Xenobiotic-metabolizing enzymes.

INTRODUCTION

Chemicals used during military deployments, and particularly interactions between such chemicals, are frequently cited as possible causative agents in deployment-related illnesses. Unfortunately, definitive evidence for or against such a role is lacking. In part, this is related to the fact that investigations of deployment-related chemicals carried out in experimental animals assume relevance only when they can be extrapolated with confidence to humans. As most exogenous chemicals can be substrates, inhibitors, and/or

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inducers of xenobiotic-metabolizing enzymes (XMEs), these enzymes are an important potential locus for interactions. In fact, human XMEs often differ dramatically from those of experimental animals, rendering such extrapolations of little value. Based on animal studies, large uncertainty factors, usually undefined and therefore used as default values, must be employed in any numerical estimate of risk. Under these circumstances, it is difficult to be certain that the best estimate of human health risk is being used. At the same time, it must be understood that many studies of toxic mechanisms can only be carried out using surrogate animals. Recent advances in molecular biology have made available techniques that are helpful in the integration of animal and human studies in toxicology and risk assessment, in particular, the use of genetic knockout and humanized mice, and have thus added new dimensions to animal studies.

Certain aspects essential in risk analysis of chemicals can only be examined in human studies. They include the determination of variation within the human population, which is highly outbred. Little of value can be learned about human variation by the use of inbred experimental animals. Many XMEs are polymorphic, and the distribution of polymorphic variants varies between population subgroups and individuals, potentially putting some individuals and subpopulations at greater or lesser risk than others.

The military need for information on human health risk from chemicals is clear. In several recent conflicts, poorly defined aggregations of health effects (most recently, Gulf War related illness) have been attributed to chemicals or to interactions between two or more chemicals (Haley and Kurt, 1997). Thus the need exists both to determine whether previous health effects are indeed chemical-related and to minimize future problems. Following the Gulf War a number of veterans reported a variety of illnesses which may have been the result of abnormal chemical exposures. Some studies of these veterans have concluded that significant correlations between perceived illnesses and chemical use exist (Haley and Kurt, 1997).

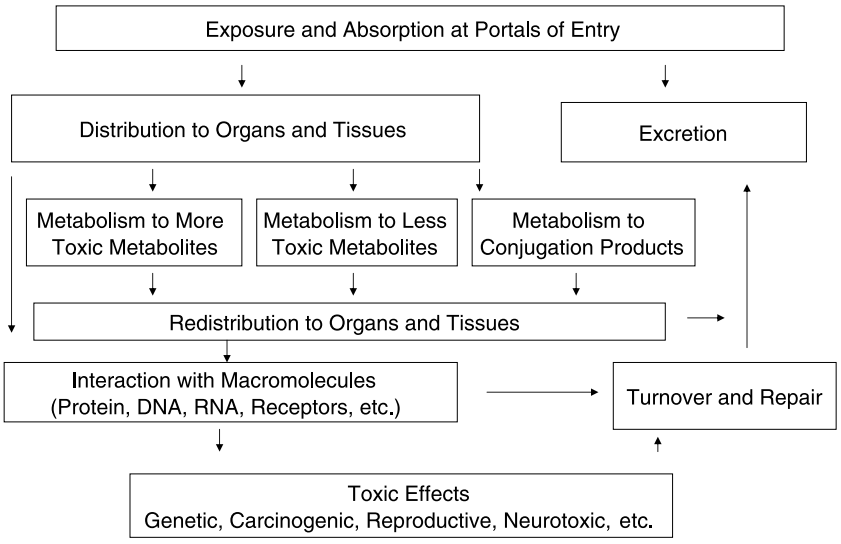

Figure 1. Mode of toxic action: a cascade of events.

Toxicity is the endpoint of a cascade of events that starts with exposure and ends with the expression of a toxic endpoint (Fig. 1). Intermediate steps include absorption, distribution, metabolism, distribution of metabolites, excretion, and/or interaction with cellular macromolecules followed by overt toxicity or repair. Although interactions can occur at any of these loci, metabolism is of critical importance, inasmuch as chemicals may be metabolically detoxified or activated to products more toxic than the parent compound. Phase I metabolism generally results in the introduction of a reactive group into the molecule, a reactive group that is subsequently conjugated with an endogenous compound during phase II metabolism. The most important phase I enzymes are the isoforms of cytochrome P450 (CYP) and the flavin-containing monooxygenase (FMO) (Hodgson and Smart, 2001). Furthermore, because XMEs have broad and often overlapping substrate specificities, metabolism is a probable locus for chemical interactions and for effects on the metabolism of endogenous substrates.

Because these activities are species, enzyme, isoform, and polymorphism specific, human studies using recombinant enzymes, subcellular preparations, and/or cultured cells are essential for the improvement of human health risk assessment, as follows:

- Identification of human XMEs, their isoforms and polymorphic forms involved in activation and/or detoxication
- Identification of reactive metabolites produced by human XMEs
- Identification of interactions between chemicals at the metabolic level
- Evaluation of the variation between individuals and identification of individuals and subpopulations at increased risk
- Provision of mechanistic insight into the results of epidemiological studies
- Overall improvement of the process of human health risk assessment with particular reference to deployment-related chemicals and deployment-related exposures

During the last few years, human subcellular preparations and recombinant enzymes have become readily available, and many new sensitive methods for the identification and quantitation of metabolites have been developed. Furthermore, noninvasive methods for the collection of human DNA combined with methods for high throughput genotyping make population studies possible.

Recent studies, as described below, have examined the role of specific human XMEs on the metabolism of a subset of chemicals important in military deployments. Carbaryl, chlorpyrifos, diethyl toluamide (DEET), permethrin, pyridostigmine bromide, sulfur mustard and its degradation products, and other chemicals of interest have been tested as substrates and inhibitors of the most important human XMEs, including cytochrome P450s (CYPs), flavin-containing monooxygenases (FMOs), alcohol and aldehyde dehydrogenases, and esterases (Choi et al., 2002, 2004; Dai et al., 2001; Tang et al., 2001, 2002; Usmani et al., 2002, 2003). A database on phase I enzymes and the metabolism of chemicals used in agriculture and public health was recently updated (Hodgson, 2003). Most XMEs exist as a number of isoforms, and these isoforms may have a number of polymorphic variants. Thus, it is important to know the relative abundance of these forms as well as their kinetic properties, because an enzyme with high activity may, in vivo, be less important than one with somewhat lower activity but with high abundance. The ability of these chemicals to act as inhibitors or enhancers of physiological (endogenous) substrates may also be of importance to the health of

exposed individuals, and effects on testosterone metabolism have been described. The potential of deployment-related chemicals to act as XME inducers has been studied to some extent in experimental animals, but studies are being initiated using human hepatocytes.

It is difficult to know exactly how many chemicals may be defined as deployment-related. Given the complexity of the noncombat deployment environment, with its wide array of public health and industrial chemicals as well as such combat-related chemicals as chemical warfare agents and combustion products, many variants of a list are possible. However, a useful starting point is the list provided in volumes 1 and 2 of ''Gulf War and Health'' (Institute of Medicine, 2003). To date, almost all studies of human in vitro xenobiotic metabolism and metabolic interactions have been carried out on clinical drugs, with few, if any, on insecticides, repellents, solvents, and industrial chemicals.

The relevance of these approaches to other occupational exposures in public health, industrial health, and agriculture is obvious, and the results should enable findings from animal studies to be extrapolated to humans with greater confidence. The results will also permit the identification of metabolic interactions specific to humans and permit the identification of populations at risk. Because the same XMEs are involved in the metabolism of clinical drugs and the xenobiotics of interest in deployments and elsewhere, interactions with clinical drugs are an aspect of considerable importance, unfortunately, an aspect that has not yet been addressed.

INSECTICIDES

Chlorpyrifos and Related Organophosphorus Insecticides

Introduction. Organophosphorus insecticides, including chlorpyrifos, are commonly used to control disease vectors as well as household and agricultural pests. They are potent inhibitors of acetylcholinesterase (AChE), resulting in a variety of symptoms including increased secretion, hyperactivity, and eventually death by respiratory failure (Echobicon, 2001). Many are organophosphorothioates, weak anticholinesterase inhibitors that are activated to their oxons, potent acetylcholinesterase inhibitors, by CYP catalyzed desulfuration. The resultant oxons are three or more orders of magnitude more potent as AChE inhibitors than the parent chemicals. Given these large differences in potency, the traces of oxons found as impurities in the parent compounds can have significant toxicological consequences (Yuknavage et al., 1997), and the effect of this oxon contamination will be impacted by the status of the individual relative to PON1 polymorphic forms (Li et al., 2000). The same enzymes involved in organophosphate activation are also important in the detoxication process. Thus, the ratio of activation to detoxication is dependent upon the individual CYP isoforms present in the tissue at a given time and will determine the amount of oxon available to cause toxicity (Levi and Hodgson, 1985).

Of the 11 insecticides shipped to the Gulf War (Institute of Medicine, 2003), five are organophosphorus chemicals. Three, chlorpyrifos, diazinon, and malathion, are phosphorothioates, containing the P═S group that are activated to their oxons by various CYP isoforms. Two others, azamethiphos and dichlorvos contain the P═O group and are potent AChE inhibitors without metabolic activation. Of these five insecticides, in terms of its use, chlorpyrifos is the most important as a deployment-related chemical.

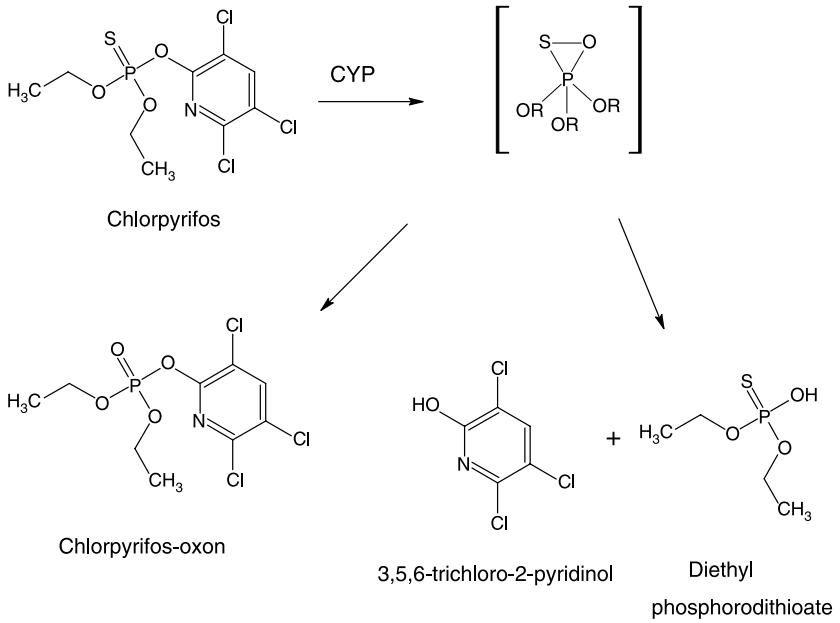

Figure 2. Metabolism of chlorpyrifos by human liver microsomes.

Numerous studies have been conducted in experimental animals [see Sultatos (1991) for references] on the metabolism of organophosphorus pesticides, including chlorpyrifos. Chlorpyrifos is metabolized to the oxon, and both chlorpyrifos and its oxon are rapidly hydrolyzed to 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) (Fig. 2). In humans, low doses of chlorpyrifos are completely metabolized and subsequently eliminated in the urine as 3,5,6-TCP or glucuronide conjugates of 3,5,6-TCP (Nolan et al., 1984). Although under these circumstances, inhibition of AChE may not be apparent, interactions with esterases other than AChE, currently of unknown significance, may occur. Other metabolites may include deethylated chlorpyrifos (*O*-ethyl trichloropyridyl phosphorothioate) as well as glutathione conjugates.

Although the liver appears to play a primary role in metabolism, resulting in a net activation of chlorpyrifos, extrahepatic tissue activation may be important in its ultimate toxicity. Although female rats produce less chlorpyrifos oxon in liver perfusion studies than do males, females are more susceptible to chlorpyrifos. This indicates that the rate of oxon production by the liver cannot adequately explain differences observed in the acute toxicities of these pesticides (Sultatos, 1991).

Organophosphorus insecticides are readily metabolized by cytochrome P450 (Fig. 2). Oxidative metabolism can result in both intoxication as a result of desulfuration or detoxication by several pathways. Esterase metabolism of organophosphates is also an important mechanism of detoxication. A human polymorphic enzyme (PON1), an esterase that hydrolyzes the oxon, in one form (Arg-192) hydroylzes paraoxon rapidly, while the other form (Gln-192) hydrolyzes paraoxon more slowly. However, the Gln-192 form rapidly hydrolyzes sarin, soman, and diazoxon in contrast with the Arg-192 form. Because the amount of PON1 is also highly variable in humans, the combination of genetic isoforms and the amount of enzyme may affect individual susceptibility to

organophosphates (Furlong et al., 1998). It has long been known that, as a result of the release of highly reactive sulfur, CYP is inhibited during oxon formation (Neal, 1980), and it was demonstrated more recently, in vivo, that chlorpyrifos is a CYP inhibitor when administered at a rate of 50 mg/kg to rats (Vodela and Dalvi, 1995).

Individual variation and the effect of polymorphisms are discussed below. It is clear, however, that there is considerable potential for differential sensitivity to chlorpyrifos to exist in the human population.

Subcellular preparations. Studies of in vitro human chlorpyrifos metabolism (Tang et al., 2001) demonstrate that pooled human liver microsomes, although effective in both desulfuration and oxidative dearylation, are less active toward this substrate than either rat or mouse microsomes. These species differences are illustrated by differences in the intrinsic clearance values (V_{max}/K_m) that have been determined for the more toxic oxon as well as the detoxication product, 3,5,6-TCP. In rats, the intrinsic clearance value for the oxon is 17-fold greater than in humans, while in mice, the intrinsic clearance for this product is only threefold greater. However, rats also have an enhanced ability to produce the detoxication products, as reflected by an eightfold greater intrinsic clearance value for the dearylation product than humans, while mice, again, show a threefold difference compared to humans for this value. Pooled human female liver microsomes were somewhat more active in chlorpyrifos metabolism than were pooled male liver microsomes.

In addition to chlorpyrifos, a number of other organophosphorus insecticides, including diazinon and parathion, are metabolized via both desulfuration and dearylation by pooled liver microsomes [see Hodgson (2003) for references].

Xenobiotic-metabolizing enzymes. Tang et al. (2001) also showed that the ability of human liver microsomes to detoxify chlorpyrifos and to activate it to its oxon was due to CYP isoforms 1A2, 2B6, 2C9, 2C19, and 3A4. The ratio of activation to detoxication varied between CYP isoforms, the ratios of desulfuration to dearylation ranged between 3.38 for the isoform most active in desulfuration (2B6) to 0.14 for the isoform least active in desulfuration (2C19). All of these isoforms are polymorphic and inducible, and thus, the potential for variation between individuals is high. More recently, Buratti et al. (2003) showed by correlation analysis that human CYP2B6 and CYP3A4 are most active in oxon production from a number of organophosphorus insecticides, including chlorpyrifos. The same CYP isoforms active in chloropyrifos oxidation are also known to be active in the metabolism of diazinon and parathion [see Hodgson (2003) for references].

Polymorphic variants. Polymorphic variants of several of the CYP isoforms active in chlorpyrifos metabolism have been examined. Of three CYP2C19 polymorphisms (Tang et al., 2001), all had reduced ability for dearylation, while no desulfuration activity could be detected.

The variability of polymorphic variants is further exemplified by studies of variants of human CYP3A4, an isoform active in both the activation and detoxication of chlorpyrifos (Dai et al., 2001). It is known that the expression of this isoform, often the most abundant CYP isoform in human liver, can, nevertheless, vary as much as 40-fold between individuals. In these studies, several new polymorphic variants of CYP3A4 were identified, sequenced, expressed, and characterized. These variants differ from the wild type in their frequency in different populations and in their ability to metabolize chlorpyrifos. Of four CYP3A4 polymorphisms examined, two had desulfuration and

dearylation activities similar to the wild type, one appeared to be defective in both activities, and the fourth was significantly more active.

CYP2B6 polymorphisms are also being examined (J. A. Goldstein, personal communication, 2004). Results to date indicate that several polymorphic variants do not differ significantly from the wild type in their ability to metabolize chlorpyrifos.

Individual livers. Potential differences in chlorpyrifos metabolism in the human population based on differences in hepatic enzymes were examined (Tang et al., 2001) using microsomes from five individual livers. Individuals with varying levels of CYP2B6, 2C19, 2D6, and 3A4 were selected to represent contrasting levels of predictable metabolic activity. Thus, as was observed, individuals with higher levels of CYP2B6 and CYP3A4 would be expected to show higher levels of desulfuration than those with lower levels of these isoforms. CYP3A4 should contribute to both desulfuration and dearylation, but, because microsomes from livers with contrasting levels of CYP2B6 and 3A4 were not available, the extent of their separate contributions to desulfuration could not be estimated. However, based on these results and those of others using parathion as a substrate (Butler and Murray, 1997; Mutch et al., 1999; Sams et al., 2000), CYP3A4 should contribute significantly to both desulfuration and dearylation. Even with a number as small as five, the variation in desulfuration activity was fivefold and that for dearylation was threefold. For parathion, the variation was 16- and 10-fold, respectively (Butler and Murray, 1997; Mutch et al., 1999). Mutch et al. (1999) demonstrated that the activation of parathion to paraoxon varied among individuals by as much as 16-fold, while the detoxication rate varied by only threefold.

Considerations of metabolic differences between individuals should also include the contributions of esterases, which, from animal studies, are known to be major factors in determining the in vivo toxicity of organophosphorus insecticides (Chambers et al., 1990; Costa et al., 1990; Maxwell et al., 1987). Unfortunately, with the exception of paraoxonase, the human esterases involved in organophosphorus insecticide metabolism have not been investigated. It is of interest (Li et al., 2000) with regard to the detoxication of diazoxon that only the level of PON1 is important, whereas for chlorpyrifos oxon, the status of the PON1 polymorphic forms as well as the level is also important, with the PON1R192 alloform providing significantly more protection than the PON1Q192 alloform. At the same time, neither alloform appears to be significant in protecting against a paraoxon exposure (Li et al., 2000).

Permethrin and Related Pyrethroids

Introduction. Of the 11 insecticides shipped to the Gulf War (Institute of Medicine, 2003), only two are pyrethroids, permethrin and d-phenothrin. Of these, permethrin is more important.

Permethrin, 3-phenoxybenzyl (±)-*cis*, *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylate, is a synthetic pyrethroid commonly used as an insecticide, including use in military deployments. The discovery of the first photostable pyrethroid, permethrin (Elliott, 1976; Elliott et al., 1973), revolutionized the use of pyrethroids as a class. Since its discovery, this class of insecticide has become one of the mainstays of chemical control due to high insecticidal efficacy, low mammalian toxicity, and lack of environmental persistance.

Permethrin has been extensively studied with respect to its toxicological properties (Ishmael and Litchfield, 1988; Litchfield, 1985; Metker et al., 1978). At high doses, permethrin produces a syndrome characterized by aggressive sparring, increased sensitivity to external stimuli, and a fine tremor that progresses to whole body tremor and prostration (Verschoyle and Barnes, 1972). At low doses, a dose-dependent decrease in locomotor activity and an increase in startle response to acoustic stimuli are observed (Crofton and Reiter, 1988). Bloomquist (1993) provides an overview of electrophysiological effects and interactions of pyrethroid insecticides with biochemical and physiological target sites in mammals and insects.

The low acute mammalian toxicity of permethrin and other pyrethroids is the result of their rapid metabolism and excretion (Elliott et al., 1976; Gaughan et al., 1977; Miyamoto, 1976). The major mammalian metabolite of permethrin, excreted as both free and conjugated forms, is 4′-hydroxy-3-phenoxybenzoic acid. This product, the result of ester hydrolysis, has been demonstrated to be a significant factor in the detoxication of permethrin in adult rats through the use of esterase synergist studies (Cantalamessa, 1993). Other minor metabolites that include hydroxylations at the acidic geminal dimethyl group and at the phenoxy group of the alcohol are the result of oxidative enzymes. Once these oxidations occur, the resulting carboxylic acids and phenols may be conjugated by a variety of conjugating enzymes. These metabolites are quickly excreted and do not persist in the tissues (Elliott et al., 1976; Gaughan et al., 1977).

Prior to recent studies (Choi et al., 2002), studies of permethrin metabolism in humans had been limited to the detection of primary metabolites in blood or urine samples (Angerer and Ritter, 1997; Asakawa et al., 1996; Hardt and Angerer, 2003; Leng et al., 1997). Although providing information as to permethrin persistence and potential routes of metabolism, actual metabolic pathways and specific enzymes involved are not provided by these studies. The more recent studies have demonstrated that while *cis*-permethrin is poorly metabolized by human liver fractions, the *trans* isomer is readily metabolized by both soluble and microsomal esterases (Choi et al., 2002). The resulting phenoxybenzyl alcohol is then readily metabolized to phenoxybenzoic acid by sequential oxidations by alcohol dehydrogenase and aldehyde dehydrogenases (Fig. 3). Although human alcohol and aldehyde dehydrogenases have been studied [e.g., Chen and Yoshida (1991), Stone et al. (1993), Wagner et al. (1983)], prior to these studies, the role of alcohol and aldehyde dehydrogenase enzymes in metabolism of pyrethroids had not been described in either humans or in mammals, although the metabolites detected would have suggested the existence of these metabolic pathways.

Subcellular Preparations. *Trans*-permethrin is metabolized to phenoxybenzyl alcohol and phenoxybenzoic acid in both pooled human liver microsomes and human liver cytosol, while the *cis* isomer does not appear to be metabolized by these preparations. The addition of NADPH to the microsomal preparations was without effect, indicating that the initial step, to phenoxybenzoic alcohol, was a hydrolysis, a finding supported by the observation that cytosol and microsomes produced the same metabolites. As noted below, this hydrolysis can be inhibited by chlorpyrifos oxon and carbaryl. The hypothesis that the metabolism of phenoxybenzyl alcohol to phenoxybenzoic acid was mediated by alcohol dehydrogenase and aldehyde dehydrogenase and that phenoxybenzaldehyde was an intermediate was tested by the use of recombinant human enzymes (Choi et al., 2002).

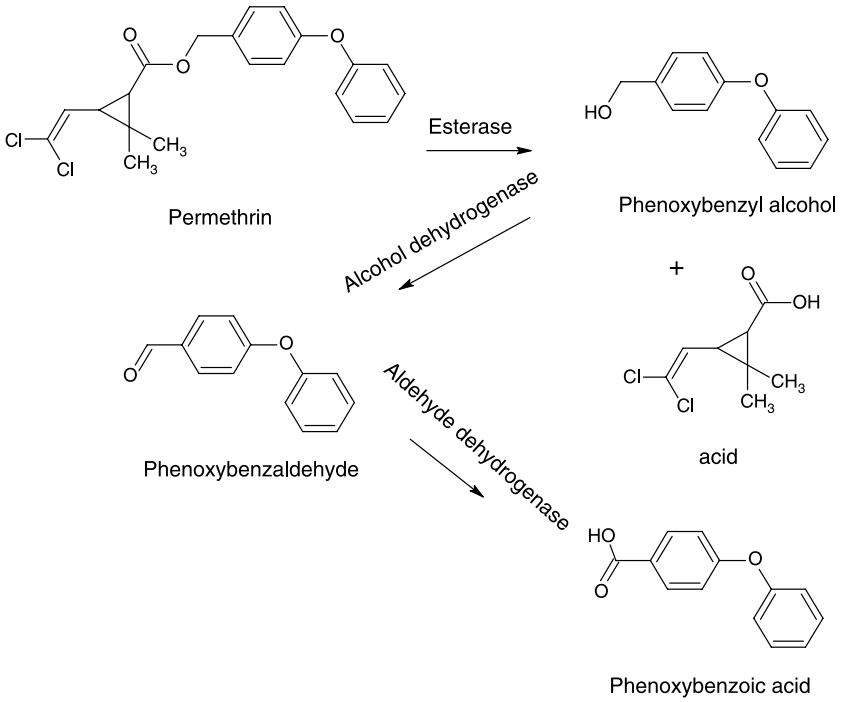

Figure 3. Metabolism of permethrin by human liver microsomes and cytosol.

Xenobiotic-metabolizing enzymes. All four purified recombinant human alcohol deydrogenases tested metabolized phenoxybenzyl alcohol to phenoxybenzaldehyde with K_m values ranging from 4 to 48 μM. These K_m values are around two orders of magnitude smaller than those for ethanol and catalytic efficiencies, indicating that phenoxybenzyl alcohol is a preferred substrate for this enzyme. Only a single isoform of human aldehyde dehydrogenase (ALDH3A1) was available for these studies (Choi et al., 2002). This isoform catalyzed the metabolism of phenoxybenzaldehyde to phenoxybenzoic acid.

Carbaryl and Related Carbamates

Introduction. Four carbamates are among the insecticides shipped to the Gulf War, carbaryl, bendiocarb, propoxur, and the carbamate oxime, methomyl (Institute of Medicine, 2003). Of these, carbaryl is the one most likely to have been used and is the most important.

Carbaryl, 1-naphthol *N*-methylcarbamate, is a broad-spectrum carbamate insecticide with a variety of agricultural and public health applications. Due to its wide use, humans may be exposed either occupationally or through food and other routes (Cranmer, 1986). The mechanism underlying the toxicity of carbamate pesticides is its action as a cholinesterase inhibitor (Fukuto, 1990). The inhibition of cholinesterase by carbamates is reversible and less persistent than that by organophosphates.

Early studies of carbamate metabolism focused on hydrolysis because of the assumption that the ester linkage was susceptible to esterase attack as well as because of the limitations of the analytical techniques then available (Dorough, 1970). However, the importance of oxidative pathways had been shown earlier with the demonstration of NADPH-dependent metabolic activity toward carbamates in rat liver microsomes (Hodgson and Casida, 1960, 1961). Like many other carbamates, carbaryl can be hydrolyzed by esterases and oxidized by cytochrome P450-mediated monooxygenases (CYP) to form both hydrolysis and hydroxylation products, respectively, that are subject to further conjugation, such as sulfate and glucuronic acid conjugation of 1-naphthol and 4-hydroxycarbaryl (Matsumura, 1975). The major hydroxylation products include 5-hydroxycarbaryl, (5-hydroxy 1-naphthyl *N*-methylcarbamate), 4-hydroxycarbaryl, (4-hydroxy 1-naphthyl *N*-methylcarbamate), and carbaryl methylol, (1-naphthyl *N*-(hydroxymethyl)carbamate) (Dorough and Casida, 1964; Dorough et al., 1963; Strother, 1972; Fig. 4).

Although the contributions of hydrolysis and hydroxylation toward total metabolism of carbaryl have yet to be elucidated, it has been suggested that hydroxylation by CYP is the more important route of carbaryl metabolism (Ehrich et al., 1992; Ward et al., 1988). It has been shown (Ehrich et al., 1992) that chickens have a higher clearance rate for carbaryl than rats, although they have lower carboxylesterase and A-esterase activities, suggesting that these esterases did not contribute to the difference between the

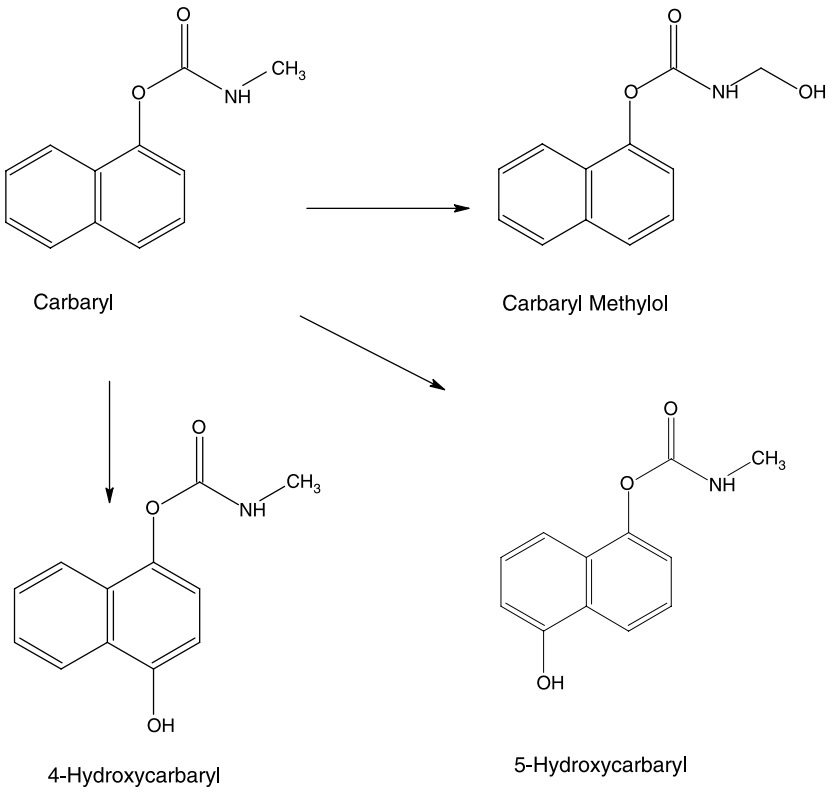

Figure 4. Metabolism of carbaryl by human liver microsomes.

two species. An inhibition study in rats and humans showed that the CYP inhibitor cimetidine reduced the metabolism of carbaryl, again suggesting that CYP plays a major role in carbaryl metabolism (Ward et al., 1988).

Although carbaryl is an anticholinesterase, some hydroxylation products have been shown to be more active than the parent compound. It has been shown (May et al., 1992) that while pretreatment with cimetidine increased the plasma concentration of carbaryl in man, the inhibition of blood cholinesterase activity was reduced, suggesting that cimetidine is blocking the production of active metabolites generated by CYP. 5-Hydroxycarbaryl has been reported to be more toxic than carbaryl (Dorough, 1970; Oonithan and Casida, 1968).

Both carbamate and organophosphorus insecticides are metabolized by CYP (Hodgson and Casida, 1960) and act as anticholinesterases (Fukuto, 1990). It is possible, therefore, that they may interact in metabolic pathways as well as in target sites. One important group of organophosphorus insecticides is the phosphorothionates, such as chlorpyrifos and malathion. Bioactivation of this group by CYP causes suicide inhibition of CYP activity (Neal, 1980), resulting in a reduction of CYP activity. A significant increase in carbaryl toxicity in red-legged partridges following malathion administration has been attributed to inhibition of carbaryl metabolism (Johnston, 1995). Although the hepatic metabolism of carbaryl in humans has been previously investigated in vitro (Chin et al., 1974; Strother, 1972), the contributions of individual CYP isoforms to the metabolic pathways had not been elucidated prior to the studies of Tang et al. (2002). Knowledge of the varying contributions of CYP isoforms to carbaryl metabolism should enable better understanding of differences in metabolism among individuals as well as among subpopulations and will provide important information relative to metabolic interactions of carbaryl with other chemicals.

Subcellular preparations. Four metabolites were detected after incubation of carbaryl with pooled human liver microsomes (Tang et al., 2002), namely, 5-hydroxycarbaryl, 4-hydroxycarbaryl, carbaryl methylol, and 1-naphthol. The first three were generated only in the presence of an NADPH-generating system and are, therefore, the products of CYP-mediated reactions. Only very small amounts of 1-naphthol, an hydrolysis product produced in the presence or absence of the NADPH-generating system, were generated in either the microsomal or the cytosolic fractions of human liver. Based on kinetic constants and clearance rates, methylol carbaryl was produced most efficiently and 5-hydroxymethylcarbaryl least efficiently, by liver microsomes.

Xenobiotic-metabolizing enzymes. Of 16 human CYP isoforms tested (Tang et al., 2002), only CYP2D6*10 and CYP4A11 were without detectable activity toward carbaryl. All other isoforms generated all three oxidative metabolites, although the activities and product ratios varied widely among isoforms. The most active isoforms were CYP1A1, 1A2, 2B6, 2C19, and 3A4. Of these, CYP2B6 had the highest activity and produced primarily (almost exclusively) the methylol derivative.

Polymorphic variants. Polymorphic variants of CYP isoforms have not been extensively investigated with respect to carbaryl metabolism. However, CYP2C9*2 is less active than the wild type 2C9, and CYP2D6*10 showed no activity toward carbaryl although 2D6*1 did, producing primarily 5-hydroxycarbaryl (Tang et al., 2002).

Individual livers. Human liver microsomes (Tang et al., 2002) from five selected individuals showed a twofold difference in the generation of either 5-hydroxycarbaryl or 4-hydroxycarbaryl with the activities not correlated with any single CYP isoform. On the other hand, the same microsomes showed a fivefold difference

in the generation of methylol carbaryl, a difference correlated with the level of CYP2B6 activity.

Other Insecticides

Other than those listed above, few other insecticides appear to have been used in military deployments, at least in recent years. An exception is lindane, listed as having been shipped to the Gulf War (Institute of Medicine, 2003).

REPELLENTS

N,N-Diethyl-*m*-Toluamide (DEET)

Introduction. DEET appears to be the only repellent used to any extent in military deployments. Moreover, every year, approximately one-third of the U.S. population, over 75 million people, use DEET-containing products with DEET concentrations ranging from 10 to 100% in a variety of liquids, lotions, gels, sprays, sticks, and impregnated materials, some 30 million packages of DEET-containing products being sold annually (Veltri et al., 1994).

Given this wide use, DEET must be considered safe when applied properly; although there are accounts of side effects, largely anecdotal, that include toxic encephalopathy, seizure, acute manic psychosis, cardiovascular toxicity, and dermatitis. A few cases of death may have resulted from extensive skin absorption (Schoenig et al., 1996). Studies of absorption and metabolism in humans suggest that a small percentage of topically applied DEET is rapidly absorbed (5–8%) and excreted. As many as six major metabolites were recovered from the urine (Selim et al., 1995).

Subcellular preparations. Studies of the oxidative metabolism of the insect repellent *N*,*N*-diethyl-*m*-toluamide (DEET) have been conducted using pooled human liver microsomes, rat liver microsomes, and mouse liver microsomes. In this first in vitro study of DEET metabolism in humans (Usmani et al., 2002), it was shown that human liver microsomes metabolized DEET primarily to two products, a ring methyl oxidation product, *N*,*N*-diethyl-*m*-hydroxymethylbenzamide and an *N*-deethylated product, *N*-ethyl-*m*-toluamide (Fig. 5).

Xenobiotic-metabolizing enzymes. Although in vitro studies of DEET metabolism using pooled human liver microsomes showed the production of two metabolites, the production of the ring methyl oxidation product, *N*,*N*-diethyl-*m*-hydroxymethylbenzamide was approximately 10-fold higher than that of the *N*-deethylated product, *N*-ethyl-*m*-toluamide. Both the affinities and intrinsic clearance of human liver microsomes (HLM) for ring methyl hydroxylation are greater than those for *N*-deethylation. An interesting observation is that the two products are formed by two different sets of CYP isoforms. Among 15 cDNA-expressed CYP enzymes examined, CYP1A2, 2B6, 2D6*1 (Val_{374}), and 2E1 metabolized DEET to the ring methyl metabolite, while CYP3A4, 3A5, 2A6, and 2C19 produced the *N*-deethylated metabolite. CYP2B6 is the principal CYP involved in the metabolism of DEET to its major ring methyl oxidation product, while CYP2C19 had the greatest activity for the formation of the *N*-deethylated product (Usmani et al., 2002).

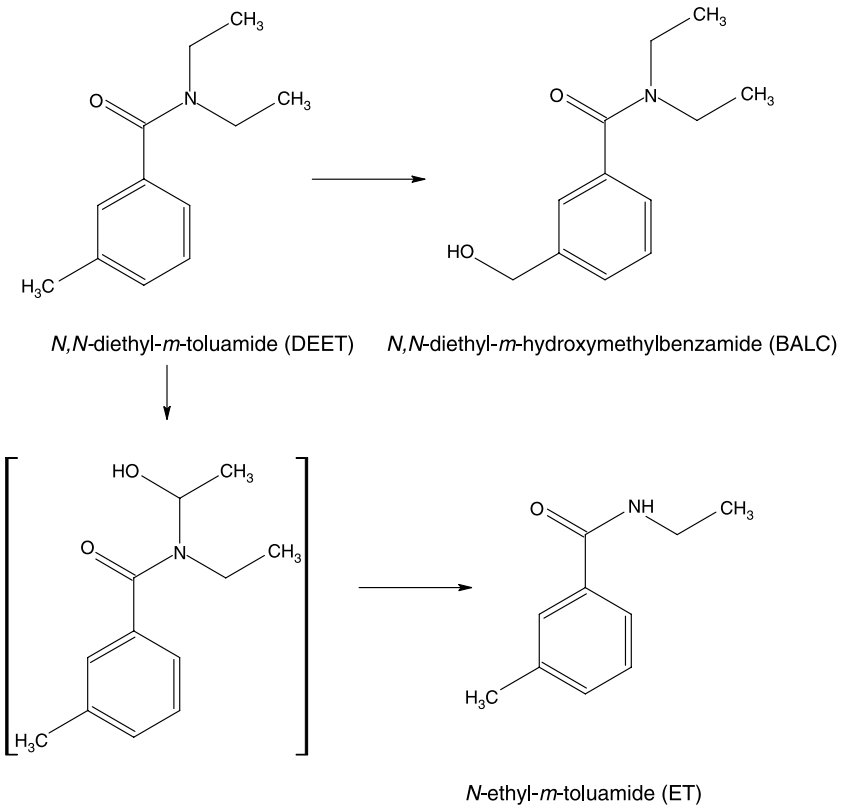

Figure 5. Metabolism of DEET by human liver microsomes.

To summarize, CYPs 1A2, 2B6, 2D6, and 2E1 produced the hydroxymethyl product, and 3A4, 3A5, 2A6, and 2C19 produced the deethylated product with no overlap between the two groups of isoforms, that is to say, there are no isoforms that produce both products. Given the different affinities observed, it is probable that at low substrate concentrations, only the ring methyl hydroxylation is likely to be observed (Usmani et al., 2002).

Individual livers. The use of phenotyped HLM demonstrated that individuals with high levels of CYP2B6, 3A4, 2C19, and 2A6 have the greatest potential to metabolize DEET. Mice treated with DEET demonstrated induced levels of the CYP2B family, increased hydroxylation, and a 2.4-fold increase in the metabolism of chlorpyrifos to chlorpyrifos-oxon, a potent anticholinesterase (Usmani et al., 2002).

SOLVENTS

Although some 53 solvents are listed as being shipped to the Gulf War (Institute of Medicine, 2003), little is known about their human metabolism in vitro. Based on industrial experience, many would be regarded as safe in most circumstances, while

others were probably not used extensively. Included in the list are the following; although arranged by functional group, many fall in several categories:

Acids
Acetic acid
Cresylic acid

Alcohols (including glycols)
2-Butoxyethanol
Butyl alcohol
Cyclohexanol
Diethylene glycol
Dipropylene glycol
Ethanol
2-Ethyl butanol
Ethylene glycol
Glycerol
Hexyl alcohol
Hexylene glycol
Isopentyl alcohol
Isopenpropyl alcohol
Methanol
Polyalkylene glycol
Propyl alcohol

Alkanes and Substituted Alkanes
Chloroform
Dichlorofluoromethane
n-Heptane
Methylene chloride (dichloromethane)
Tetrachloroethylene
1,1,1-Trichloroethylene
1,1,2-Trichloro-1,2,2-trifluoroethane

Aldehydes and Ketones
Acetone
Amyl acetate
Cyclohexanone
Ethyl acetate
Isoamyl acetate
1-Methoxy-2-propanol acetate
Methyl ethyl ketone
Methyl isoamyl ketone
Methyl isobutyl ketone
Methyl propyl ketone

Amines
Diethylene triamine

Aromatics
Benzene
Camphor
Cresol
Morpholine
Phenol
Toluene
Xylene

Ethers
Diethylene glycol monobutyl ether
Ethylene glycol monoethyl ether
Ethylene glycol monomethyl ether
Ethyl ether

Phosphorus esters
Tricresyl phosphate

Mixtures
Naphtha
Stoddard solvent

It is clear, from the extensive compilation of Bruckner and Warren (2001), that essentially all populations in any industrialized society are exposed to solvents and vapors and that the many factors that govern exposure, metabolism, and potential metabolic interactions in general are all relevant to solvent exposure. Most of the chemicals listed (Institute of Medicine, 2003) are used in connection with transportation either as solvents, as listed, or as antifreezes or deicers. Their use in specific military applications, such as in military jet planes and fighting vehicles, should not be significantly different from their use in commercial jets, heavy road transport, or earth-moving equipment.

Although few studies of the in vitro metabolism of any of the above chemicals have been carried out in humans, most have been investigated in surrogate animals, particularly rodents. It is apparent from these studies that metabolic interactions are possible, indeed probable, in humans. For example, many small, lipophilic molecules are substrates for CYP2E1, a cytochrome P450 isoform found in both hepatic and extrahepatic tissues. It has been shown (Guengerich et al., 1991) that human CYP2E1 metabolized a number of halogenated aromatic and aliphatic hydrocarbons, including several listed above, such as trichloethylene, chloroform, and benzene. It is also known that in individual human liver microsomes, increased ability to metabolize chlorofluorocarbons is correlated with high CYP2E1 levels, and that individuals with chemical exposures (e.g., to ethanol) that increase CYP2E1 levels may be at increased risk from chlorofluorocarbon poisoning (Surbrook and Olson, 1992). In the present context, it is

important to note that oxidation by CYP2E1 may produce reactive metabolites capable of causing cytotoxicity and mutagenicity (Raucy et al., 1993).

Alcohols

In addition to occupational exposure, ethanol is frequently ingested in moderate to excessive amounts. Although the psychomotor effects of ethanol are of prime importance due to their effect on performance of essential tasks, it should be noted that ethanol can have other effects that may be of considerable importance. First, ethanol is metabolized to acetaldehyde, a reactive product, by CYP2E1, but also with the concomitant release of reactive oxygen radicals (Lieber, 1997). Ethanol is also a potent inducer of CYP2E1, possibly increasing the production of reactive intermediates from other substrates (Nakajima et al., 1985). Ethanol is also oxidized by alcohol dehydrogenase (Nakajima et al., 1985), followed by aldehyde dehydrogenase, producing first acetaldehyde and subsequently acetate. As these two enzymes are now known to be important in the metabolism of permethrin in humans (Choi et al., 2002), alcohol–permethrin interactions are possible. The potential utility of physiologically based pharmacokinetic models for the investigation of ethanol interactions is suggested by a recently published pharmacokinetic model for ethanol in humans that incorporates information on isoforms and polymorphisms in human alcohol dehydrogenase (Sultatos et al., 2004).

Methanol is metabolized to formaldehyde by alcohol dehydrogenase in primates or by catalase in rodents. The formaldehyde is further metabolized in both rodents and primates to formate by aldehyde dehydrogenase [see Bruckner and Warren (2001) for details]. Both formaldehyde and formate are toxic. The formate produced is metabolized to carbon dioxide more rapidly in rodents than in primates. Thus, methanol, whether used as a solvent or as a gasoline additive, may be involved in metabolic interactions in humans.

Less is known about the metabolism in experimental animals of the other alcohols listed above, and virtually nothing is known about their metabolism in humans. Given the broad specificity of both CYP isoforms (including CYP2E1) and alcohol and aldehyde dehydrogenases, it seems likely that these enzymes are involved in both surrogate animals and humans and that metabolic interactions with other xenobiotics are possible.

Glycols

These chemicals are widely used as antifreeze agents and deicers as well as for other industrial uses. The widely used ethylene glycol is acutely toxic to humans, toxicity that not infrequently results in death. The involvement of alcohol dehydrogenase may also be a cause of concern for reasons stated above with reference to alcohols. Glycol ethers represent a similar, but somewhat more complex, situation.

Chlorinated Hydrocarbons

Many of these are important solvents, and their metabolism has been studied in experimental animals. Although the pathways differ significantly from one chemical to

another and, in some cases, from one species to another, they usually involve cytochrome P450 isoforms as well as glutathione S-transferases. In both cases, reactive intermediates are common, and the potential for metabolic interactions is high. Although much less is known about their metabolism in humans, in view of the often considerable exposures, they must be a cause for concern.

Aromatic Hydrocarbons

These chemicals, particularly benzene, are important toxicants and may also be involved in metabolic interactions. There is a considerable body of information on the hematopoietic toxicity of benzene to humans (Bruckner and Warren, 2001). Benzene toxicity is more likely to be due to the toxicity of its metabolites than to benzene itself. Some metabolites identified include phenol, catechol, hydroquinine, 1,2,4-trihydroxybenzene, 1-phenylmercapturic acid, and *trans*-muconic acid. Benzene metabolism involves cytochrome P450 and glutathione S-transferase as well as glucuronide and sulfate conjugation pathways (Bruckner and Warren, 2001). Further studies in humans are needed before the extent of such interactions and the risk associated with them becomes clear.

Phosphorus Esters

While most of the concern about phosphorus esters centers around organophosphorus insecticides and chemical warfare agents, there are some lubricants and fuel additives of concern, including tricresyl (tritolyl) phosphate. This chemical has been studied intensively as a cause of organophosphate-induced delayed neuropathy (OPIDN), a human toxic endpoint that, although known earlier, first came to prominence during prohibition in the United States as ginger jake paralysis, caused by the addition of tolyl ester contaminated alcoholic extracts of Jamaican ginger (Echobicon, 2001). Tricresyl phosphate is an important component of jet fuel lubricants that can enter the cabin area through the bleed air when engine seals fail (Winder and Balouet, 2002). Winder and Balouet (2002) stated that the risk of tricresyl phosphate exposure is frequently seriously underestimated.

JET FUEL COMPONENTS

Military personnel may be frequently, even daily, exposed to jet fuels (Pleil et al., 2000), the components of which may be absorbed either through dermal absorption or inhalation. In animal studies, JP-8 jet fuel has been shown to have effects on several organ systems including the liver and skin, as well as the immune, nervous, respiratory, and reproductive systems (Allen et al., 2000, 2001; Cooper and Mattie, 1996; Dudley et al., 2001; Grant et al., 2000; Harris et al., 2000; Kabbar et al., 2001; Kanikkannan et al., 2002; Monteiro-Riviere et al., 2001; Ramos et al., 2002; Rhyne et al., 2002; Ritchie et al., 2001; Robledo et al., 2000; Ullrich and Lyons, 2000). Although not as extensively studied in humans, it is known to cause irritant dermatitis and to have some neurological effects (Smith et al., 1997; Zeiger and Smith, 1998). The toxicology of the jet fuel JP-8 has recently been summarized (National Research Council, 2003).

However, the extent to which jet fuel components are metabolized in humans is not well known, and, therefore, their potential to interact at the metabolic level with other deployment-related chemicals is unknown.

Although not discussed in this review, it should be noted that considerations of the toxicity and metabolism of the components of automotive and other diesel fuels should not be significantly different from those for jet fuel components. Most of the constituents of these fuels are common to all.

Because jet fuels are complex mixtures, it is not possible to use jet fuel itself in studies of the metabolism of jet fuel components. However, jet fuels can be used in preliminary studies in such areas as induction of XMEs or inhibition of the metabolism of either exogenous or endogenous chemicals. Initial studies of metabolism and interaction of components can be carried out using known components representative of different chemical classes. For example, dodecane and naphthalene are JP-8 components that have been used in studies of dermal absorption (Baynes et al., 2000; McDougal et al., 2000; Riviere et al., 1999).

Naphthalene metabolism has been studied in mammalian systems and is known to yield a number of metabolites, including oxidation products and their conjugates (Fig. 6). The initial step appears to be CYP dependent and to yield the arene epoxide, 1,2-naphthalene oxide. 1,2-Naphthalene oxide can spontaneously rearrange to form 1- and 2-naphthol, compounds that can be further hydroxylated to di-, tri-, and tetrahydroxylated products (Horning et al., 1980). Some of these metabolites may be conjugated

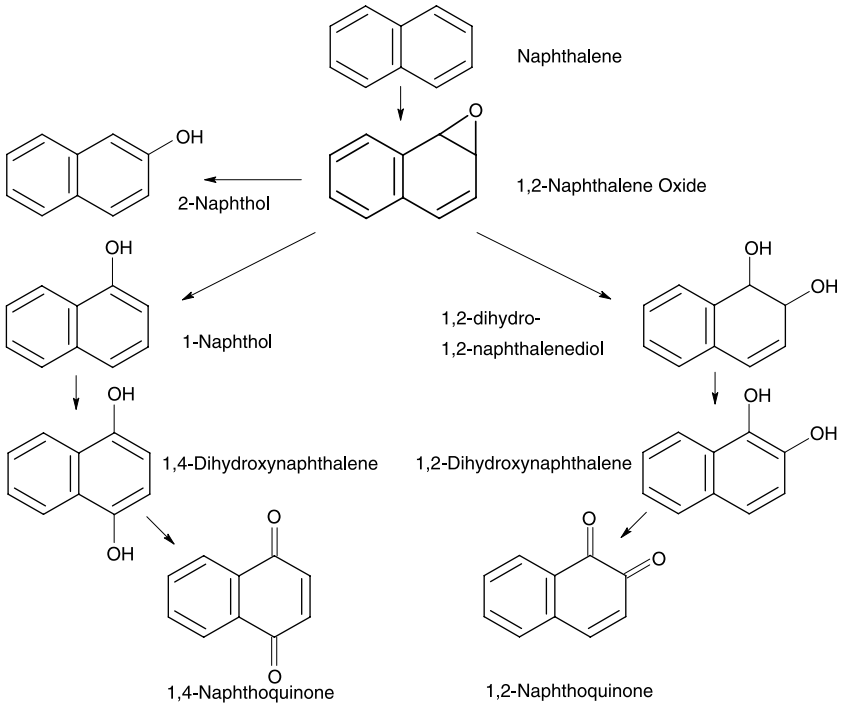

Figure 6. Metabolism of naphthalene by human liver and lung microsomes.

with glutathione, glucuronic acid, or sulfate, with the glutathione conjugates eventually giving rise to mercapturic acids (ATSDR, 1995; Pakenham et al., 2002; USEPA, 1998). An alternative pathway involves the action of epoxide hydrolase on the arene oxide to form 1,2-dihydro-dihydroxynaphthalene, and then, by a series of reactions, 1,2- and 1,4-naphthoquinone are formed (USEPA, 1998).

It is apparent from the small number of human studies that these pathways operate, in whole or in part, in humans. Human lung microsomes exposed in vitro to naphthalene produced dihydro-1,2-naphthalenediol and several of its glutathione derivatives (Buckpitt and Bahnson, 1986; Buckpitt and Richieri, 1984; Buonarati et al., 1990; USEPA, 1998). In human liver microsomes (Tingle et al., 1993), the primary stable metabolite was 1,2-dihydro-1,2-naphthalenediol, while 1-naphthol was a minor product. Earlier urine analyses (Mackell et al., 1951) indicated that both 1,2- and 1,4-naphthoquinone are formed, in vivo, in humans exposed to naphthalene. Few studies appear to have been carried out specifically on CYP, on CYP isoforms, or on their polymorphisms in either humans or experimental animals, although naphthalene was one of the substrates used in a study of atypical kinetics (non-Michaelis-Menton) shown by several human CYP isoforms (Korzekwa et al., 1998). In this study, naphthalene was shown to be metabolized to 1-naphthol by human CYPs 2B6, 2C8, 2C9 3A4, and 3A5 and that the atypical kinetics could be explained by simultaneous binding of more than one substrate to the active site. Other products, known to be produced in humans, were not investigated. However, this mechanism would clearly increase the possibility of interactions at the metabolic level between naphthalene and other CYP substrates.

As it is clear, from the above studies, that humans generate several highly reactive intermediates from naphthalene (the arene oxide as well as naphthoquinones), further investigation of the metabolism of naphthalene (and possibly other polyaromatic jet fuel constituents) in humans and metabolic interactions of naphthalene with other exogenous chemicals and endogenous metabolites is of great importance in the toxicology of deployment-related chemicals. Ongoing studies (Cho et al., 2004) show that several human CYP isoforms produce 1-naphthol, 2-naphthol, and *trans*-dihydroxydihydrodiol naphthalene, the ratio of products varying from one isoform to another.

To date, there appear to have been no studies of dodecane metabolism in humans, although it has been suggested that the dodecane moiety of fatty acids that are substrates for omega-1 hydroxylation by CYP2E1 is involved in binding to the substrate binding site (Adas et al., 1998). Early studies indicated that dodecane, a known cocarcinogen, is an inducer of benzo(a)pyrene metabolism in the isolated, perfused rabbit lung (Warshawsky et al., 1977), raising the possibility of its involvement in metabolic interactions with other chemicals.

Recent studies of nonane metabolism in humans (Edwards et al., 2004a) have indicated that nonane is metabolized to 2-nonanol and 2-nonanone by human liver microsomes, and this activity is due to cytochrome P450, with CYP1A2, -2B6, and -2E1 being the principal isoforms involved. Ongoing studies (Edwards et al., 2004a,b) indicate that 2-nonanol can also be metabolized to 2-nonanone by human alcohol dehydrogenase and that 2-nonanone can be metabolized to the corresponding acid by human aldehyde dehydrogenase.

The risks associated with jet engine lubricants have been mentioned above with regard to tricresyl phosphate, but there may be other chemicals of concern, including *N*-phenyl-1-naphthylamine, a skin sensitizer (Winder and Balouet, 2002).

CHEMICAL WARFARE AGENTS

Sulfur Mustard and Metabolites

Metabolism of sulfur mustard has been investigated in rats, humans, mice, and guinea pigs (Fig. 7). In rats exposed intraperitoneally, approximately 60% of the dose was excreted in the urine by 24 h. Many metabolites were present, nine of which were identified and characterized by mass spectrometry (Black et al., 1992a,b). The majority of metabolites were formed by conjugation with glutathione. Three major pathways are suggested. In both rats and humans, thiodiglycol sulfoxide was excreted in much higher concentrations than thiodiglycol (Black and Read, 1995). In mice and guinea pigs, sulfur mustard and thiodiglycol sulfoxide were not detected in several tissues, even after massive doses. Thiodiglycol was found only in urine samples (Tripathi et al., 1995). Studies in progress have indicated no metabolism of thiodiglycol, the hydrolysis product of sulfur mustard, by any of the human CYPs tested.

Evaluation of the metabolism of thiodiglycol by alcohol dehydrogenase from horse liver using a novel nuclear magnetic resonance (NMR) technique has indicated the formation of two previously unreported metabolites in the form of the monoaldehyde and

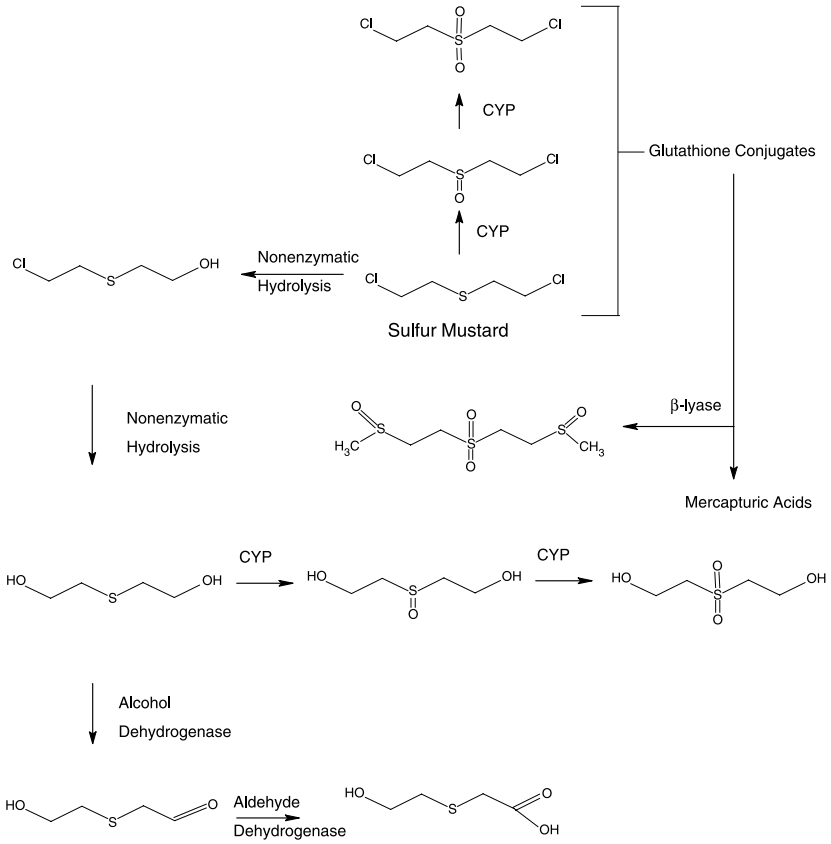

Figure 7. Some metabolic reactions of sulfur mustard.

the monoacid of thiodiglycol (Brimfield et al., 2002). There is a strong likelihood that human isoforms will perform similar oxidative transformations although the reaction has only been followed by nicotine adenine dinucleotide (NAD) reduction up to this point, not by product formation (Dudley et al., 2000).

Induction of cytochrome P450 in mouse liver using 5%, 10%, and 20% of the respective LD50 concentrations of thiodiglycol and sulfur mustard have been carried out (Brimfield, personal communication). The results indicated a dose-related increase in liver levels of P450 after thiodiglycol administration and reduced levels of P450 in the livers of mice treated with sulfur mustard. The apparent reduction of CYP concentration following treatment with sulfur mustard is of interest from the point of view of its potential effect on the metabolism of other compounds involved in coexposure. Sulfur mustard has been reported to alter the activity of several CYP isoforms (Pons et al., 2001). Additionally, sulfur mustard and thiodiglycol have been implicated in the inhibition of protein phosphatase activity (Brimfield, 1995), which is necessary for the induction of CYP2Bs and CYP3Bs in the presence of phenobarbital (Joannard et al., 2000; Kawamura et al., 1999).

Sarin

Little appears to be known of the overall metabolism of sarin and related phosphorus-containing nerve agents such as soman, tabun, and VX (Szinicz and Baskin, 1999), in either experimental animals or humans. However, it appears that, in the rat, the principal route of metabolism is hydrolysis, and that the principal metabolite is an alkylmethylphosphonic acid, isopropylmethylphosphonic acid in the case of sarin (Shih et al., 1994). Metabolism in humans appears to resemble that in rats, and isopropylmethylphosphonic acid has been found in human urine following sarin intoxication (Minami et al., 1997, 1998).

One of the esterases hydrolyzing sarin is paraoxonase (PON1), an enzyme known to be polymorphic in humans (Furlong et al., 1993). The resulting R allozyme (Arg-192) hydrolyzes paraxon rapidly but has low activity toward nerve agents such as sarin and soman (Davies et al., 1996). The reverse is true of the Q allozyme that has high activity toward soman and low activity toward paraoxon. Thus, individuals with the Q allozyme would be expected to detoxify sarin more rapidly than those with the R allozyme, and the PON1 status may be important in human poisoning, although it has been suggested that the catalytic efficiency of PON1 toward sarin may be inadequate to provide significant in vivo protection (Furlong, 2000; Richter and Furlong, 1999). A recent study (Haley et al., 1999) suggested that the R genotype may be a risk factor for illness in Gulf War veterans. Unfortunately, the number of subjects involved was too small to provide any certainty that this was the case.

PROPHYLACTIC DRUGS

Pyridostigmine Bromide

Although the pharmacokinetics of pyridostigmine bromide have been studied (Marino et al., 1998), there are, as yet, no studies that indicate metabolism of this drug in

humans. A study by Leo (1997) provided evidence that pyridostigmine bromide is not metabolized in humans, and specifically, that it is not a substrate for human CYPs 1A1, 2C9, 2E1, 2D6, and 3A4. More recently it has been shown (Usmani et al., 2004) that pyridostigmine bromide is not metabolized by pooled human liver microsomes, rat liver microsomes, pooled human liver cytosol, rat liver cytosol, pooled human liver S9, or rat serum.

METABOLISM-BASED INTERACTIONS

Introduction

Studies of chemical interactions are relatively recent in origin and often provide conflicting data. For example, an acute oral study involving pyridostigmine bromide, permethrin, and DEET in the rat indicated that animals fed combinations of these chemicals at LD16 dose levels suffered greater than additive mortality (McCain et al., 1997). Another study indicating that significant synergistic interactions are possible is that of Chaney et al. (1997), where the lethal effects of pyridostigmine bromide in mice were strongly potentiated by application of other adrenergic drugs (isoproteronol, salbutamol, phentolamine, etc.) or caffeine. (An interesting question raised by this study is whether the interaction is via metabolic perturbations or interactions with the anticholinesterase properties of PB.) Abou-Donia et al. (1996) demonstrated that coadministration of binary or tertiary combinations of pyridostigmine bromide, DEET, and chlorpyrifos to hens resulted in significant increases in neurotoxicity. These increases in neurotoxicity were associated with increases in inhibition of brain acetylcholinesterase and neurotoxicity target esterases.

However, other studies contradict the idea of synergistic interactions. For example, in a study examining chemical uptake into the central nervous system (CNS), application of pyridostigmine bromide was found to reduce tissue levels of permethrin by 30% (Buchholz et al., 1997). Similarly, a study of pesticide absorption through mouse, rat, and pig skin suggests that application of DEET inhibits insecticide absorption for permethrin and carbaryl (Baynes et al., 1997).

Inhibition and Activation of Xenobiotic-Metabolizing Enzymes

Inhibition and activation of xenobiotic substrates

Inhibition and activation of DEET metabolism. The effects on DEET metabolism of chlorpyrifos, permethrin, and pyridostigmine bromide alone or in combination have been investigated in human microsomes (Usmani et al., 2002). The greatest effect shown was the inhibition of the metabolism of DEET to the ring methylol derivative by chlorpyrifos either alone or in combination with any other compound. None of the three compounds tested had any significant inhibitory effect on the production of the *N*-deethylated metabolite. These inhibitory effects were also seen to a greater or lesser extent in microsomes from rat and mouse liver and in microsomes from mice pretreated with DEET, phenobarbital, or 3-methylcholanthrene. Human liver microsomes differed from those from both rats and mice in that pyridostigmine and permethrin, alone or in combination, showed an activation effect for the production of both of the major products of DEET metabolism.

Inhibition of carbaryl metabolism. More recent studies (Tang et al., 2002) indicate that chlorpyrifos inhibited the metabolism of carbaryl by human liver microsomes, preferentially inhibiting the formation of the methylol derivative. This is correlated with the findings first, that the production of this metabolite is primarily the result of CYP2B6 activity and that this is the same isoform that produces the oxon from chlorpyrifos, and second, that CYP isoforms are inhibited during oxon production. This interpretation was confirmed by the results of experiments carried out directly on CYP 2B6.

Inhibition of permethrin metabolism. Because the initial step in the metabolism of *trans*-permethrin in human liver is hydrolysis, it appeared important to test the ability of other chemicals to inhibit this reaction (Choi et al., 2004). Two inhibitors, chlorpyrifos oxon and carbaryl, were examined in some detail.

Trans-permethrin hydrolysis in human liver fractions was inhibited more effectively by chlorpyrifos oxon than by carbaryl. Under similar assay conditions, the IC50s of chlorpyrifos oxon in human liver cytosolic and microsomal fractions were 35 nM and 60 nM, respectively. Above 60 nM (cytosol) or 150 nM (microsomes) levels, *trans*-permethrin hydrolysis was completely inhibited by chlorpyrifos oxon.

When the cytosolic fraction was pre-incubated for 5 min with chlorpyrifos oxon, the V_{max} value was significantly reduced, while K_m values stayed approximately the same. Five minutes preincubation with chlorpyrifos oxon in the microsomal fraction again resulted in a significant decrease in V_{max} value with only an insignificant decrease in K_m. Based on these observations, noncompetitive or irreversible inhibition was assumed for chlorpyrifos oxon inhibition of permethrin hydrolysis in human liver fractions. The inhibition constants (K_i) for chlorpyrifos oxon, an indicator of inhibitor affinity for the target enzyme, were 21 nM for the cytosolic fraction and 95 nM in the microsomal fraction as calculated from V_{max} and K_m values. These values are approximately 100 times lower than those for carbaryl, indicating a higher inhibitory potential of chlorpyrifos oxon.

In range finding assays, carbaryl showed IC50 values of 10 μM in both microsomal and cytosolic fractions. The most noticeable difference from chlorpyrifos oxon was that *trans*-permethrin hydrolysis in either the microsomal and the cytosolic fractions was not completely inhibited by a wide range of carbaryl concentrations. This observation led to an assumption that the esterases involved in *trans*-permethrin hydrolysis in both the microsomal and the cytosolic fractions are composed of at least two different entities, which have differential susceptibilities to carbaryl and chlorpyrifos oxon inhibition.

K_i values for carbaryl were 2.49 μM in the cytosolic fraction and 11.08 μM in the microsomal fraction. In contrast to chlorpyrifos oxon, in the assay to determine the inhibition type, carbaryl appeared to act as a noncompetitive inhibitor.

These studies demonstrate that there are potentially important interactions between permethrin and chlorpyrifos in humans. Chlorpyrifos, which has been used in military deployments in conjunction with permethrin, is a potent inhibitor of *trans*-permethrin hydrolysis after metabolic activation to chlorpyrifos oxon. This observation implies that coexposure to chlorpyrifos might potentiate the toxicity of permethrin by deactivating the metabolic detoxification pathway for permethrin. In a related study, coexposure of a small number of human volunteers to both cyfluthrin and methyl parathion appeared to have significantly increased the half-life of cyfluthrin (Leng et al., 1999). Other deployment-related compounds, an insect repellent (*N*,*N*-diethyl-*m*-toluamide, DEET) and a nerve gas

prophylactic (pyridostigmine bromide) did not cause the inhibition of *trans*-permethrin hydrolysis, regardless of the presence of an NADPH regeneration system.

Chlorpyrifos oxon completely inhibited *trans*-permethrin hydrolysis in both cytosolic and microsomal human liver fractions with very low K_i values, indicating that B-esterases are responsible for *trans*-permethrin hydrolysis in human liver fractions. Compared to chlorpyrifos oxon, the parent compound, chlorpyrifos and the other major chlorpyrifos metabolite (3,5,6-trichloro-2-pyridinol) showed minimal levels of inhibition in either fraction. The observation that pre-incubation with NADPH in the microsomal fraction substantially increased chlorpyrifos inhibition capability confirmed that chlorpyrifos oxon is the chemical species responsible for the inhibition of *trans*-permethrin hydrolysis.

The mechanism of chlorpyrifos oxon inhibition of esterases is *trans*-esterification, in which a covalent bond is formed between the oxon and the alcohol functional group of a serine residue in the active site of the esterase. With a normal substrate, a transient bond is formed in place of the covalent bond and readily cleaved by deacylation (Chambers et al., 1990). The observed inhibition kinetics (reduced V_{max} and constant K_m) and the irreversible nature of inhibition strongly implies that the inhibition of the human liver esterases hydrolyzing permethrin is mediated by the same mechanism described above.

Carbaryl shows a different pattern of inhibition from chlorpyrifos oxon, typical noncompetitive inhibition. This result is in accord with the fact that carbamate compounds are reversible and less-persistent inhibitors compared to organophosphorus compounds, and that carbamate compounds can be hydrolyzed by esterases. This also explains why K_i values for carbaryl are two orders of magnitude higher than those for chlorpyrifos oxon.

Another important observation is that, in contrast to chlorpyrifos oxon, carbaryl cannot completely inhibit *trans*-permethrin hydrolysis, even at high concentrations. Incomplete inhibition at high concentrations of carbaryl suggests that there are multiple hydrolytic enzymes involved in *trans*-permethrin hydrolysis, a finding that was not revealed by chlorpyrifos oxon inhibition. It is deduced that in *trans*-permethrin hydrolysis in human liver fractions, at least two species (or groups) of B-esterases are involved, both sensitive to chlorpyrifos oxon inhibition but one with higher sensitivity to carbaryl inhibition and the other with lower or no sensitivity to carbaryl.

In conclusion, we reported that in human liver fractions, hydrolysis, the key step in *trans*-permethrin detoxification, is strongly inhibited by chlorpyrifos oxon. The differential inhibition pattern of chlorpyrifos oxon and carbaryl indicates that multiple B-esterases are involved in the hydrolysis of *trans*-permethrin in human liver fractions.

Inhibition of endogenous substrates

Inhibition of testosterone metabolism. Many pesticides are known to have significant endocrine disrupting effects. There are several avenues by which endocrine disruption may occur, including interference with synthesis, secretion, transport, binding, or elimination of natural hormones that are responsible for homeostasis and reproductive development. Although some pesticides are known to interact directly with the hormone receptors, the methods by which many cause endocrine disruption is still poorly understood. It is suspected that many pesticides with endocrine disrupting potential may do this by interfering with the normal hormone synthesis and degradation. In this regard, we have demonstrated in mice that subchronic administration

of some pesticides and polychlorinated biphenyl compounds can significantly increase metabolism of testosterone and estradiol (Gillette et al., 2002). These changes in hormone metabolism as observed in mice were mediated primarily by induction of CYP isoforms by the pesticides.

Cytochrome P450 monoxygenases are not only major catalysts involved in the metabolism of xenobiotics but also in the oxidative metabolism of endogenous substrates such as testosterone. Major testosterone metabolites formed by human liver microsomes include 6β-hydroxytestosterone, 2β-hydroxytestosterone, and 15β-hydroxytestosterone (Fig. 8). A screen of 16 cDNA expressed human CYPs demonstrated (Usmani et al., 2003) that 94% of all testosterone metabolites are produced by members of the CYP3A subfamily, with 6β-testosterone accounting for 84% of all testosterone metabolites. While similar K_m values were observed with human liver microsomes, regardless of which metabolite is measured, V_{max} and intrinsic clearance are both much higher for 6β-testosterone than for any other metabolite.

A recent study of effects on endocrine metabolism (Usmani et al., 2003) indicates effects, often dramatic, of deployment-related chemicals on the oxidative metabolism of testosterone. Preincubation of human liver microsomes with a variety of ligands, including the deployment-related test chemicals used throughout this project, resulted in varying levels of inhibition or activation of testosterone metabolism. The greatest inhibition of testosterone metabolism in human liver microsomes was seen following

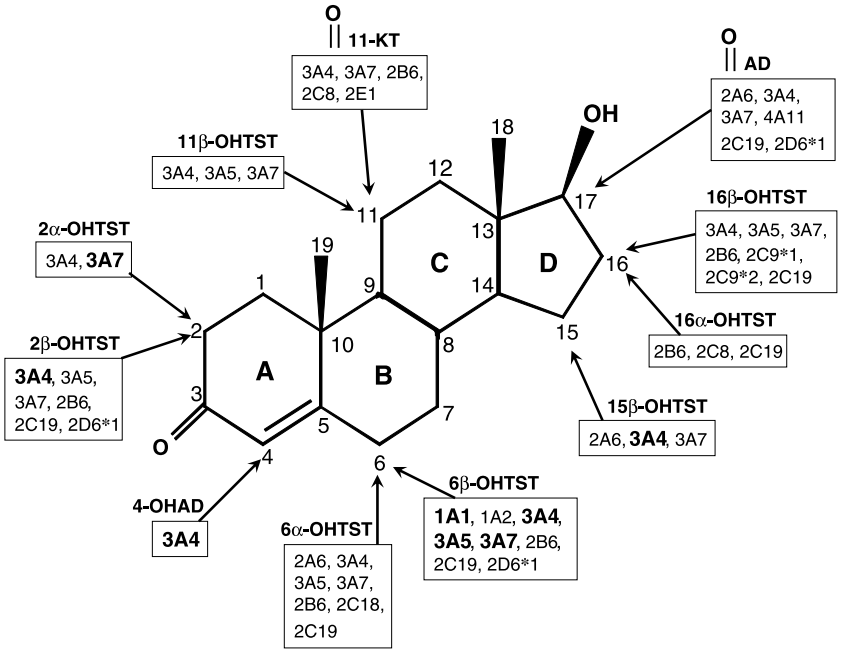

Figure 8. Metabolism of testosterone by human CYP isoforms. Boldface indicates isoforms with either high activity or distinct regioselectivity. The following abbreviations are used: 6α- or 6β-OHTST for 6α- or 6β-hydroxytestosterone; 15β-OHTST for 15β-testosterone; 16α- or 16β-OHTST for 16α- or 16β-hydroxytestosterone; 11-KT for 11-ketotestosterone; 11β-OHST for 11β-hydroxytestosterone; 2α- or 2β-OHTST for 2α- or 2β-hydroxytestosterone; AD for androstenedione; 4-OHAD for 4-hydroxyandrostenedione.

preincubation with organophosphorus compounds, including chlorpyrifos, phorate, and fonofos, with up to 80% inhibition of the formation of several metabolites, including 6β-testosterone. Preincubation of CYP3A4 with chlorpyrifos, but not chlorpyrifos oxon, resulted in 98% inhibition of testosterone metabolism. Kinetic analysis indicated that chlorpyrifos is one of the most potent inhibitors of testosterone metabolism to be discovered to date, and that phorate and fonofos were also potent inhibitors. In all cases, the inhibition is noncompetitive and irreversible. Conversely, preincubation of CYP3A4 with pyridostigime bromide increased the metabolism of testosterone to the 6β- and 2β-derivatives. Preincubation of aromatase (CYP19) with the test chemicals had no effect on the production of the endogenous estrogen, 17β-estradiol. The significance of inhibition of testosterone metabolism in vivo in humans has not yet been studied.

Inhibition of estrodiol metabolism. To date, similar studies have not been carried out on estradiol metabolism. We are currently carrying out such studies using human preparations and the methods developed during studies involving perturbations of estradiol metabolism by polychlorinated biphenyls (PCBs) in rodents (Gillette et al., 2002)

Induction of Xenobiotic-Metabolizing Enzymes

Although many aspects of pesticide and drug metabolism can be easily studied using human liver microsomal and cytosolic preparations, it is nearly impossible to study the inducing effects of pesticide or drug exposure using these systems. Induction of XMEs requires a complex mechanism that cannot be duplicated in vitro. As a consequence, most induction studies in the past have been conducted in vivo using experimental animals, generally rodents. While such studies are useful in pointing to the potential of induction to bring about metabolic interactions, definitive findings from human systems are necessary to extrapolate the results to humans with increased confidence. Human hepatocytes represent one possible approach to this dilemma. Their cellular systems are intact, and, at least for some time, they are responsive to XME inducers. Many difficulties remain, however; they can be used only in primary culture, and due to the innate variability of humans, the results are frequently highly variable.

Induction studies in human hepatocytes. Many pesticides and drugs are known to induce the metabolism of other coadministered drugs as well as to induce their own metabolism. The potential of drugs to induce their own metabolism as well as the metabolism of other drugs has prompted many pharmaceutical companies to conduct elaborate screening protocols to verify the lack of potential harmful interactions between new drug candidates prior to releasing drugs to the marketplace. Because the liver is the primary organ of drug metabolism, use of primary cultures of human hepatocytes is one of the best methods for the study of potential drug or pesticide interactions. Human hepatocyte cultures have been demonstrated to retain many aspects of liver function, including CYP-mediated oxidation of drugs and CYP induction (Donato et al., 2000; Li et al., 1997; Robertson et al., 2000).

The branched DNA (bDNA) assay is a new technique that allows for quantitative determinations of messenger RNA (mRNA) levels from hepatocyte tissues. This technique allows for the evaluation of a variety of chosen genes simultaneously at the level of mRNA. Recent experience in our laboratories with the bDNA assay, through collaboration with Dr. N. Cherrington (University of Arizona), has demonstrated the

utility of this assay to quantitate levels of induction following hepatocyte treatment with several inducers, including some pesticides.

The bDNA assay resembles the well-established enzyme-linked immunosorbent assay (ELISA) in principle but uses multioligonucleotides not only to capture the mRNA of interest, but also to link it to an enzyme that produces a chemiluminescent signal on addition of substrate. This technology is thoroughly explained by Hartley and Klaassen (2000). The primary value of the bDNA assay lies in its ability to assess the differential expression of a chosen set of genes in response to a chemical stimulus. For a targeted gene sequence, such as a series of metabolizing enzymes, one total RNA sample may be split among several different probe sets for quantitative analysis. Gene expression for many genes can, therefore, be monitored simultaneously in parallel wells. Results are reproducible and reflect other assays routinely used to monitor gene expression, including Northern blot analysis, in situ hybridization, quantitative PCR, etc.

Preliminary assays (Rose and Cherrington, 2004) conducted with human hepatocytes in combination with the bDNA assay suggest that several CYP isoforms are induced by permethrin, chlorpyrifos, and DEET. Chlorpyrifos was surprisingly efficient in its induction of CYP1A1, 1A2, and 2B6. Other isoforms induced by chlorpyrifos include 2A6 and possibly 3A4. Our previous determinations of metabolic activity by CYPs had demonstrated that CYP2B6 was involved in the activation of chlorpyrifos to chlorpyrifos oxon. Data with mice also suggested that CYP2B10, the mouse phenobarbital inducible isoform analogous to CYP2B6, was also inducible by chlorpyrifos. It is of interest that permethrin also strongly induced CYP2B6 and CYP2A6. Neither of these enzymes had been implicated in permethrin metabolism using microsomes and the purified CYP isoforms.

Recently acquired data examining CYP3A4 induction using western blot analysis has demonstrated that the best induction of CYP3A4 was with rifampicin (an established CYP3A4 inducer) and DEET. These data were also corroborated with testosterone metabolism data using hepatocyte S9 preparations that were exposed to rifampicin, permethrin, chlorpyrifos, and DEET. A western blot of CYP1A1 protein did not provide confirming evidence for protein induction by chlorpyrifos as might have been expected based upon the bDNA assays performed. As these results are preliminary, it is not known if the absence of correlation is the result of protein destabilization (a possibility, because chlorpyrifos acts as a suicide inhibitor) or whether insufficient time between mRNA induction and protein synthesis might explain the result. Permethrin also strongly induced CYP2B6 along with CYP2A6.

DISCUSSION

Methodology

The development of new high-performance liquid chromatography (HPLC) methods and/or modification and validation of available methods for analysis of deployment-related chemicals and their metabolites and appropriate endogenous chemicals has greatly facilitated research in this area. Methods include those for chlorpyrifos and its metabolites (Dai et al., 2001; Tang et al., 2001), DEET and its metabolites (Usmani et al., 2002), permethrin and its metabolites (Choi et al., 2002), and pyridostigmine bromide and its potential metabolites (Leo, 1997; Usmani et al., 2004).

The analysis of sulfur mustard and its degradation products has always presented an analytical challenge. Their water solubility and their lack of suitable chromophores in the UV/visible region make them difficult to detect by commonly used techniques at concentrations useful for evaluating enzymatic metabolism. Brimfield et al. (personal communication) are currently having success with a fluorimetric method based on derivatization with 2-(4-carboxyphenyl)-6-*N*,*N*-diethylaminobenzofuran (Assaf et al., 2000) using a carbodiimide-catalyzed esterification that reacts at the alcoholic hydroxyl groups common to all the oxidative metabolites. Unfortunately, the fluorescent reagent is not commercially available and must be synthesized. Derivative structures are confirmed by HPLC/mass spectroscopy. Separation of the derivatized compounds is readily accomplished using a 150×4 mm C18, reverse-phase HPLC column and gradient elution. Detection is by flourescence with excitation at 387 nm and emission at 537 nm. Sensitivity is in the picogram/microliter range, a level that makes metabolite detection feasible. Thiodiglycol, thiodiglycol sulfoxide, thiodiglycol sulfone, and 2-hydroxyethyl thioacetic acid are easily derivatized with this system. Thiodipropanol is a commercially available, easily derivatized internal standard.

An improved HPLC method for the separation of testosterone and its metabolites has been developed (Usmani et al., 2003) based on the method of Purdon and Lehman-McKeeman (1997) and used in a study of testosterone metabolism and its inhibition by deployment-related chemicals (Usmani et al., 2003).

Significance of Xenobiotic-Metabolizing Enzyme Distribution

Phenotyping of liver microsomes from individual livers shows a wide variation in the expression of different CYP isoforms and that this variation has dramatic effects on the metabolism of the test chemicals. This is clearly evident in our studies of chlorpyrifos metabolism (Dai et al., 2001; Tang et al., 2001) as well as DEET (Usmani et al., 2002) and carbaryl (Tang et al., 2002). It may be inferred from our studies on human permethrin metabolism that variations in expression of alcohol and aldehyde dehydrogenases will have similar effects on permethrin metabolism. Because the expression of XMEs can depend not only on the genotype of the individual but also on induction factors such as coexposure to other toxicants and/or to clinical drugs will be of significance in the assessment of risk from deployment-related chemicals.

The almost 10-fold variation in the production of chlorpyrifos oxon by individual human liver microsomes appears to be related to the levels of expression of CYP2B6, CYP3A4, and CYP2C19 (Dai et al., 2001; Tang et al., 2001). The use of phenotyped human liver microsomes (Usmani et al., 2002) showed that individuals with high CYPs 3A4, 2C19, and 2B6 activities have the greatest potential to metabolize DEET. Similarly, these individual phenotyped microsomes showed differential activity toward carbaryl, an insecticide that is metabolized to three different primary metabolites by human microsomes and human recombinant CYPs (Tang et al., 2002).

Significance of Polymorphisms in Human Xenobiotic-Metabolizing Enzymes

Polymorphisms have been identified in human XMEs, particularly in CYP isoforms but also in enzymes of interest in the current studies, such as alcohol dehydrogenase and

aldehyde dehydrogenase. These heritable variants of normal or wild-type genes usually express proteins of lower activity, and individuals expressing these variant enzymes will have a reduced ability to metabolize any xenobiotic metabolized by that isoform. This is clear in our studies on chlorpyrifos and other chemicals, as indicated above. Thus, the genetic constitution as well as the chemical milieu will not only determine the outcome of a particular exposure but will also determine who is at greater or lesser risk. These polymorphisms in XMEs are not associated with particular ethnic groups but are seen in small percentages of individuals from all ethnic groups, although the gene frequencies may vary from one group to another. This is illustrated by studies carried out in the laboratory of a collaborator, [J. A. Goldstein (NIEHS), personal communication] in which polymorphic forms of CYP3A5 are shown to have very different abilities to oxidize testosterone and the drug, nifedipine, but occur in all ethnic groups examined.

CYP isoforms CYP2C19, 3A4, and 2B6 each have the ability to produce activation or detoxication products from chlorpyrifos, varying significantly in the ratio of the products.

CYP2C19 is one of the isoforms with the greatest potential for detoxication. Three naturally occuring polymorphic alleles of CYP2C19 exhibited markedly reduced ability to detoxify chlorpyrifos, compared to the wild-type enzyme and no ability to produce the oxon (Tang et al., 2001). Individuals carrying these polymorphisms could therefore be at greater risk to chlorpyrifos.

CYP3A4 both activates and detoxifies chlorpyrifos. Four CYP3A4 alleles were tested, one was essentially inactive and one was more active (Dai et al., 2001). Individuals carrying an inactive allele might be more susceptible to chlorpyrifos.

CYP2B6 appeared to be by far the best enzyme at activating chlorpyrifos to the toxic oxon (Tang et al., 2001). Activation in microsomes correlated well with CYP2B6 activity toward other substrates, indicating it is very important in producing the toxic product. Future studies will examine the effect of several newly discovered polymorphic CYP2B6 alleles in man (Goldstein, personal communication).

The use of microsomes with varying CYP3A4, CYP2B6, CYP2C19, CYP2C9, etc., activity (due to induction or polymorphisms in the individuals) is also an excellent way to determine whether particular isozymes are important in toxication/detoxication and whether polymorphisms affect this process. Consideration should also be given to the PON1 status, because, for example, enhanced ability to activate chlorpyrifos combined with low PON1 status could have significant toxicological consequences. This method can also be used to address the role of isoforms and polymorphisms on metabolism of other deployment-related chemicals.

Genotyping Studies

Genotyping studies will be able to address the relative proportions of various CYP alleles for enzymes that are important in metabolism of deployment-related chemicals in representative military populations. These will include the CYP2B6 alleles, CYP2C19 alleles, CYP3A4 alleles, and those for other CYP enzymes identified as vital to the metabolism of these chemicals in man.

In a recent major study (Garcia-Closas et al., 2001) carried out as a preliminary to the Agricultural Health Study (NCI, NIEHS, EPA, etc.) of the use of buccal samples for the collection of genomic DNA, the authors stated ''In conclusion, although DNA

isolated from either mouthwash or cytobrush samples collected by mail from adults is adequate for a wide range of PCR-based assays, a single mouthwash sample provides substantially larger samples and higher molecular weight DNA than two cytobrush samples.'' As more information is obtained with respect to risks associated with metabolic deficiencies, these samples will be valuable in the testing of hypotheses related to chemical exposure and subsequent health effects.

Interactions Based on Inhibition

As indicated above, there are numerous interactions between the chemicals of interest in this study that are based on the inhibition, by one chemical, of the metabolism of another. For example, chlorpyrifos is a potent inhibitor of DEET, carbaryl, and testosterone metabolism. Because the mechanism of this inhibition is almost certainly due to the formation of highly reactive sulfur during the oxidative desulfuration of chlorpyrifos followed by the interaction of this sulfur with the heme iron of cytochrome P450, this interaction will occur in the presence of chlorpyrifos whenever another chemical is metabolized by a CYP isoform that carries out the desulfuration reaction. It should also be a general interaction of any organophosphorus compound that has a P=S group in the molecule, and our studies have shown that, although chlorpyrifos is the most potent inhibitor, other organophosphorus chemicals act in the same way. Other interactions noted are the inhibition of permethrin metabolism by chlorpyrifos oxon and by carbaryl, presumably by inhibition of the B-esterases involved.

Interactions Based on Induction

Until recently, it was necessary to measure induction in experimental animals and extrapolate possible effects to humans, so it is difficult to assess the importance of this source of interaction in humans. However, we have recently been establishing procedures and baseline conditions for measuring induction in human hepatocytes.

Genotyping and Individuals and Populations at Increased Risk

Identification of individuals at increased risk will depend on a knowledge of their genotype with respect to XMEs, as well as a knowledge of the other chemicals expected to be used during a particular circumstance and clinical drugs prescribed for the individual. Using these results and appropriate paradigms, it will be possible to avoid individual or simultaneous exposures that might result in increased toxicity.

GENERAL CONCLUSIONS

HPLC analytical methods for carbaryl, chlorpyrifos, DEET, permethrin, pyridostigmine and their metabolites, sulfur mustard metabolites, testosterone, estradiol, and other endogenous metabolites have been brought on line in recent years and have been used in a number of human metabolic studies. The human cytochrome P450 isoforms metabolizing carbaryl, chlorpyrifos, and DEET have been identified and include forms known to be inducible and forms known to be polymorphic. It has been established that pyridostigmine and permethrin are not significant substrates for human

monooxygenases. While pyridostigmine does not appear to be readily metabolized, permethrin has been shown to be metabolized, in humans, through a series of reactions involving hydrolytic enzymes, followed by alcohol dehydrogenase and aldehyde dehydrogenase. Specific purified isoforms of human alcohol and aldehyde dehydrogenases have been shown to be active in these reactions. The reactive metabolite of chlorpyrifos, chlorpyrifos oxon, is a potent inhibitor of permethrin hydrolysis, while carbaryl is a less potent inhibitor of this reaction. Chlorpyrifos has been shown to be a potent inhibitor of the metabolism of DEET, carbaryl, and testosterone. It is clear that chlorpyrifos and, by implication, other organophosphorus compounds, may be significant both in health-related interactions between different deployment-related chemicals and in the determination of subpopulations and individuals at increased risk from anticholinergic chemicals.

Even within the very small subset of deployment-related chemicals examined to date, essentially all interact with at least one other or with an endogenous metabolite at the metabolic level: chlorpyrifos inhibits the metabolism of DEET, testosterone, and carbaryl; chlorpyrifos oxon, the principal reactive metabolite of chlorpyrifos, inhibits the hydrolysis of permethrin; and DEET is an inducer of XMEs in human hepatocytes. The potential for interactions is obvious, and it should also be noted that essentially all of the XMEs involved are polymorphic, and several are inducible, making human variation, both genotypic and phenotypic, important in the expression of toxicity. The potential for interactions with clinical drugs is one of great importance, and it is critical that investigations in this area be initiated.

These studies permit more confident extrapolation of past and future animal studies to humans and have permitted identification of interactions not apparent from animal studies. Perhaps even more important, they open the way to molecular genetic studies that will permit identification of human subpopulations at greater risk from specific toxicants, whether from genetic, environmental, or combined considerations, and will produce specific analytic methodologies for assessment of future exposures.

It is becoming increasingly apparent that induction studies previously requiring the use of experimental animals may be accomplished directly with human materials. Newer techniques that maintain the capacity for induction in cultured human hepatocytes combined with microarray techniques for determination of gene expression and repression may substitute for animal studies. Preliminary studies indicate that this is a viable approach that can be used to advantage in lieu of hepatocytes from experimental animals. Thus, it is possible to conclude the following:

- Human XMEs are important loci for toxic interactions of chemicals used in military deployments.
- Such interactions are of importance in the underlying causes of deployment-related illnesses.
- Selection of suitable animal models and extrapolation of animal-derived data to humans requires knowledge of human metabolism.
- Risk assessment for chemicals utilized in future deployments can be improved by the use of human data.
- Individuals and populations at increased risk can be defined by the use of human data on XME levels of expression and polymorphisms.

- Questions of diet and/or clinical drug exposure interactions with deployment-related chemicals can also be addressed by these approaches.
- These studies are of equal significance in industrial health, public health, agriculture, and, indeed, in the human health risk analysis of any group with significant environmental chemical exposure.

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