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INTRODUCTION

Chemicals used during 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. Since most exogenous chemicals can be substrates, inhibitors and/or inducers of xenobiotic-metabolizing enzymes (XMEs), these enzymes are an important potential locus for interactions. Many XMEs are polymorphic, potentially putting some individuals at greater risk than others. We are examining the role of specific human XMEs on the metabolism of a subset of chemicals important in military deployments. Chlorpyrifos, diethyl toluamide (DEET), permethrin, pyridostigmine bromide, sulfur mustard and its degradation products are being 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. 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 are being investigated. The potential of the test chemicals to act as XME inducers is also being tested in mice with some initial studies using human hepatocytes. HPLC methods have been either adapted or developed to facilitate these studies. The results will enable findings from animal studies to be extrapolated to humans with confidence, permit the identification of populations at risk and provide analytical methods for exposure assessment.

BODY OF REPORT

Since this is a final report covering a three year grant and many of the tasks overlap from year to year, the sequence has been realigned in order to facilitate reporting and improve readability. The new, overall, headings are as follows:

1. Development of new HPLC methods and/or modification and validation of available methods for analysis of the test chemicals* and their metabolites and appropriate endogenous chemicals.
2. Determination of substrate specificity of recombinant CYP, FMO and other human XME isoforms for the test chemicals and variations between test compound metabolism in phenotyped human liver microsomes. Interactions based on inhibition of test compound metabolism by xenobiotics and the metabolism of endogenous chemicals by the test chemicals.
3. Metabolism of pyridostigmine bromide.
4. Induction of XMEs by the test chemicals.
5. Reevaluation of relevance to human risk from the test chemicals: significance of XME isoforms and polymorphisms; interactions based on XME inhibition; interactions based on XME induction; identification of individuals and populations at increased risk.

*The deployment-related test chemicals are as follows: chlorpyrifos; DEET; permethrin; pyridostigmine bromide; sulfur mustard and metabolites; related chemicals as appropriate. The endogenous hormone involved was testosterone.

1. *Development of new HPLC methods and/or modification and validation of available methods for analysis of the test chemicals and their metabolites and appropriate endogenous chemicals.*

Chlorpyrifos:

The HPLC method we developed for the analysis of chlorpyrifos and its metabolites is described, in detail, in the attached reprints. This method has proven most useful, resulting in two peer-reviewed papers (Tang et al., 2001 and Dai et al., 2001 - copies attached) and is currently in use in this and other laboratories.

Chlorpyrifos studies were also supported, in part, by the North Carolina Environmental Trust Fund.

DEET:

The method developed for the analysis of DEET and its metabolites (*N*-ethyl-*m*-toluamide (ET) and *N*, *N*-diethyl-*m*-hydroxymethylbenzamide (BALC) utilized the same Shimadzu HPLC system (Kyoto, Japan), and the same column, as that used for chlorpyrifos. It is described in detail in the attached reprint (Usmani et al., 2002).

Permethrin:

The HPLC method for the separation and quantitation of permethrin and its metabolites has been utilized in the published study of the in vitro metabolism of permethrin by human enzymes and their isoforms (Choi et al., 2002, copy attached). Since substantial revisions in the procedure were found to be necessary due to poor resolution of phenoxybenzoic acid we conducted extensive studies to optimize the procedure with respect to run time, detection wavelength and pH for resolution of each metabolite. These are summarized below and described in detail in the attached reprint (Choi et al., 2002).

The optimal conditions for separation and resolution of permethrin and its metabolites involved the use of a gradient system. Two solvents (solvent A: 90% acetonitrile and 10% H₂O, solvent B: 100% H₂O adjusted to pH 1.7 with 85% phosphoric acid) were used for gradient elution (flow rate: 1ml/min). The HPLC gradient system reliably resolved and detected the two permethrin isomers and three major metabolites, phenoxybenzyl alcohol, phenoxybenzoic acid and phenoxybenzaldehyde; 230 nm was the most appropriate detection wavelength.

Since phenoxybenzoic acid is ionized at a neutral pH, the effect of mobile phase pH on the protonation of this and other metabolites was determined. As the protonation of phenoxybenzoic acid proceeded with increasing hydrogen ion concentration in the mobile phase, a separate peak

of phenoxybenzoic acid gradually became distinguishable, forming a clearly identifiable peak at a mobile phase pH of 1.7.

Pyridostigmine bromide:

A reverse phase high pressure liquid chromatography (HPLC) method was developed for the separation and detection of pyridostigmine bromide and its potential metabolites based on the HPLC method of Leo (1997). The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of 2 pumps (LC-10AT VP) a Shimadzu auto injector (SIL-10AD VP), and a Shimadzu UV/VIS detector (SPD-10A VP). All system components were controlled through the Shimadzu powerline firmware. Data was collected via a Shimadzu system controller (SCL-10A VP) and analyzed using CLASS-VP 7.0 software. The mobile phase consisted of 5% methanol, 5% acetonitrile, 0.1% triethylamine, and 89.9% ammonium acetate buffer, pH 4.0 (adjusted with acetic acid) running at 1.0 ml/min. Metabolites were separated by a Synergi Max column (Synergi 4 μ , 150 x 4.60 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 260 nm.

Sulfur Mustard and its Degradation Products:

These compounds have 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. The methods under development early in the project proved too insensitive for metabolite analysis. We are having success with a fluorimetric method based on derivatization with 2-(4-carboxyphenyl)-6-N,N-diethylaminobenzofuran (Assaf et al., J. Chromatog. A, 869:243, 2000) using a carbodiimide-catalysed esterification that reacts at the alcoholic hydroxyl groups common to all the oxidative metabolites. The fluorescent reagent is not commercially available and had to be synthesized. Derivative structures are being confirmed by HPLC/Mass Spectroscopy. Separation of the derivatized compounds is readily accomplished using a 150 x 4 mm C18, reverse phase HPLC column and gradient elution. Detection is by fluorescence 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.

Testosterone

An improved HPLC Method for the separation of testosterone and its metabolites was developed based on the method of Purdon and Lehman-McKeeman (1997). Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan). The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of pump (LC-10AT VP), a solvent proportioning valve (FCV-10AL VP), a degasser (DUG-14A), a Shimadzu auto injector (SIL-10AD VP), and a Shimadzu UV/VIS detector (SPD-10A VP). Data was collected via a Shimadzu system controller (SCL-10A VP) and analyzed using CLASS-VP 7.0 software. The mobile phase for pump A was 5%

tetrahydrofuran, 95% water, and for pump B 100% methanol. A linear system was employed in the following manner: 0-6 min (30%B), 6-50 min (30-60% B), 50-55 min (60-90% B), 55-57 min (90% B), 57-58 min (90-30%B), and 58-60 min (30% B). The flow rate was 0.5 ml/min. Metabolites were separated by a C18 column (Luna 5 μ , 150 x 3 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 247 nm. A representative separation is shown in Figure 1. The method has been used in a study of testosterone metabolism and its inhibition by the test chemicals (Usmani et al., 2003, copy attached).

2. Determination of substrate specificity of recombinant CYP, FMO and other human XME isoforms for the test chemicals and variations between test compound metabolism in phenotyped human liver microsomes. Interactions based on inhibition of test compound metabolism by xenobiotics and the metabolism of endogenous chemicals by the test chemicals.

Chlorpyrifos:

The studies of human chlorpyrifos metabolism have been published (Tang et al., 2001, copy attached). These are the most complete in vitro human metabolism studies of an organophosphorus xenobiotic published to date and, as indicated, have important implications for possible interactions and for the definition of individuals and sub-populations at increased risk.

This latter point is further exemplified by the studies of variants of human CYP3A4, an isoform active in both the activation and detoxication of chlorpyrifos (Dai et al., 2001, copy attached). 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 the current studies several new polymorphic variants of CYP3A4 have been 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.

In ongoing studies supported by another agency we have characterized the human metabolism of the carbamate insecticide, carbaryl, identifying the isoforms responsible for the production of the three major oxidative metabolites. Of relevance to this report, however, is the observation that chlorpyrifos is a potent inhibitor of carbaryl metabolism. Sample data sets are shown in the following tables (Tables 1 - 4). Note that inhibition at 0 time preincubation is due to a combination of irreversible and competitive inhibition taking place during the incubation period.

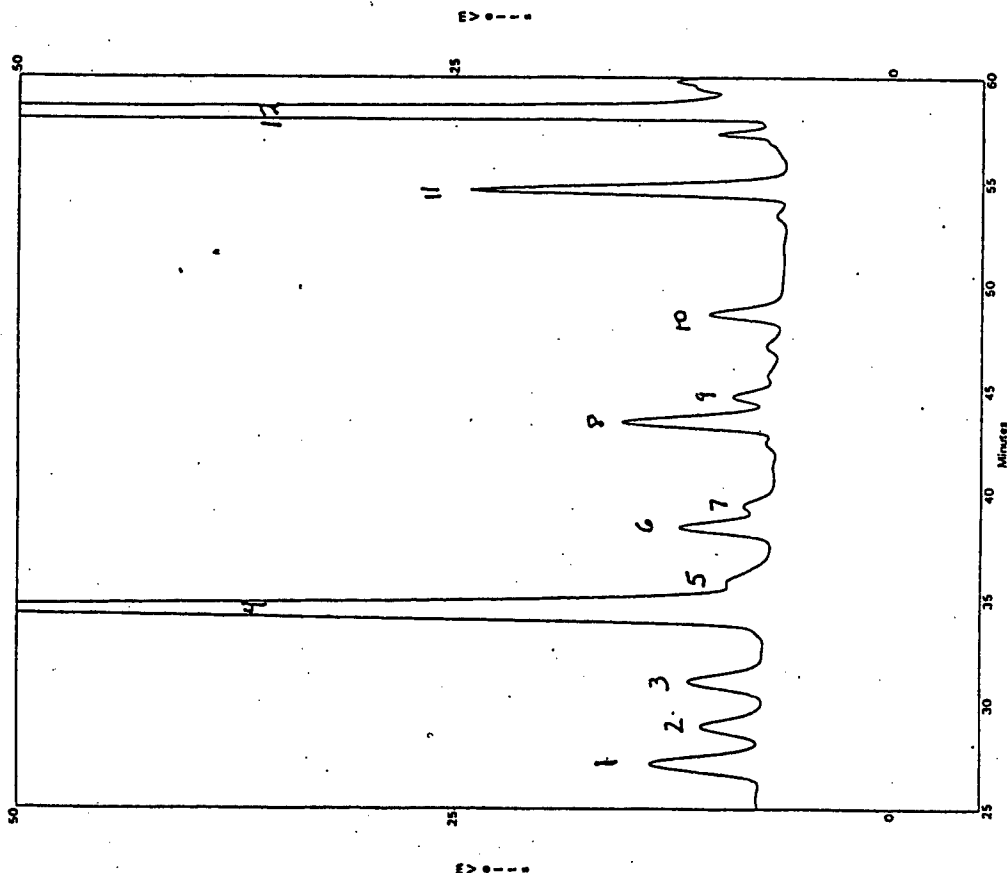


Fig 1. Representative chromatogram for testosterone metabolism in male CD-1 mice form Phenobarbital-induced microsomes*.

- 1) 7 β -Hydroxytestosterone
- 2) 6 α -Hydroxytestosterone
- 3) 15 β -Hydroxytestosterone
- 4) 6 β -Hydroxytestosterone
- 5) 15 α -Hydroxytestosterone
- 6) 7 α -Hydroxytestosterone
- 7) 16-ketotestosterone
- 8) 16 α -Hydroxytestosterone
- 9) 2 α -Hydroxytestosterone
- 10) 2 β -Hydroxytestosterone
- 11) Androstenedione
- 12) Testosterone

* Tentative identification of testosterone metabolites were made based on Purdon and Lehman-McKeeman (1997) chromatograms. Further improvement in the separation of testosterone metabolites is underway.

Table 1. Inhibition of carbaryl metabolism by pre-incubation of pooled human liver microsomes with chlorpyrifos.

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	%inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
5	0	6	52
15	14	37	69
30	43	56	80

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and microsomal protein 1 mg in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.021 nmol/mg protein/min, 4-hydroxycarbaryl 0.45, carbaryl methylol 0.301.

Table 2. Inhibition of carbaryl metabolism by pre-incubation of human CYP1A1 with chlorpyrifos

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	%inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
0	87	89	33
30	96	95	58

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and 17.86 pmol CYP1A1 in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.002 nmol/mg protein/min, 4-hydroxycarbaryl 0.0039, carbaryl methylol 0.00012.

Table 3. Inhibition of carbaryl metabolism by pre-incubation of human CYP2B6 with chlorpyrifos.

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	% inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
5	100	100	100
15	100	100	100
30	100	100	100

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and 22.73 pmol CYP2B6 in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.0002 nmol/mg protein/min, 4-hydroxycarbaryl 0.0012, carbaryl methylol 0.0135.

Table 4. Inhibition of carbaryl metabolism by pre-incubation of human CYP3A4 with chlorpyrifos.

pre-incubation time (min)	% inhibition of 5-hydroxy carbaryl formation	% inhibition of 4-hydroxy carbaryl formation	% inhibition of N-methylol carbaryl formation
0	96	94	86
5	89	92	93
30	99	95	100

Note: 1) Final concentrations for chlorpyrifos were 50 μ M, carbaryl 500 μ M, and 25 pmol CYP3A4 in 500 μ l reaction volume. Incubation time with carbaryl was 15 min.
2) Control (i.e. without chlorpyrifos) values: 5-hydroxycarbaryl 0.0014 nmol/mg protein/min, 4-hydroxycarbaryl 0.00444, carbaryl methylol 0.00059.

As discussed in the manuscript on DEET (Appendix 3), chlorpyrifos is also an inhibitor of the metabolism of DEET by human liver microsomes.

Chlorpyrifos studies were also supported, in part, by the North Carolina Environmental Trust Fund.

DEET:

The studies of the oxidative metabolism of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) by pooled human liver microsomes (HLM), rat liver microsomes (RLM), and mouse liver microsomes (MLM) have been published (Usmani et al., 2002, copy attached). These studies are described in detail in the published work but are summarized, for the sake of continuity, at this point.

DEET is metabolized by cytochrome P450s (CYPs) leading to the production of a ring methyl oxidation product, *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) and an *N*-deethylated product, *N*-ethyl-*m*-toluamide (ET). Both the affinities and intrinsic clearance of HLM for ring hydroxylation are greater than those for *N*-deethylation. Among 15 cDNA-expressed CYP enzymes examined, CYP1A2, 2B6, 2D6*1 (Val₃₇₄), and 2E1 metabolized DEET to the BALC metabolite while CYP3A4, 3A5, 2A6, and 2C19 produced the ET metabolite. CYP2B6 is the principal CYP involved in the metabolism of DEET to its major BALC metabolite while CYP2C19 had the greatest activity for the formation of the ET metabolite. 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. Preincubation of human CYP2B6 with chlorpyrifos completely inhibited the metabolism of DEET. Preincubation of human or rodent microsomes with chlorpyrifos, permethrin, and pyridostigmine bromide, alone or in combination, can lead to either stimulation or inhibition of DEET metabolism.

Permethrin:

Permethrin is a pyrethroid insecticide used in agriculture, public health and in military deployments, for control of disease vectors and nuisance insects. Because of this use pattern it was important to understand the basic enzymatic pathways for its metabolism in humans as a preliminary to the study of interactions with implications for human health. In these studies we have determined that *trans*-permethrin is metabolized by human liver fractions, producing phenoxybenzyl alcohol by hydrolysis and subsequently phenoxybenzoic acid. Alcohol and aldehyde dehydrogenases were identified as the enzymes responsible for the metabolism of phenoxybenzyl alcohol to phenoxybenzoic acid, with phenoxybenzaldehyde as an intermediate. *Cis*-permethrin was not significantly metabolized by human liver fractions and CYP isoforms were not involved either in the hydrolysis of *trans*-permethrin or in the oxidation of phenoxybenzyl alcohol to phenoxybenzoic acid. Purified alcohol dehydrogenase isoforms oxidized phenoxybenzyl alcohol to phenoxybenzyl aldehyde and was a preferred substrate to ethanol. Purified aldehyde dehydrogenase was responsible for the formation of phenoxybenzoic acid from phenoxybenzyl aldehyde. These studies are described in detail in the attached reprint (Choi et al., 2002).

Since 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. As these studies are not yet published they are described in detail as follows:

Range finding assays were conducted for chlorpyrifos oxon (CFO) and carbaryl inhibition of *trans*-permethrin hydrolysis resulting in a final *trans*-permethrin concentration of 200 μ M. Varying concentrations of CFO (5-400 nM) and carbaryl (0.5-200 μ M) were mixed with either microsomal (60 μ g protein) or cytosolic (200 μ g) fractions, and pre-incubated for 5 minutes at room temperature. The assays were started when *trans*-permethrin (200 mM final concentration) was added to the mixtures and placed in a water bath set at 37°C. The assay was conducted in Tris buffer (pH 7.4, 5 mM $MgCl_2$ and 3 mM EDTA) containing 5 mM $MgCl_2$ and 3 mM EDTA with a total volume of 250 μ l. The reaction mixtures were incubated for 20 min and cold acetonitrile was added to stop the reactions.

When necessary, 13 μ l of NADPH regenerating system solution A, 5 μ l of NADPH generation system solution B were added to the reaction mixture. Solution A of the NADPH regenerating system contained 26.0 mM $NADP^+$ and 66 mM of glucose-6-phosphate in phosphate buffer (pH 7.4). Solution B of the NADPH regenerating system contained 40 units/ml of glucose-6-phosphate dehydrogenase in phosphate buffer (pH 7.4).

Based on IC50 values, appropriate concentration levels (32 nM CFO for cytosol, 80 nM for microsomes and 10 mM carbaryl for both fractions) were selected for inhibition kinetics. Using these selected concentrations, CFO and carbaryl effects on permethrin hydrolysis in

human liver fractions were examined with a series of *trans*-permethrin concentrations. Human liver fractions were mixed with either CFO or carbaryl at room temperature and pre-incubated for 5 minutes. Then the mixtures were transferred to a water bath set at 37°C and 20 minute incubations were started as *trans*-permethrin was added to the mixtures. After the incubation was completed, 250 µl of cold acetonitrile was added to each reaction mixture to stop enzymatic reactions. The reaction mixtures were then centrifuged at 20,000 g for 5 minutes using a microcentrifuge. The supernatant was sampled and stored at 4°C until HPLC analysis.

To distinguish between non-competitive and irreversible inhibition, increasing amounts of microsomal or cytosolic proteins were incubated with a high enough concentration (200 mM) of *trans*-permethrin to bring about V_{max} in the absence or presence of either CFO or carbaryl. If V_{max} linearly increases from a protein level of zero to higher protein levels, it will indicate non-competitive inhibition. On the other hand, if the V_{max} remains at zero up to a certain protein level and then begins to linearly increase after that, irreversible inhibition will be assumed.

V_{max} and apparent K_m values were determined by non-linear regression method using GraphPad software (GraphPad Software, Inc., San Diego, CA) and K_i values were determined using a formula based on V_{max} and K_m of particular inhibition type (Segel, 1976). For inhibition kinetics graphs, all values are expressed as mean ± SEM (n = 3). Statistical analyses were performed using GraphPad Software (San Diego, CA). Significant statistical differences for V_{max} and K_m were apparent when 95 % confidence intervals did not overlap. The type of inhibition was determined based on whether there were any statistically significant differences in V_{max} or K_m values after inhibitor treatments. The level of statistical significance for all tests was p < 0.05.

CFO inhibition of trans-permethrin hydrolysis

Trans-permethrin hydrolysis in human liver fractions was inhibited more effectively by CFO than by carbaryl. In given assay conditions, IC₅₀s of CFO 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 CFO.

When the cytosolic fraction was pre-incubated for 5 min with 32 nM CFO, the V_{max} value was significantly reduced from 0.92 nmole/mg/min to 0.38 nmole/mg/min while K_m values stayed within 95 % of confidence interval (Table 5). Five minutes pre-incubation with 80 nM CFO in the microsomal fraction resulted in a significant decrease in V_{max} value (8.45 to 4.60) with an insignificant drop in K_m values (Table 5). The same pattern of kinetic parameter changes, decreased V_{max} with no changes in K_m, were found in both fractions and based on this observation, non-competitive or irreversible type of inhibition was assumed for CFO in human liver fractions. The inhibition constant (K_i), an indicator of inhibitor affinity to target enzyme was calculated for CFO from V_{max} and K_m values. They were 21.39 nM for the cytosolic fraction and 95.00 nM in the microsomal fraction, respectively. These values were

approximately one hundred times lower than those for carbaryl, indicating a higher inhibitory potential of CFO.

The parent compound, chlorpyrifos was not inhibitory but co-incubation with an NADPH regenerating system and the microsomal fraction led to inhibitory effects confirming that CFO, the reactive metabolite of chlorpyrifos, is the chemical responsible for the inhibition. In the assay to further clarify whether CFO inhibition of permethrin hydrolysis is non-competitive or irreversible; no hydrolysis products were produced up to 30 μg level and this indicates that human liver esterases involved in *trans*-permethrin hydrolysis is irreversibly inhibited by CFO (Fig. 2).

Carbaryl inhibition of trans-permethrin hydrolysis.

In the range finding assays, carbaryl showed IC₅₀ values of 10 μM in both microsomal and cytosolic fractions. The most noticeable difference from CFO 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 had 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 different susceptibility to carbaryl inhibition.

Five minutes pre-incubation of 10 μM carbaryl with either fraction resulted in the same pattern of inhibition, irreversible or non-competitive inhibition as indicated in Table 6. Carbaryl caused significant decreases in V_{max} and no significant changes in K_m (Table 6 and Fig. 3). K_i values for carbaryl were 2.49 μM in the cytosolic fraction and 11.08 μM in the microsomal fraction. In contrast to CFO, in the assay to determine the inhibition type, V_{max} linearly increased from zero protein level indicating that carbaryl acts as a non-competitive inhibitor (Fig. 3).

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 very potent inhibitor of *trans*-permethrin hydrolysis after metabolic activation to CFO. This observation implies that co-exposure to chlorpyrifos might potentiate the toxicity of permethrin by deactivating the metabolic detoxification pathway for permethrin. Other deployment related compounds, an insect repellent (N,N-diethyl-*m*-toluamide) a nerve gas prophylactic (pyridostigmine bromide) did not cause the inhibition of *trans*-permethrin hydrolysis regardless of the presence of an NADPH regeneration system.

CFO completely inhibited *trans*-permethrin hydrolysis in both 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 CFO 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 CFO is the chemical species responsible for the inhibition of *trans*-permethrin hydrolysis.

The mechanism of CFO inhibition is trans-esterification, which forms a strong covalent bond between oxon and alcohol functional group of a serine residue in the active site of esterases. With normal substrate, a transient bond is formed in place of the covalent bond and readily cleaved by deacylation (Chambers, 1992). 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 CFO, typical non-competitive inhibition. This result is in an 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 CFO.

Another important observation that, in contrast to CFO, 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 CFO 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 CFO inhibition but one with higher sensitivity to carbaryl inhibition and the other with lower or no sensitivity to carbaryl.

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

Table 5. Chlorpyrifos oxon (CFO) inhibition kinetics in human liver fractions.

	V_{max} (nmole/mg/min)	K_m (μM)	K_i (nM)
Cytosol w/o CFO	0.92	32.4	NA
Cytosol w/ CFO (32 nM)	0.38	11.6	21.4
Microsomes w/o CFO	8.45	61.3	NA
Microsomes w/CFO (80 nM)	4.60	29.4	95.0

Table 6. Carbaryl inhibition kinetics in human liver fractions.

	V _{max} (μ mole/mg/min)	K _m (μ M)	K _i (μ M)
Cytosol w/o carbaryl	1.65	46.0	NA
Cytosol w/ carbaryl (10 μ M)	0.33	80.1	2.49
Microsomes w/o carbaryl	3.81	16.4	NA
Microsomes w/ carbaryl(10 μ M)	2.01	25.7	11.1

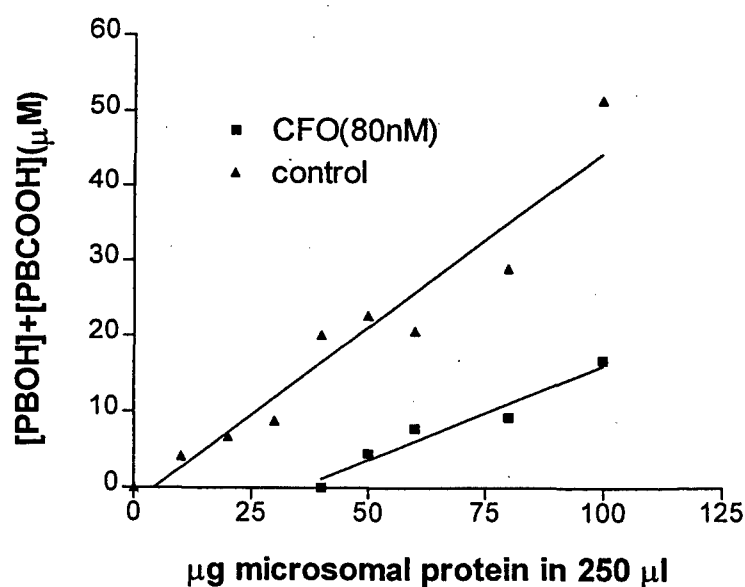


Figure 2. Non-competitive vs. irreversible inhibition. A series of different amount of human liver microsomes were pre-incubated with chlorpyrifos oxon (80 nM) or carrier for 5 minutes, and 200 μ M trans-permethrin was added and incubated for 20 minutes at 37°C.

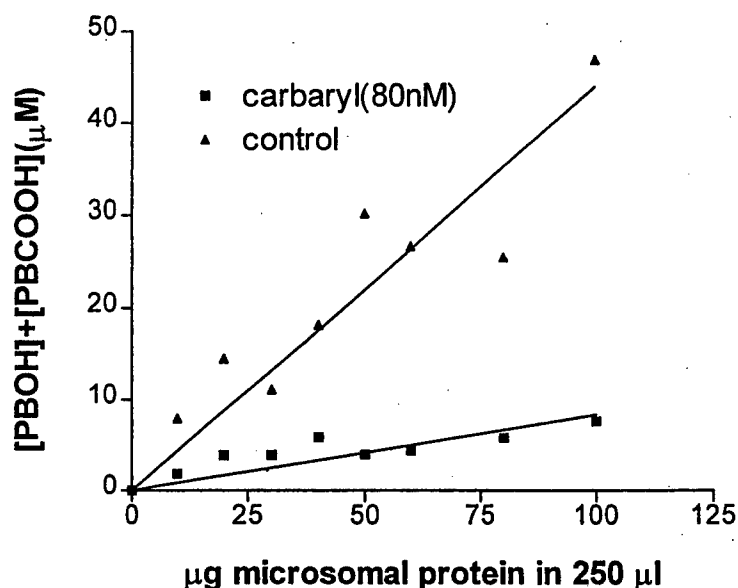


Figure 3. Non-competitive vs. irreversible inhibition. A series of different amount of human liver microsomes were pre-incubated with carbaryl (80 nM) or carrier for 5 minutes, and 200 μ M transpermethrin was added and incubated for 20 minutes at 37°C.

Inhibition of testosterone metabolism.

One of the possible mechanisms for toxic effects on human health is the inhibition of the metabolism of endogenous metabolites, particularly metabolites with significant effects on physiological processes or control mechanisms, such as hormones. Since there have been no such studies utilizing human materials we felt it important to gain insight into the potential for this mechanism in humans by conducting some initial experiments on the effects of deployment-related chemicals on the metabolism of testosterone by human oxidative enzymes (Usmani et al., 2003, copy attached).

Cytochrome P450 (CYP) monooxygenases 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 6 β -hydroxytestosterone, 2 β -hydroxytestosterone and 15 β -hydroxytestosterone. Utilizing the HPLC method developed (described above) a screen of 16 cDNA expressed human CYPs demonstrated that 94% of all testosterone metabolites are produced by members of the CYP3A subfamily with 6 β -testosterone accounting for 84% of all testosterone metabolites. While similar K_m values were observed with HLM, regardless of which metabolite is measured, V_{max} and Cl_{int} were much higher for 6 β -testosterone than for any other metabolite.

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 preincubation with organophosphorus compounds, including chlorpyrifos, phorate and fonofos, with up to 80% inhibition of the formation of several metabolites, including 6 β -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 metabolite 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 pyridostigmine bromide increased the metabolism of testosterone. Preincubation of aromatase (CYP19) with the test chemicals had no effect on the production of the endogenous estrogen, 17 β -estradiol.

Details of the methodology, kinetic constants, etc., are to be found in the attached reprint (Usmani, et al., 2003)

3. Metabolism of Pyridostigmine bromide:

Pyridostigmine bromide metabolism activity assays were performed by incubation of 50 mM pyridostigmine bromide with pooled human liver microsomes, pooled human liver cytosol, pooled human liver S9, rat liver microsomes, rat liver cytosol, and rat serum for 60 min. The microsomal protein concentrations used in the assays were 1.0 mg/ml in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 5 mM MgCl₂ and 3 mM EDTA. Reactions were stopped by the addition of methanol followed by centrifugation at 15,000 rpm. The supernatant was analyzed for pyridostigmine bromide metabolites by HPLC.

Our data indicate 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. An earlier study by Leo et al., (1977) 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. This is confirmed and extended by our results.

4. Induction of XMEs by the test chemicals.

Adult male CD-1 mice, 28-30 grams, were obtained from Charles River Laboratories and acclimated for 4 days. Doses for pyridostigmine bromide, trans- and cis-permethrin, and chlorpyrifos were selected such that the highest administered dose did not exceed doses known to produce physiological effects. The doses administered by intraperitoneal injection for each of three days as follows: pyridostigmine bromide (0.1, 0.5, and 1 mg/kg/day) in 100 μ l water, trans-permethrin (1, 10, and 100 mg/kg/day) in 100 μ l corn oil, cis-permethrin (1, 10, and 20 mg/kg/day) in 100 μ l corn oil, chlorpyrifos (0.1, 1, and 10 mg/kg/day) in 100 μ l corn oil. Doses approximating LD₁₀ values for phenobarbital (80 mg/kg/day) in 100 μ l water or 3-

methylcholanthrene (20 mg/kg/day) in 100 µl corn oil were also administered intraperitoneally, to separate groups of mice, daily for 3 days. Controls were given corn oil only or water.

Microsomes were prepared from livers of fed mice on the fourth day according to the method of Cook and Hodgson (1983). Total cytochrome P450 content was determined by the CO-difference spectrum method of Omura and Sato (1964). Protein concentration was determined using the BioRad protein assay with bovine serum albumin (BSA) as standard (Bradford, 1976).

The following substrates were used as indicators of the activities for the following isozymes: ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) for CYP1A1/2 (Burke and Mayer, 1974; Nerurkar et al., 1993), and pentoxyresorufin O-dealkylation (PROD) for CYP2B10 (Lubet et al., 1985). Assays were conducted as described by Pohl and Fouts (1980). Product formation for EROD, MROD and PROD activities were determined spectrofluorimetrically (RF-5301PC Personal Fluorescence spectrophotometer, Shimadzu, Kyoto, Japan) by comparison with a standard curve generated with resorufin.

Mice treated with PB, 3-MC, and varying concentration of pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos showed no significant differences in liver weight. PB treated mouse liver microsomes showed significantly higher P450 content than control or pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos treated mouse liver microsomes. No significant differences were observed in P450 content between control and pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos treated mouse liver microsomes. Mice treated with varying concentrations of pyridostigmine bromide, *trans*-permethrin, *cis*-permethrin, and chlorpyrifos exhibited no significant levels of induction toward EROD, MROD, and PROD. Phenobarbital treatment as a positive control significantly induced PROD activity (12-fold). In a similar manner, treatment with 3-methylcholanthrene also resulted in significant induction of MROD (8-fold) and EROD (28-fold) as expected.

Induction Studies in Human Hepatocytes

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. 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. Since 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 (Li et al., 1997).

The branched DNA (bDNA) assay is a new technique which allows for quantitative determinations of messenger RNA levels from hepatocyte tissues. This technique allows for the evaluation of a variety of chosen genes simultaneously at the level of mRNA. Recent experience 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 multi-oligonucleotides not only to capture the mRNA of interest, but also link it to an enzyme which 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 conducted with human hepatocytes in combination with the bDNA assay suggest that several CYP isoforms are induced by permethrin, chlorpyrifos and DEET (Figure 4). 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 had also suggested that CYP2B6, 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. This data was also corroborated with testosterone metabolism data using hepatocyte S9 preparations which had been exposed to rifampicin, permethrin, chlorpyrifos and DEET (Figure 5). A similar western blot of CYP1A1 activity did not provide confirming evidence for protein induction by chlorpyrifos as might have been expected based upon the bDNA assays performed. Since these results are preliminary it is not known if the absence of correlation is the result of protein destabilization (a possibility since chlorpyrifos acts as a suicide inhibitor) or whether sufficient time between mRNA induction and protein synthesis might explain the result. By the same token, induction of CYP3A4 by DEET may actually be greater if we had had the opportunity to explore the proper time frame for protein synthesis. Permethrin also strongly induced CYP2B6 along with CYP2A6.

Use of human hepatocytes for induction studies is also sponsored, in part, by a grant from

Induction in Mice by Sulfur Mustard and its Degradation Products.

The first round of the induction of mice with thiodiglycol was a range finding experiment with 5 mice per dose level using ip injections of 5, 10, and 20% of an LD₅₀ dose daily for three days followed by liver microsome preparation on the fourth day and freezing of the microsomes at high protein concentration in phosphate buffer containing 0.25 M sucrose (Sabourin et al., 1984). The animals exhibited no signs of discomfort at the doses employed. There was evidence of liver hypertrophy at the highest dose.

Subsequent induction studies in mice with thiodiglycol and sulfur mustard were carried out to identify the possible participation of cytochromes P450 in their metabolism as well as the metabolism of other xenobiotics. Adult CD1 males 31 – 32 g were obtained from Charles River Laboratories, acclimated for 10 days, grouped to give equivalent average weights and dosed ip with thiodiglycol in saline and with sulfur mustard in corn oil at 5 mice per group. Treatments were administered based on average group weights. Treatment levels using sulfur mustard were vehicle control, 5% LD50 (100 µl at 0.25 mg/ml), 10% LD50 (100 µl at 0.5 mg/ml), and 20% LD50 (100 µl at 1.0 mg/ml). Dosage was based on published mouse ip LD50s for sulfur mustard of 7.7 mg/kg (McMaster and Hogeboom, 1942) to 19 mg/kg (Friedberg et al., 1983).

The mouse LD50 for thiodiglycol is 5.3 g/kg (Anslow et al., 1948). It was administered using ip saline injected animals (250 µl/mouse) as controls and 5% LD50 (75 µl at 120 mg/ml), 10% LD50 (150 µl at 120 mg/ml) and 20% LD50 (280 µl at 120 mg/ml). The animals received injections for three days and were euthanized on day four. Liver microsomes were prepared and stored as described in Sabourin et al., 1984. Liver cytosolic preparations (thiodiglycol induced animals) were prepared as outlined in Dudley et al. (2000). Protein determinations were carried out using the Bradford method (1976). CYP levels were measured by CO difference spectrum as outlined by Omura and Sato (1964) (Table 7).

CYP isoforms present in the mouse microsomal preparations were analyzed by Western blot as described in Emoto et al., (2000), using commercially available antibodies (BD Gentest, Woburn, MA) with specificity for rat cytochrome P450s that have been shown to cross react with their mouse counterparts. Microsomes from the thiodiglycol and sulfur mustard treated animals and untreated controls were tested for the presence of CYP 3A2, 2B1/2B2, 2E1, 2C11, 2C13, 2C6, 1A1, 1A2, 1B1 and 4A1/4A3. Samples consisted of aliquots of the treated and control microsomal preparations and mouse "Supersome" standards. The results are summarized in Table 8.

Enzymatic activity toward isoform specific substrates has been tested in thiodiglycol and sulfur mustard treated microsomes. Table 9 gives the results for catechol formation by *p*-nitrophenol hydroxylase activity. These data were generated using the method outlined in literature from BD

Figure 4. Induction of mRNA by Selected Pesticides and DEET as Measured by the bDNA Assay.

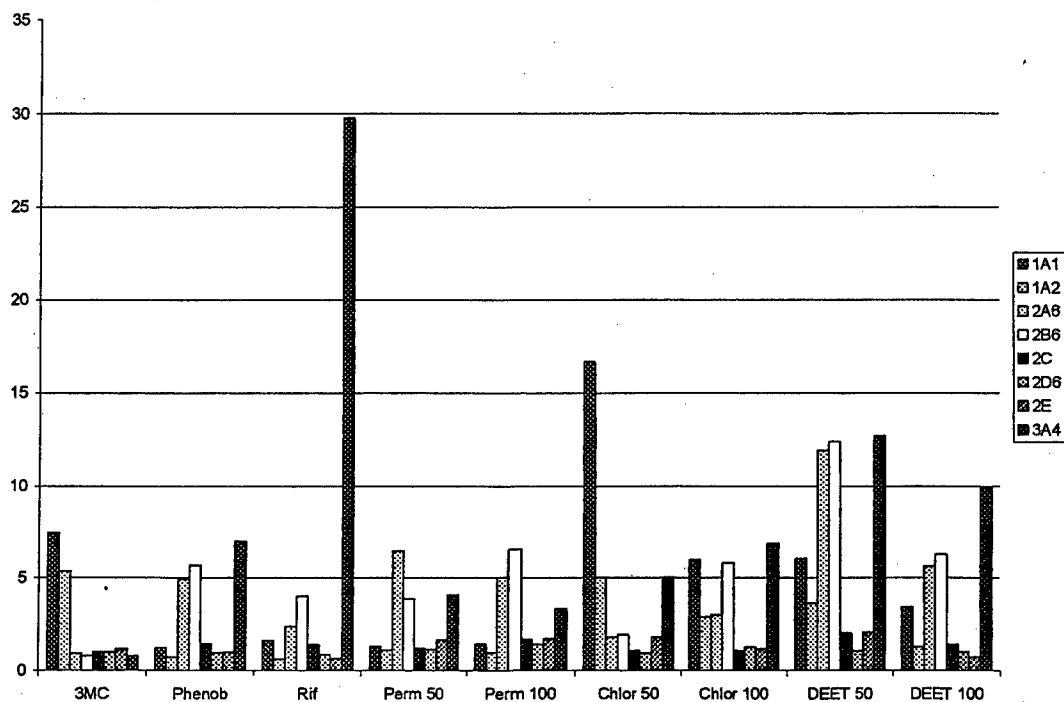
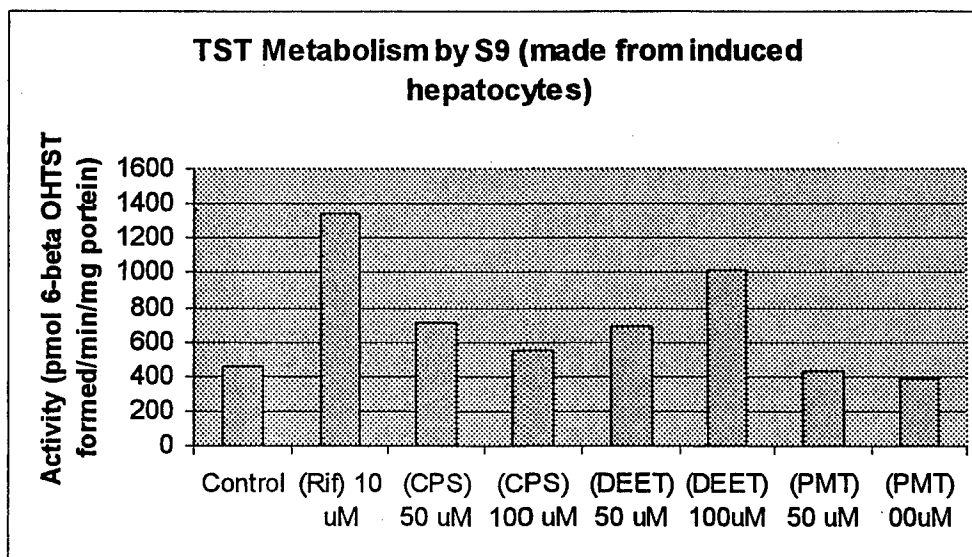


Figure 5.



Gentest (Woburn, MA) which is based on the published spectrophotometric (Beckman DU 70, Beckman Instruments, Palo Alto, CA) method of Koop (1986) measuring absorbance at 535 nm and using an extinction coefficient for 4-nitrocatechol of $11.2 \text{ mM}^{-1}\text{cm}^{-1}$ supplied by Gentest. Table 10 shows the outcome of pentoxyresorufin-O-dealkylation determined spectrofluorimetrically (RF-5310PC. Personal Fluorescence spectrophotometer, Shimadzu, Kyoto, Japan) by comparison with a resorufin standard curve according to the method of Pohl and Fouts (1980).

Thiodiglycol Oxidation by Alcohol Dehydrogenase: Cytosolic preparations made with the microsomes were evaluated for alcohol dehydrogenase activity using ethanol and thiodiglycol. These preparations readily metabolized thiodiglycol as measured spectrophotometrically by following the change in OD at 340 nm as described by Dudley et al. (2000). However, there was no difference among the thiodiglycol or mustard treatment groups that would indicate increasing activity as a function of the treatments. Attempts to evaluate the actual metabolic products arising from the action of purified human recombinant alcohol dehydrogenase isoforms on thiodiglycol using NMR (Brimfield et al., 2002) have been unsuccessful. This may be due to the presence of large quantities of glycerol present in the purified enzyme preparations or to damage to the protein during removal of the glycerol by column chromatography.

Table 7. Total Cytochrome P450 Concentrations in Thiodiglycol and Sulfur Mustard Induced Mouse Liver Microsomes.

Thiodiglycol Induction	
Control	0.23 nmol/mg protein
5% LD50	0.31 nmol/mg protein
10% LD50	0.37 nmol/mg protein
20% LD 50	0.47 nmol/mg protein
Sulfur Mustard Induction	
Control	0.22 nmol/mg protein
5% LD50	could not be determined
10% LD50	0.20 nmol/mg protein
20% LD50	0.19 nmol/mg protein

Table 8. Identification of P450 Isoforms in Microsomes from Thiodiglycol and Sulfur Mustard Treated CD1 Mice.

Thiodiglycol Induction	3A2	2B1/2B2	2E1	2C11/2C13	2C6	1A1	1A2	1B1	4A
Control	++	faint	+++	+++	+++	+	faint	faint	++
5% LD50	++	+	+++	++	++	+	faint	faint	++
10% LD50	++	++	+++	++	+	faint	-	faint	++
20% LD50	++	++	+++	++	+	faint	-	faint	++

Mustard Induction									
Control	+++	++	++	+	--	--	faint	faint	+
5% LD50	++	++	++	+	--	--	faint	faint	faint
10% LD50	++	++	++	+	--	--	faint	faint	+
20% LD50	+	++	++	+	--	--	faint	--	++

Table 9. *p*-Nitrophenol Hydroxylation by Mouse Liver Microsomes from Thiodiglycol and Sulfur Mustard Treated CD1 Mice.

Thiodiglycol Induction	
Control	0.25 nmol/min/μg protein
5% LD50	0.25 nmol/min/μg protein
10% LD50	0.21 nmol/min/μg protein
20% LD50	0.20 nmol/min/μg protein

Sulfur Mustard Induction	
Control	0.16 nmol/min/μg protein
5% LD50	0.14 nmol/min/μg protein
10% LD50	0.11 nmol/min/μg protein
20% LD50	0.11 nmol/min/μg protein

Table 10. Pentoxoresorufin-O-dealkylase by Mouse Liver Microsomes from Thiodiglycol and Sulfur Mustard Treated CD1 Mice.

Sulfur Mustard Induction	
Control	0.022 pmol/min/μg protein
5% LD50	0.037 pmol/min/μg protein
10% LD50	0.042 pmol/min/μg protein
20% LD50	0.027 pmol/min/μg protein
Thiodiglycol Induction	
Control	0.05 pmol/min/μg protein
5% LD50	0.049 pmol/min/μg protein
10% LD50	0.048 pmol/min/μg protein
20% LD50	0.047 pmol/min/μg protein

5. Reevaluation of relevance to human risk from the test chemicals: significance of XME isoforms and polymorphisms; interactions based on XME inhibition; interactions based on XME induction; identification of individuals and populations at increased risk.

a. Significance of XME isoform 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 (Tang et al., 2001, Dai et al., 2001) as well as DEET (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. Since the expression of XMEs can depend not only on the genotype of the individual but also on induction such factors as co-exposure to other toxicants and/or to clinical drugs will be of significance in the assessment of risk from deployment-related chemicals.

b. Significance of polymorphisms in human XME genes.

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 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. 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.

c. Interactions based on XME 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. Since 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 inhibitors, other organophosphorus chemicals act in the same way. Other interactions noted are the inhibition of permethrin metabolism by chlorpyrifos oxon and by carbaryl.

d. Interactions based on XME induction.

Since until recently it was necessary to measure induction in experimental animals and extrapolate possible effects to humans, 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 (see above) and, based on our initial results, we expect to make rapid progress in this area in the future.

e. Identification of 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.

We are currently planning a major review on human metabolism and metabolic interactions in humans of deployment-related chemicals.

KEY RESEARCH ACCOMPLISHMENTS

- Analytical methods developed for the test compounds, chlorpyrifos, DEET, permethrin, pyridostigmine bromide, sulfur mustard metabolites and testosterone are available and have been utilized in carrying out the extensive experiments described in this report.
- The most complete description to date of the human metabolism of an organophosphorus toxicant has been carried out and has been published. Additional studies of human CYP3A4 polymorphisms involved in detoxication and activation of chlorpyrifos, have also been published.
- The first description of DEET metabolism by human enzymes has been accomplished and published, including identification of the CYP isoforms involved in the production of the two principal metabolites. Induction of XMEs by DEET and inhibition of DEET metabolism is also reported.
- A detailed outline of the human metabolism of permethrin has been developed and published. Monooxygenases do not appear to be involved, metabolism being initiated by hydrolysis, followed by metabolism of the resultant alcohol to the aldehyde by alcohol dehydrogenase and then to the acid by aldehyde dehydrogenase. This pathway has been confirmed by the use of purified human enzymes.
- In further studies of human metabolism of permethrin we have shown that chlorpyrifos oxon, the principal reactive metabolite of chlorpyrifos, is an inhibitor of permethrin hydrolysis in human liver preparations and that carbaryl, while less potent than chlorpyrifos, is also an inhibitor of this hydrolysis.
- It has become clear that chlorpyrifos and, by implication, other organophosphorus compounds are potent inhibitors of the metabolism of other xenobiotics. Chlorpyrifos is shown to be a potent inhibitor of the human metabolism of DEET and the insecticide carbaryl.
- We have shown that chlorpyrifos and other organophosphorus chemicals are also potent inhibitors of the metabolism of testosterone by human liver microsomes and by human CYP3A4.
- Studies of induction in mice by DEET indicate that a number of monooxygenase reactions are induced, including the activation of chlorpyrifos to chlorpyrifos oxon.

- Initial studies of induction of XMEs based on the use of human hepatocytes, indicate that this technique will be of considerable use in the investigation of metabolic interactions based on induction.
- As a result of all of the studies carried out, it is now possible to construct an overall inclusive picture of the possible metabolic interactions of a small subset of deployment-related chemicals that is based on human studies. It will be possible, based on this beginning, to expand this to include more chemicals and, perhaps of most importance, to predict possible interactions from chemicals proposed for future use.

REPORTABLE OUTCOMES

Publications (see appendices).

Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Dai, D., Goldstein, J. A. and Hodgson, E. (2001) Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse and rat liver microsomes. *Drug Metabol. Disp.* 29:12-1-1204.

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Usmani, K. A., Rose, R. L., Goldstein, J. A., Taylor, W. G., Brimfield, A. A. and Hodgson, E. (2002). In vitro human metabolism and interactions of the repellent, N,N-diethyl-m-toluamide (DEET). *Drug Metabol. Disp.* 30:289-294.

Choi, J., Rose, R. L., and Hodgson, E. (2002). In vitro human metabolism of permethrin: the role of human alcohol and aldehyde dehydrogenases. *Pesticide Biochem. Physiol.* 73:117-128.

Usmani, K. A., Rose, R. L. and Hodgson, E. (2003). Inhibition and activation of the human liver and human cytochrome P450 3A4 metabolism of testosterone by deployment-related chemicals. *Drug. Metabol. Disp.* 31:384-391.

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Choi, J., R. L. Rose and E. Hodgson. (2003). Chlorpyrifos oxon and carbaryl inhibition of trans-permethrin hydrolysis in human liver fractions. In preparation.

Choi, J., Rose, R. L. and Hodgson, E. (2003). Chlorpyrifos oxon and carbaryl inhibition of trans-permethrin hydrolysis in human liver fractions. In preparation.

Presentations.

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J. Tang, Y. Cao, R.L. Rose, A.A. Brimfield, J.A. Goldstein, and E. Hodgson. Differential activities of chlorpyrifos metabolism in human liver microsomes and cytochrome P450 isoforms. Conference on Illnesses Among Gulf War Veterans: A Decade of Scientific Research. Alexandria, VA. Jan. 24-26, 2001.

Rose, R.L., N.J. Cherrington, J. Tang, Y. Cao, D.P. Hartley, C.D. Klaassen, and E. Hodgson. Induction of CYP isoforms by pesticides and DEET as monitored by the bDNA assay. The Toxicologist 66:599.

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CONCLUSIONS

HPLC analytical methods for chlorpyrifos, DEET, permethrin, pyridostigmine and their metabolites, sulfur mustard metabolites, and testosterone have been brought on line and have been used in several metabolic studies. The human cytochrome P450 isoforms metabolizing 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 is shown to be a potent inhibitor of DEET metabolism, the metabolism of the insecticide, carbaryl, and is a potent inhibitor of the metabolism of 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 sub-populations and individuals at increased risk from anticholinergic chemicals.

Even within this very small subset of deployment-related chemicals, 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; 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 of toxicity.

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 sub-populations at greater risk from specific xenobiotic toxicants 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. We continue to explore the possibility that 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 the proposed animal studies. Our preliminary studies indicate that this is a viable approach, and it will be used, in lieu of hepatocytes from experimental animals in the continuation study recently approved for funding.

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METABOLISM OF CHLORPYRIFOS BY HUMAN CYTOCHROME P450 ISOFORMS AND HUMAN, MOUSE, AND RAT LIVER MICROSOMES

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ABSTRACT:

One of the factors determining the toxicity of chlorpyrifos (CPS), an organophosphorus (OP) insecticide, is its biotransformation. CPS can be activated by cytochrome P450 (CYP) through a desulfuration reaction to form chlorpyrifos-oxon (CPO), a potent anticholinesterase. CPS can also be detoxified by CYP through a dearylation reaction. Using pooled human liver microsomes (HLM), a $K_{m,app}$ of 30.2 μ M and $V_{max,app}$ of 0.4 nmol/min/mg of protein was obtained for desulfuration, and a $K_{m,app}$ of 14.2 μ M and a $V_{max,app}$ of 0.7 nmol/min/mg of protein was obtained for dearylation. These activities are lower than those obtained from rat liver microsomes. Gender differences in humans were also observed with female HLM possessing greater activity than male HLM. Use of human CYP isoforms expressed in human lymphoblastoma cells demonstrated

that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 are involved in CPS metabolism. CYP2B6 has the highest desulfuration activity, whereas dearylation activity is highest for 2C19. CYP3A4 has high activity for both dearylation and desulfuration. The use of phenotyped individual HLM demonstrated that predictions of metabolic activation and/or detoxication could be made based on relative amounts of CYP2B6, 2C19, and 3A4 in the microsomes. Thus, individuals with high CYP2C19 but low 3A4 and 2B6 are more active in dearylation than in desulfuration. Similarly, individuals possessing high levels of CYP2B6 and 3A4 have the greatest potential to form the activation product. These differences between individuals suggest that differential sensitivities to CPS may exist in the human population.

Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] (CPS¹) is a widely used organophosphorus (OP) insecticide. It has numerous agricultural applications and, until recently, has been used for termite control in foundations and for the control of nuisance insects and disease vectors in homes and during military deployments. The extensive use of CPS inevitably results in human exposure and has the potential to cause toxic effects.

The *in vivo* toxicity of CPS is a result of its bioactivation by cytochrome P450 (CYP)-mediated monooxygenases to a more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO). This oxidation reaction, which proceeds through a possible phosphooxythiiran intermediate, can result in either a desulfuration reaction that generates the

oxon or a dearylation reaction that degrades the parent compound (Chambers, 1992) (Fig. 1).

Studies of parathion, a related organophosphate, have shown that parathion oxidation is catalyzed by human CYP1A2, 2B6, and 3A4 and that its oxidation is highly correlated to CYP3A4 activity in human liver microsomes (HLM) (Butler and Murray, 1997; Mutch et

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¹ Abbreviations used are: CPS, chlorpyrifos; CPO, chlorpyrifos-oxon; TCP, 3,5,6-trichloro-2-pyridinol; OP, organophosphorus; HLM, human liver microsomes; RLM, rat liver microsomes; MLM, mouse liver microsomes; CYP, cytochrome P450; HPLC, high-performance liquid chromatography.

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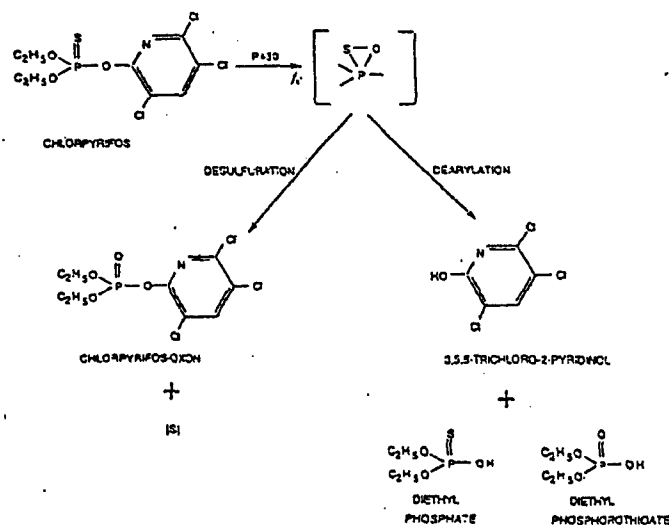


FIG. 1. Cytochrome P450-dependent metabolism of phosphorothioates illustrated with chlorpyrifos.

TABLE 1

Dearylation and desulfuration activities toward chlorpyrifos in HLM, RLM, or MLM

Activities are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations), means in RLM (pooled from three male rats) and MLM (pooled from three male mice) significantly different than HLM (pooled from 10 donors) are indicated by * $p < 0.05$ or ** $p < 0.01$.

	Desulfuration			Dearylation		
	K_m μM	V_{max} nmol/mg protein/min	V_{max}/K_m	K_m μM	V_{max} nmol/mg protein/min	V_{max}/K_m
HLM	30.2 ± 1.7	0.4 ± 0.1	0.01	14.2 ± 2.2	0.7 ± 0.1	0.05
RLM	$6.1 \pm 1.1^{**}$	$1.0 \pm 0.2^*$	0.17	$4.8 \pm 1.8^*$	$2.1 \pm 0.4^{**}$	0.43
MLM	24.4 ± 2.4	0.7 ± 0.1	0.03	14.9 ± 3.0	$2.7 \pm 0.1^{**}$	0.18

al., 1999). Other studies using specific chemical inhibitors for human CYP isoforms demonstrated that both CYP2D6 and CYP3A4 were active in the desulfuration of parathion, CPS, and diazinon (Sams et al., 2000).

The present study was designed to 1) determine oxidation activities toward CPS in human, mouse, and rat liver microsomes in the same assay system, 2) identify the human CYP isoforms and CYP polymorphic forms responsible for CPS oxidation, and 3) examine the differences in CPS oxidation activities among liver microsomes from selected individual humans.

Materials and Methods

Chemicals. CPS, CPO, and 3,5,6-trichloro-2-pyridinol (TCP) were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO).

Rodent Liver Microsome Preparation. Rat liver microsomes (RLM) and mouse liver microsomes (MLM) were prepared from adult male Long-Evans rats and adult male CD-1 mice (Charles River Laboratories, Raleigh, NC), respectively, according to the method of Cook and Hodgson (1983). Briefly, immediately after sacrificing the animals, the fresh livers were removed, weighed, minced, and then homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 1.15% potassium chloride. The homogenate was centrifuged at 10,000g for 15 min. The supernatant was filtered through glass wool and centrifuged at 100,000g for 1 h. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 0.25 M sucrose. All processes were performed at 0 to 4°C. The microsomal preparation was aliquoted and stored at -80°C until use. Protein concentration was determined using a BCA kit (Pierce, Rockford, IL).

Human Liver Microsomes and Human Cytochrome P450 Isoforms. Pooled HLM (pooled from 10 donors), individual HLM, and human lymphoblast-expressed CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1 (Arg₁₄₄, Ile₃₅₀), 2C9*2 (Cys₁₄₄), 2C19, 2D6*1 (2D6-Val), 2E1, 3A4, and 4A11 were purchased from GENTEST (Woburn, MA). Pooled male and pooled female HLM (pooled from 10 male donors and 10 female donors, respectively) were purchased from XenoTech, LLC (Kansas City, KS). Different mutant alleles of human CYP2C19 were expressed in *Escherichia coli*, according to the method of Luo et al. (1998). NADPH-CYP reductase was obtained from Oxford Biomedical Sciences (Oxford, MD).

In Vitro Chlorpyrifos Metabolism. Enzyme kinetic assays for microsomes were performed by incubation of serial concentrations of CPS (final concentration range, 2–100 μM) with microsomes in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 5 mM MgCl₂ and 3 mM EDTA for 5 min. The microsomal protein concentrations used in assays were 1.5 mg/ml for HLM, 0.5 mg/ml for RLM, and 1 mg/ml for MLM. After preincubation at 37°C for 3 min, reactions were started by the addition of an NADPH-generating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). The controls were identical except for the absence of an NADPH-generating system. Reactions were terminated by adding an equal volume of ice-cold methanol and vortexing. After 5 min of centrifugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for CPO and TCP concentrations by HPLC.

Metabolic activity assays for human lymphoblast-expressed CYP isoforms

were performed by incubation of CPS (final concentration, 100 μM) with CYP isoforms (final protein concentration, 0.9 mg/ml; final P450 contents, 23.4–180 pmol/ml) for 20 min in CYP-specific buffers recommended by the supplier (GENTEST). For CYP1A1, 1A2, 2D6, and 3A4, 100 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For CYP2B6, 2C8, 2C19, and 2E1, 50 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For CYP2C9*1, 2C9*2, and 4A11, the buffer was 100 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.5), whereas for CYP2A6, 50 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.4) was used.

The metabolic activity assays for *E. coli*-expressed human CYP2C19s were performed according to the method of Klose et al. (1998). Briefly, L- α -phosphatidylcholine (0.3 $\mu\text{g}/\text{pmol}$ of P450), CYP reductase (4 pmol/pmol of P450), and CYP2C19 (24 pmol) were combined and preincubated at 37°C for 5 min. This mixture then was incubated with 100 μM CPS in 50 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) for 10 min. The reaction was initiated with NADPH-generating system as described previously. Assays of individual HLM (final protein concentration, 1.5 mg/ml) with CPS (final concentration, 100 μM) were described previously.

Analysis of Metabolites by HPLC. The HPLC system used in this study consisted of two Shimadzu (Kyoto, Japan) pumps (LC-10AT), a Shimadzu auto injector (SIL-10AD VP), and a Waters 486 tunable absorbance detector (Milford, MA). The mobile phase for pump A was 10% acetonitrile, 89% water, and 1% phosphoric acid, whereas that for pump B was 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min. Metabolites were separated by a C₁₈ column (Luna 5 μ , 150 $\times$ 3 mm; Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm. Using this system, the retention times obtained for TCP, CPO, and CPS were 8.5, 12, and 17 min, respectively. The limits of detection for TCP and CPO were 0.03 and 0.04 μM , respectively, at an injection volume of 15 μl . Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. K_m and V_{max} were obtained using a Hanes-Woolf plot (Segel, 1975).

Statistics. Significant differences between data sets were determined by one-way analysis of variance, and multiple comparisons were performed with the Tukey-Kramer method using an SAS program (SAS, 1989).

Results

The protein concentrations and incubation times used in the assays were within linear ranges determined in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

HLM displayed lower affinity (i.e., higher K_m) and lower reaction velocity toward CPS for both desulfuration and dearylation than RLM (Table 1). Compared with HLM, MLM exhibited similar affinities but a higher reaction velocity toward CPS (Table 1). Both RLM and MLM have higher values of clearance terms (V_{max}/K_m) than HLM. Pooled female HLM showed significantly higher metabolic activity toward CPS than pooled male HLM (Table 2).

A screen of several human CYP isoforms demonstrated that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 were involved in CPS metabolism (Table 3), whereas no oxidation activity toward CPS was detected using CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Desulfuration and dearylation activities were greatest for CYP2B6

TABLE 2

Metabolic activities toward chlorpyrifos in pooled male and female HLM

Activities are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations), means in female HLM (pooled from 10 donors) significantly different than male HLM (pooled from 10 donors) are indicated by * $p < 0.05$ or ** $p < 0.01$. Protein concentration and cytochrome P450 content in microsomes are provided by the supplier (XenoTech, LLC).

	Desulfuration		Dearylation	
	nmol/mg protein/ min	nmol/nmol P450/ min	nmol/mg protein/ min	nmol/nmol P450/min
Male	0.24 $\pm$ 0.01	0.54 $\pm$ 0.01	0.47 $\pm$ 0.01	1.05 $\pm$ 0.03
Female	0.56 $\pm$ 0.02**	0.89 $\pm$ 0.02**	0.78 $\pm$ 0.02**	1.23 $\pm$ 0.04*

TABLE 3

Metabolic activities toward chlorpyrifos in human lymphoblast-expressed cytochrome P450 isoforms

Activities are expressed as nanomoles of product per nanomoles of P450 per minute $\pm$ S.E.M. ($n = 3$ determinations).

	Desulfuration*	Dearylation*	Desulfuration/Dearylation
1A2	0.39 $\pm$ 0.06*	0.23 $\pm$ 0.05*	1.70
2B6	1.59 $\pm$ 0.12*	0.47 $\pm$ 0.03*	3.38
2C9	0.32 $\pm$ 0.06*	0.90 $\pm$ 0.10 ^{ab}	0.36
2C19	0.30 $\pm$ 0.08*	2.09 $\pm$ 0.42 ^b	0.14
3A4	0.52 $\pm$ 0.13*	0.79 $\pm$ 0.24 ^{ab}	0.66

* Means in the same column followed by the same letter are not significantly different, $p < 0.01$.

and CYP2C19, respectively. Marked decreases in metabolic activity toward CPS were observed with different polymorphic alleles of CYP2C19 (Table 4).

To determine the variation range of CPS metabolism between individuals, we examined CPS metabolism from five individuals representing contrasting activities of some important CYP isoforms (Table 5). Individuals with high levels of CYP2B6 and 3A4 (HG042 and 112) had high-desulfuration activity; individuals with low levels of CYP2B6 and 3A4 (HG006, 023 and 043) had low-desulfuration activity. The dearylation pathway was more predominant in the individual (HG043) with high-CYP2C19 but low-3A4 levels. No particular increase in either metabolite was observed in the individual (HG023) with high levels of CYP2D6.

Discussion

The metabolic intrinsic clearance rates (V_{max}/K_m) indicate that liver microsomes from all three species more readily produce a detoxication product (i.e., TCP) than an activation product (i.e., CPO). These observations are similar to previous reports on rodents (Sultatos and Murphy, 1983; Ma and Chambers, 1995). The clearance rates also demonstrate that pooled HLM are less active than RLM and MLM in both desulfuration and dearylation, suggesting that less TCP and CPO are generated in the human liver than in the rodent liver immediately after exposure to CPS.

Consistent with data on other substrates provided by the supplier (XenoTech, LLC) regarding gender differences in CYP activity, pooled female HLM showed higher activities in both desulfuration and dearylation of CPS than pooled male HLM. Note that this gender difference was demonstrated using only one pool of 10 males and 10 females, respectively. It is not known whether this difference would also be true in a larger population. These data contrast with CYP activities in rats because males are more active in CPS desulfuration than females (Chambers and Chambers, 1989; Sultatos, 1991).

Our results show that human lymphoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 are responsible for both dearylation and desulfuration of CPS, whereas CYP1A1, 2A6, 2C8, 2C9*2, 2D6*1,

TABLE 4

Metabolic activities toward chlorpyrifos in CYP2C19 expressed in E. Coli

Activities are expressed as nanomoles of product per nanomoles of P450 per minute $\pm$ S.E.M., ($n = 3$ determinations). Means in the same column followed by the same letter are not significantly different, $p < 0.01$.

	Desulfuration	Dearylation
	nmol product/nmol P450	
2C19*1B	0.68 $\pm$ 0.03	6.11 $\pm$ 0.28*
2C19*8	not detected	1.66 $\pm$ 0.07 ^b
2C19*6	not detected	0.69 $\pm$ 0.05*
2C19*5	not detected	0.68 $\pm$ 0.06*

TABLE 5

Metabolic activities toward chlorpyrifos in individual human liver microsomes

Activities are expressed as mean $\pm$ S.E.M., ($n = 3-4$ determinations). Individual human liver microsomes (protein concentration, 20 mg/ml), CYP2B6, 2C19, 2D6, and 3A4 activities (pmol/mg of protein/min), represented by (S)-mephenytoin *N*-demethylase, (S)-mephenytoin 4'-hydroxylase, bufuralol 1'-hydroxylase, and testosterone 6 β -hydroxylase catalytic activities, respectively, are 3.1, 36, not detectable and 2990 for HG006 (16-year-old male), 12.2, 78.1, 160 and 4050 for HG023 (25-year-old male), 140, 3.5, 110 and 14330 for HG042 (48-year-old female), 7.4, 212, 10.6 and 3408 for HG043 (23-year-old female), and 59.1, 260.0, 23 and 17519 for HG112 (2-year-old female) (data were provided by GENTEST).

	Desulfuration*	Dearylation*
	nmol/mg protein/min	
HG006	0.09 $\pm$ 0.01*	0.35 $\pm$ 0.03*
HG023	0.16 $\pm$ 0.01*	0.31 $\pm$ 0.04*
HG042	0.74 $\pm$ 0.10 ^b *	0.67 $\pm$ 0.07 ^{ab}
HG043	0.08 $\pm$ 0.01*	0.61 $\pm$ 0.04 ^{ab}
HG112	0.67 $\pm$ 0.08 ^b	0.91 $\pm$ 0.10 ^b

* Means in the same column followed by the same letter are not significantly different, $p < 0.01$.

2E1, and 4A11 did not display detectable activities toward CPS oxidation. These results are similar to those of a parathion metabolism study (Butler and Murray, 1997), where CYP1A2, 2B6, and 3A4 were shown to have high-desulfuration activities toward parathion. CYP2B6 more readily generates the oxon, similar to phenobarbital-induced CYP2B isoforms in rodents (Fabrizi et al., 1999; Levi et al., 1988). CYP2C19 exhibits the greatest dearylation activity and relatively low-desulfuration activity. Genetic polymorphisms have been identified in CYP2C19 (Demorais et al., 1994), and differential metabolic activities toward CPS by different variants of CYP2C19 were observed in this study. Dearylation by the polymorphic CYP2C19 alleles was significantly less than that of the wild-type forms, which could influence the in vivo toxicity of CPS in individuals possessing these alleles.

CYP3A4 is also a highly active, although not the most active, isoform in CPS metabolism. The fact that CYP3A4 is the most abundant CYP isoform in human liver (Shimada et al., 1994) suggests that this isoform plays a significant in vivo role in both desulfuration and dearylation. CYP2C9*1 (Arg₁₄₄) showed some activities in both desulfuration and dearylation of CPS, whereas CYP2C9*2 (Cys₁₄₄), a single amino acid difference, showed no detectable activity toward CPS. CYP2C9*1 and 1A2, although not the most active isoforms for either desulfuration or dearylation, may play a role in vivo. A previous study using specific chemical inhibitors and human lymphoblastoid cell-expressed CYP2D6 suggested that CYP2D6 plays a significant role in desulfuration of parathion, CPS, and diazinon (Sams et al., 2000). Our work with CPS did not reveal any metabolites using human lymphoblast-expressed CYP2D6, nor did HLM with high-CYP2D6 activity (HG023) produce a significant amount of the oxon metabolite.

Potential differences in the human population with respect to CPS metabolism were further examined using different individual HLM.

Individuals with varying levels of CYP2B6, 2C19, 2D6, and 3A4 were selected to represent contrasting levels of predicted metabolic activity. Thus, individuals (HG042 and HG112) possessing high levels of CYP2B6 and CYP3A4 would be expected to possess greater ability to form the desulfuration product than those (HG006, HG023, and HG043) with lower levels of these isoforms, as observed (Table 5). Similarly, individuals with greater levels of CYP2C19 or CYP3A4, such as HG042, HG043, and HG112, would also be expected to produce more of the dearylation product than those with significantly lower levels of these isoforms (HG006 and HG023). Because individuals with contrasting levels of CYP2B6 and 3A4 were not available, we were unable to differentiate the extent of their contribution to desulfuration separately. However, based on its content in human liver, CYP3A4 should contribute significantly to both desulfuration and dearylation, as has been previously observed (Butler and Murray, 1997; Mutch et al., 1999; Sams et al., 2000).

Using our selection of five individuals, the variations between individual HLM in desulfuration of CPS were around 8-fold and that for dearylation was 3-fold and would presumably be greater if more samples were examined. For parathion, the difference between individual HLM in dearylation and desulfuration is as great as 10- and 16-fold, respectively (Butler and Murray, 1997; Mutch et al., 1999). Considerations of metabolic differences between individuals should also consider the contributions of esterases, which are also major factors determining the in vivo toxicities of OP compounds (Maxwell et al., 1987; Chambers et al., 1990; Costa et al., 1990).

In conclusion, HLM use the same pathways as RLM and MLM to metabolize CPS, although activities were generally lower in humans than in rodents. From pools of 10 individuals, female HLM displayed a higher activity in CPS metabolism than male HLM. Human lymphoblast-expressed CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 showed oxidation activities toward CPS, whereas no activities were detected for CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Although CYP2C19 and 2B6 displayed the greatest dearylation and desulfuration activities, respectively, 3A4 was highly active in both reactions. Activities of CYP2B6, 2C19, and 3A4 greatly affect CPS metabolism in HLM.

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Identification of Variants of CYP3A4 and Characterization of Their Abilities to Metabolize Testosterone and Chlorpyrifos

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ABSTRACT

CYP3A4 is the most abundant isoform of cytochrome P450 (CYP) in adult human liver. It metabolizes numerous clinically, physiologically, and toxicologically important compounds. The expression of CYP3A4 varies 40-fold in individual human livers, and metabolism of CYP3A4 substrates varies at least 10-fold in vivo. Single nucleotide polymorphisms (SNPs) in CYP3A4 were identified by direct sequencing of genomic DNA in 72 individuals from three different ethnic groups, including Caucasians, Blacks (African-Americans and African pygmies), and Asians. A total of 28 SNPs were identified, including five which produced coding changes M445T (CYP3A4*3), R162Q (CYP3A4*15), F189S (CYP3A4*17), L293P (CYP3A4*18), and P467S (CYP3A4*19). The latter four represent new allelic variants. Racial variability was observed for the frequency of individual SNPs. CYP3A R162Q was identified only in Black populations

with an allelic frequency of 4%. CYP3A4 F189S and CYP3A4 M445T were identified in Caucasians with allelic frequencies 2% and 4%, respectively. L293P and P467S were only observed in Asians at allelic frequencies of 2%. The cDNAs for the F189S, L293P, M445T, and P467S mutant alleles were constructed by site-directed mutagenesis and expressed in an *Escherichia coli* expression system. Testosterone and the insecticide chlorpyrifos were used to assess the catalytic activities of the most common CYP3A4 allele (CYP3A4*1) and its allelic variants. CYP3A4 F189S exhibited lower turnover numbers for testosterone and chlorpyrifos, while CYP3A4 L293P had higher turnover numbers for both substrates. The turnover numbers of the CYP3A4 M445T and P467S alleles to metabolize these compounds were not significantly different from those of wild-type CYP3A4.

The CYP3A genes encode the most abundant CYP enzymes in humans including CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (de Wildt et al., 1999; Gellner et al., 2001). Hepatic CYP3A4 has been estimated to metabolize ~50% of currently used drugs as well as a number of steroids, environmental chemicals, and carcinogens (Aoyama et al., 1989; Shimada et al., 1994; Thummel et al., 1996; Rebbeck et al., 1998; Guengerich, 1999). CYP3A4 is considered to be the predominant form in adult human liver. CYP3A5, a polymorphic form, is present to a variable extent in adult livers. CYP3A5 is believed to be present in the livers of approximately 20% of Caucasians, but a recent study suggests that CYP3A5 is

expressed and may predominate in more than 50% of African-Americans (Lown et al., 1994; de Wildt et al., 1999; Wandel et al., 2000; Kuehl et al., 2001). Both CYP3A4 and CYP3A5 are distributed in multiple tissues including not only liver, but also intestine and kidney (Thummel and Wilkinson, 1998; Guengerich, 1999). CYP3A7 is an isoform found in intestine, reproductive organs, and infant liver but is also present in some adult livers (Kitada et al., 1985; Schuetz et al., 1994). Recently, a new CYP3A member (CYP3A43) has been identified. CYP3A43 mRNA is found predominantly in adult prostate and is also present in multiple tissues, including liver, where it is inducible by rifampicin (Gellner et al., 2001). However, Westlind et al. (2001) using heterologous expression systems including yeast, COS-1 cells, mouse hepatic H2.35 cells, and human embryonic kidney 293 cells suggested that CYP3A43 was a non-functional isoform.

CYP3A levels fluctuate in the liver throughout the life span of an individual (Shimada et al., 1994; Oesterheld,

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ABBREVIATIONS: CYP, cytochrome P450; SNP, single nucleotide polymorphism; OPs, organophosphorus; TLC, thin layer chromatography; TCP, trichloropyridinol; CPO, chlorpyrifos-oxon; PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography; NADPH, β -nicotinamide adenine dinucleotide phosphate, reduced form; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

1998). Up to 40-fold interindividual variations in expression levels of CYP3A4 have been observed in human liver. There is an approximately 10-fold variation in metabolism of CYP3A4 substrates in vivo including the antibiotics rifampicin and ketoconazole, the calcium blocker nifedipine, and the immunosuppressant cyclosporine (Thummel and Wilkinson, 1998; Guengerich, 1999). This variation can affect drug efficacy and toxicity. CYP3A4 is inducible by drugs such as rifampicin (Kolars et al., 1992). The variable expression of CYP3A4 is at least partially due to multiple factors, including induction by drugs, endogenous compounds, and environmental chemicals, but also includes genetic factors. Recent evidence suggests that the coding region of CYP3A4 is also genetically variable (Sata et al., 2000; Eiselt et al., 2001).

CYP3A4 has also been shown to be important in the metabolism of organophosphate pesticides (OPs), such as chlorpyrifos (Tang et al., 2001) and parathion (Butler and Murray, 1997; Eaton, 2000). Chlorpyrifos is a widely used broad-spectrum OP insecticide that elicits toxicity through inhibition of acetylcholinesterase (Chambers, 1992). OPs inhibit acetylcholinesterase and exert their toxicity by causing the accumulation of the neurotransmitter acetylcholine at nerve synapses and neuromuscular junctions. These OPs are used as the phosphorothioate ($P = S$), which is a very weak inhibitor of acetylcholinesterase. However, OPs are converted in vivo from ($P = S$) to an active phosphate ester or oxon ($P = O$), which is a potent acetylcholinesterase inhibitor (Chambers, 1992) by CYP enzymes.

Genetic variations of CYP3A4 have recently been reported. A mutation in the 5'-upstream region termed *CYP3A4*1B* (A290G) was observed in 52% of African-Americans and 9.6% of Caucasians, but has not been identified in Asians (Ball et al., 1999; Rebbeck, 2000; Sata et al., 2000; Gellner et al., 2001). It was suggested to be associated with advanced stage prostate cancer in men (Rebbeck et al., 1998), yet has protective effects for secondary cancer caused by chemotherapeutic drugs for leukemia metabolized by CYP3A4, such as epipodophyllotoxins (Felix et al., 1998). However, this polymorphism does not appear to affect constitutive levels of CYP3A4 (Wandel et al., 2000). Gonzales and coworkers (Sata et al., 2000) have described two coding SNPs including *CYP3A4*2* (S222P) found only in Finnish Caucasians with an allelic frequency of 2.7%, and a single case of *CYP3A4*3* (M445T) in a Chinese population of 178 individuals. Baculovirus expressed *CYP3A4*2* protein exhibited an increase in the K_m for nifedipine but not for testosterone compared with *CYP3A4*1*. There has been limited information about the effects of a new M445T allele on metabolism (Sata et al., 2000). *CYP3A4*4* (I118V), *CYP3A4*5* (P218R), and *CYP3A4*6* (a stop codon at 285) were reported in a Chinese population with allelic frequencies of 1.4%, 0.98%, and 0.5%, and all of these variant alleles were associated with lower ratios of 6 β -hydroxycortisol to free cortisol in an in vivo study (Hsieh et al., 2001). A very recent study has identified seven new polymorphisms in European Caucasians (Eiselt et al., 2001).

To identify CYP3A4 polymorphisms, we screened for single nucleotide polymorphisms among 72 individuals from three different racial groups including ethnically diverse Caucasians, Blacks (African-Americans and African pygmies) and ethnically diverse Asians. We identified four new coding polymorphisms in CYP3A4. A bacterial cDNA expression system was used. Catalytic activities of wild-type CYP3A4

and allelic variants were compared using testosterone and the insecticide chlorpyrifos as prototypic 3A4 substrates.

Materials and Methods

Testosterone, β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), isopropyl β -D-thiogalactopyranoside, δ -aminolevulinic acid, phenylmethylsulfonyl fluoride, phosphatidylcholine, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). ^{14} C-Testosterone was purchased from Invitrogen (Boston, MA). Chlorpyrifos, chlorpyrifos-oxon (CPO), and 3,5,6-trichloro-2-pyridinol (TCP) were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Human NADPH reductase was obtained from Oxford Biomedical Research (Oxford, MI). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). *Taq* polymerase was purchased from QIAGEN (Valencia, CA). Anti-CYP3A4 antibody was obtained from GENTEST (Woburn, MA).

Direct Sequencing. Genomic DNA was obtained from 72 different human lymphoblastoid cell lines (Cornell Institute, Camden, NJ). The individuals contain the following varied racial and ethnic ancestries: 24 Africans (16 African-Americans and 8 African pygmies), 24 Asians (5 native Taiwanese, 5 mainland Chinese, 4 Melanesian, 4 Indo-Pakistani, 3 Cambodian, and 3 Japanese), and 24 Caucasians (9 from Utah, 5 Druze [Lebanon], 5 eastern European, and 5 from Moscow).

Variant Identification. The exons plus splice junctions and the 5' and 3' regions of CYP3A4 were sequenced as previously described by Shen et al. (1998). Briefly, PCR primers were located so that amplification of the genomic sequence is initiated approximately 50 nucleotides from each intron-exon boundary. This is sufficient distance for high quality sequence data to be obtained before reaching the intron/exon splice site. Appended to the 5'-end of each of the PCR primers were sequences containing the primer binding sites for the forward or reverse energy transfer DNA sequencing primers (Amersham Pharmacia Biotech, Cleveland, OH). The amplification products are directly sequenced according to the manufacturer's instructions using the DYEnamic Direct cycle sequencing kit with the DYEnamic energy transfer primers (Amersham Pharmacia Biotech). The denatured products are loaded onto ABI Prism 377 stretch DNA sequencers (Foster City, CA). "PolyPhred" (version 2.1), a software package that utilizes the output from Phred, Phrap, and Consed, was used to identify single nucleotide substitutions in heterozygous individuals (Nickerson et al., 1997; Rieder et al., 1998). A nucleotide sequence analysis program (<http://genomic.sanger.ac.uk/gf/gf1.html>) was used to predict possible new splice sites introduced by any new mutations.

Modification of CYP3A4 cDNA. CYP3A4 wild-type cDNA in the vector pUC19 was generously supplied by Frank Gonzales (National Cancer Institute, National Institute of Health). N-Terminal modification of CYP3A4 cDNA included removal of the initial 10 amino acids and conversion of the first eight amino acids of CYP3A4 into those of bovine 17 α -hydroxylase (MALLAVF). This was accomplished by PCR using sense primer: 5'-TTAGGAGGTCATATG-GCTCTGTTATTAGCAGTCTTTCTGGTGCTCCTCTAT-3', which introduced a unique restriction site for *NdeI*. The antisense primer (5'-AGCAGAAGTCTCTAGAAAAATTCAGGCTCCACTTACGGTGC-3') was used to introduce an *EcoRI* site. *NdeI* and *EcoRI* sites are unique for the expression vector pCW. Amplification of CYP3A4 ORF was accomplished by PCR with Pfu polymerase using primers described above. PCR products containing an open reading frame of CYP3A4 were digested by *NdeI* and *EcoRI* and then were subcloned into pCW. Fidelity of PCR was verified by complete sequencing of CYP3A4. Sequentially, the plasmids were transformed into *E. coli* XL1 Blue cells.

Site-Directed Mutagenesis. Five mutations containing R162Q, F189S, L293P, M445T, and P467S were made using site-directed mutagenesis. A Chameleon double-stranded site-directed mutagen-

esis kit from Stratagene (La Jolla, CA) was used to introduce single nucleotide changes (indicated in lower case and boldface): primer 5'-GGTGAGAAATCTGAGGCaGGAAGCAGAGACAGG-3', was used to produce the substitution R162Q. The primer 5'-GTGATCACTAG-CACATCATcTGGAGTGAACATCGACTC-3' was used to substitute individual nucleotide changes coding for the F189S substitution in exon 7. Primer 5'-CAAAGCTCTGTCCGATCcGGAGCTCGTG-GCCCAATC-3' introduced the substitution L293P in exon 10. Primer 5'-CTGCATTGGCAcGAGGTTTGCTCTC-3' introduced M445T in exon 12. Primer 5'-CAGAACTTCTCCTTCAAAcCTTGTA-AAGAAACACAGATCCC-3' introduces P467S in exon 12. The entire coding region, including the mutated sites, was verified by sequencing with an ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences, Foster City, CA). The entire cDNA was then excised and subcloned into a new pCW plasmid to avoid any accidental mutations in the plasmid caused by the mutagenesis procedure and expressed in *E. coli* XL1 Blue.

Expression and Partial Purification of CYP3A4s. Wild-type and variant CYP3A4 alleles were expressed in *E. coli* XL1 Blue and the allelic proteins were purified as previously described (Dai et al., 2001). Cytochrome P450 content was monitored by the reduced CO spectrum using a DW-2000 Spectrophotometer. Protein concentration was determined by the method of Lowry (Dawson and Heatlie, 1984).

Western Blot Analysis. SDS-polyacrylamide gel electrophoresis was used to separate the recombinant proteins, followed by transferring the proteins onto nitrocellulose membranes. Nonspecific binding was blocked by 10% nonfat milk for 1 h. The membranes were incubated with anti-CYP3A4 primary antibody for 1 h at room temperature. An enhanced chemiluminescent kit (Pierce, Rockford, IL) was used for immunodetection.

Testosterone Metabolism. Metabolism of testosterone by the recombinant wild-type and mutant CYP3A4 alleles was characterized. The purified recombinant CYP3A4 proteins (10 pmol) were reconstituted in with 0.4% CHAPS, 1 μ g of dioleoylphosphatidylcholine, and 40 pmol of human NADPH reductase (Oxford Biomedical Research, Oxford MI) and 20 pmol of cytochrome b_5 in 1 $\times$ HEPES buffer, pH 7.6 (50 mM HEPES, 15 mM $MgCl_2$, and 0.1 mM EDTA) a 10- μ l volume. The reconstitution mixture was preincubated at 37°C for 5 min and then diluted to a final volume of 100 μ l with 1 $\times$ HEPES containing 10 μ g of dioleoylphosphatidylcholine. The optimal conditions for this substrate were generously provided by Drs. Halpert and He at the University of Texas Medical School in Galveston Texas. The reaction mixture was preincubated at 37°C for 5 min, and the reaction initiated by addition of 10 μ l of 10 mM NADPH and terminated with 50 μ l of tetrahydrofuran. All incubations were performed in triplicate. Samples were analyzed by thin layer chromatography (TLC) using a solvent system of dichloromethane/acetone (4:1, v/v). Finally, the TLC plate was exposed to radioautography and analyzed. Turnover numbers for CYP3A4*1 and mutants were determined by counting the radioactivity of the TLC spots.

Chlorpyrifos Metabolism. CYP3A4s (100 pmol) were reconstituted with dioleoylphosphatidylcholine (3 μ g/10 pmol P450), NADPH reductase (400 pmol), and cytochrome b_5 (200 pmol) added in this order. The reaction was initiated by adding 100 μ M chlorpyrifos in 100 mM potassium phosphate buffer with 3.3 mM $MgCl_2$ (pH 7.4) with the NADPH generating system (the final concentration was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). The final assay volume was 500 μ l. The 30-min incubation was terminated by the addition of 500 μ l of ice-cold acetonitrile and vortexing. After 5 min of centrifugation at 15,000 rpm, the supernatant was analyzed for chlorpyrifos-oxon and trichloropyridinol concentrations by HPLC. The HPLC system used in this study consisted of two Shimadzu pumps (LC-10AT; Kyoto, Japan) and a Shimadzu auto injector (SIL-10AD VP). The mobile phase for pump A was 10% acetonitrile, 89% water, and 1% phosphoric acid, whereas for pump B it was 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min.

Metabolites were separated by a C_{12} column (Synergi Max 4 μ , 150 $\times$ 4.6 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm by a Waters 486 tunable absorbance detector (Milford, MA). Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve.

Statistical Analysis. All enzymatic data were analyzed by analysis of variance followed by Student's *t* test. *N* is the number of samples used in study. Differences were considered significant at *P* < 0.05.

Molecular Modeling. A molecular model was developed for the human CYP 3A4 wild-type protein using the technique of comparative/homology modeling. The polymorphism residue side chains were identified and modified in the completed wild-type model. The template structure used for development of the homology model was the solved mammalian microsomal rabbit cytochrome P450 2C5/2C3 chimeric structure (protein database entry: 1DT6) (Williams et al., 2000). The Molecular Simulations homology modeling package was used in a manual mode for development of the homology model. All molecular dynamics studies of the protein were performed using the Discover, Lifson and Hagler, Consistent Valence Force Field. The model was developed based on a multiple sequence alignment (Fig. 4) of the solved crystal structure (protein database: 1DT6) sequence with human cytochrome P450 2C8, 2C9, 2C18, 2C19, and 3A4 sequences. The multiple sequence alignment was performed manually based on the published P450 alignments of Gotoh (1992; Lewis, 1998) and D. Nelson (<http://drnelson.utmem.edu>). The model required insertion of seven small loops ranging from three to nine residues inserted using the loop generation program present within the Molecular Simulations homology program. There were no deletions. Discontinuities, steric bumps, and overlaps were resolved with molecular dynamics.

Results

Direct Sequencing. Genomic sequencing of all exons and intron-exon junctions was performed on DNA from 72 different human lymphoblastoid cell lines selected from individuals of varied racial and ethnic ancestries [24 individuals with African ancestry (16 African-Americans and 8 African pygmies), 24 Asians (5 Indo-Pakistani, 5 native Taiwanese, 5 mainland Chinese, 3 Cambodians, 3 Japanese, 3 Melanesian) and 24 Caucasians (10 from Utah in the United States, 5 Druze (Lebanon), 5 eastern Europeans, and 5 Russians)]. Twenty-eight SNPs were identified in these regions of CYP3A4 (Table 1). Eight SNPs were located in the exonic regions: R162Q, F189S, I193I, L293P, A297A, T346T, M445T, and P467S. The remaining SNPs were distributed in the 5'-upstream, introns, and 3'-flanking region. Sequencing results showed that R162Q was only detected in Black populations with an allelic frequency of 4% (African-Americans 7.1%, pygmies 0%). F189S was detected only in Caucasians with an allelic frequency of 2% (ethnic frequencies 10% in Eastern Europeans, not found in other Caucasian groups). Two SNPs were only found in Asians. L293 was found in Asians with a frequency of 2% (ethnic frequencies of 10% in Chinese, 0% in other Asian groups). P467S was also found in Asians with an allelic frequency of 2% (ethnic frequencies were 12% in Indo-Pakistani and 0% in other Asian ethnic groups). M445T was only detected in Caucasians in our study with an allelic frequency of 4% (Eastern Europeans: frequency of 10%, Caucasians from Utah 5.6%, not detected in other Caucasian ethnic groups). None of the samples was homozygous for the coding SNPs. No new putative splice sites were introduced by any of these coding, noncoding, or intron SNPs.

TABLE 1
CYP3A4 SNPs^a

SNPs	Site	Amino Acid Substitution	Nucleotide Substitution	Allelic Frequencies			Location in CYP3A4 Genome
				Africans 24	Caucasians 24	Asians 24	
				%			
1	5'-UTR		aatcc(A/G)acagc	0	0	2.1	148874
2	Intron 2		tttca(T/C)tggt	14.6	0	0	154915
3	Intron 3		agctc(T/A)tgta	0	0	2.1	155157
4	Intron 4		aactg(A/T)gttag	0	0	2.1	162802
5	Intron 5		tggtg(T/G)tggt	0	0	2.1	163198
6	Intron 6		ccagc(T/G)gcctg	0	4.2	0	163355
7	Exon 6	R162Q	gaggg(G/A)ggaag	4.2	0	0	163267
8	Intron 7		atctt(T/G)ctctc	50.0	2.1	2.2	164751
9	Intron 7		tgaga(T/C)ataaa	8.7	0	0	164781
10	Intron 7		attca(T/G)ccact	2.2	0	0	164802
11	Intron 7		tgtag(T/C)acatt	6.5	0	0	164835
12	Exon 7	F189S	atcat(T/C)tgag	0	2.1	0	164613
13	Exon 7	Silent	aacat(C/T)gactc	4.2	0	0	164626
14	Intron 9		gacac(AT/-)gtttg ^b	0	0	4.2	166813
15	Intron 10		ggatg(G/A)ataat	73	14.6	37.5	169228
16	Intron 10		cttag(C/T)aaaaa	8.3	0	0	169263
17	Intron 10		aaaaa(G/C)cataa	10.4	0	0	169307
18	Intron 10		gtttc(G/A)ttctt	2.1	0	0	170793
19	Exon 10	L293P	cgatc(T/C)ggagc	0	0	2.1	169068
20	Exon 10	Silent	gtggc(C/T)caatc	2.1	0	0	169081
21	Intron 11		aagaa(A/G)cccta	0	2.1	0	172022
22	Intron 11		accaa(C/T)gtgga	21	0	2.1	172079
23	Exon 11	Silent	ccca(C/A)tatga	0	0	2.1	170821
24	Exon 12	M445T	tgaca(T/C)gaggt	0	4.2	0	172170
25	Exon 12	P467S	tcaaa(C/T)cttgt	0	0	2.1	172235
26	3'-UTR		aaata(A/T)ccggg	4.2	0	0	175065
27	3'-UTR		gtaca(T/G)gcatt	2.1	0	0	175082
28	3'-UTR		ctgca(C/T)attaa	2.1	0	0	175416

UTR, untranslated region.

^a Accession number of CYP3A4 genomic DNA in GenBank is NG_000004.^b Deletion 2 bases (AT).

Expression and Enzymatic Assay of CYP3A4 Recombinant Alleles. The CYP3A4 alleles were inserted into the pCW expression vector, expressed in *E. coli*, and partially purified with the exception of the newly discovered R162Q allele, which we are currently attempting to express. Comparison of Western blotting (data not shown) and CO spectra of the mutants indicated that all were present as the holo-protein. Both CYP3A4*1 and all mutant CYP3A4s metabolized radioactive testosterone into 6 β -OH testosterone as the only detectable metabolite (Fig. 1). CYP3A4-P189S exhibited a lower turnover number (1.9 nmol/min/nmol) than CYP3A4*1 (7.03 nmol/min/nmol) ($P < 0.05$). Conversely, CYP3A4-L293P metabolized testosterone at a higher rate

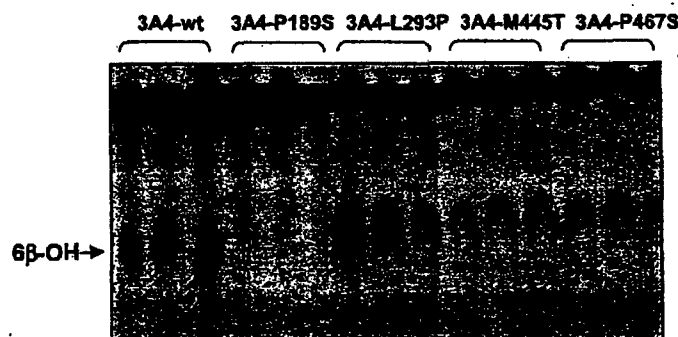


Fig. 1. Metabolism of testosterone by CYP3A4s. TLC profile of testosterone hydroxylation assay. Purified recombinant CYP3A4 wild-type and mutant proteins (10 pmol) were reconstituted and incubated with ¹⁴C-testosterone (25 μ M) for 5 min as described under Materials and Methods. Each assay was run in triplicate. 6 β -OH testosterone was the only metabolite detected in significant amounts.

(12.4 nmol/min/nmol) than CYP3A4*1 ($P < 0.05$) (Fig. 2). The turnover numbers for M445T and P457S were 5.8 and 5.9 nmol/min/nmol, respectively.

Chlorpyrifos Metabolism. The active metabolite chlorpyrifos-oxon and the inactive product trichloropyridinol were the major metabolites of chlorpyrifos by CYP3A4 (Figs. 3 and 4). The mutant allele L293P exhibited an increased turnover number for both metabolites ($P < 0.01$). In contrast, the F189S allele exhibited a lower turnover number for formation of both CPO

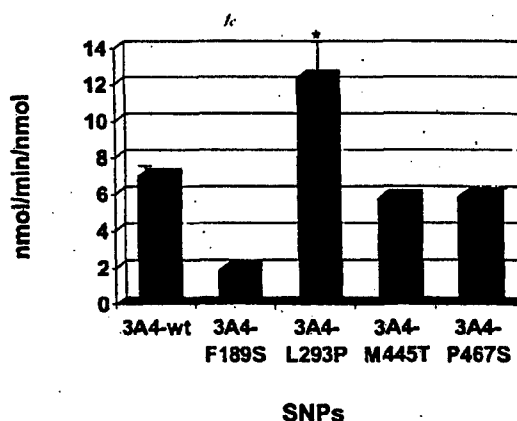


Fig. 2. Metabolism of testosterone. 6 β -OH-testosterone is the principle metabolite produced by CYP3A4*1 and its allelic variants. Purified recombinant CYP3A4 wild-type and mutant proteins (10 pmol) were reconstituted and incubated with testosterone (25 μ M) for 5 min as described under Materials and Methods. The turnover numbers for CYP3A4*1, F189S, L293P, M445T, and P457S are 7.03, 1.9 ($P < 0.05$), 12.4 ($P < 0.05$), 5.8, and 5.9 nmol/min/nmol, respectively.

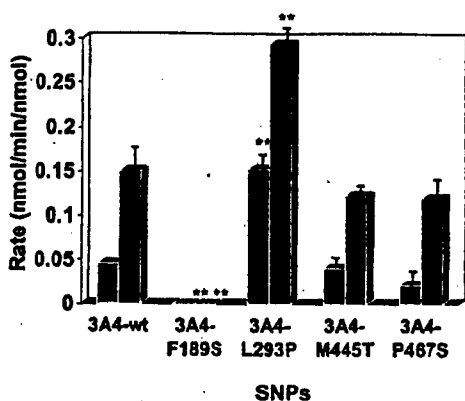


Fig. 3. Metabolism of chlorpyrifos CYP3A4 metabolizes chlorpyrifos into TCP (□) and CPO (■). CYP3A4s (100 pmol) were reconstituted and were then incubated with 100 μ M chlorpyrifos in 100 mM potassium phosphate buffer with 3.3 mM $MgCl_2$ (pH 7.4) with the NADPH generating system (the final concentration was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) for 30 min. The metabolites of chlorpyrifos-oxon (CPO) and trichloropyridinol (TCP) were analyzed by HPLC.

and TCP. The remaining two alleles did not significantly change the turnover numbers for either metabolite.

Model of CYP3A4. CYP3A4 was aligned with rabbit CYP2C5. Figure 5 shows the results of modeling CYP3A4 based on the known crystal structure of CYP2C5 (protein database entry: 1DT6). Mutations are indicated in blue. The heme is indicated in purple and the substrate testosterone in gray. The identified variants are predicted from the model to be located near the following identified structural features; R162Q is located at the end of helix D, F189S at the end of helix E, L293P is at the beginning of helix I, M445T at the beginning at helix L and P467S in a β -sheet near the C terminus of the protein. The M445T SNP is located on the other side of the heme from the ligand binding site and helix I. Side chains of residues F189, L292, and P467 are largely buried and packed into the interior of the protein. Side chains of residues R162 and M445 are largely surface exposed.

Discussion

CYP3A4 is known to metabolize many clinically important drugs, such as rifampicin, cyclosporine, and ritonavir (Kolars et al., 1992; Boxenbaum, 1999; Guengerich, 1999; Hesse et al., 2001) as well as endogenous compounds such as testosterone (Wang et al., 1997). The distribution of metabolism of CYP3A substrates is unimodal but metabolism of these sub-

strates shows at least a 10-fold variability in vivo (Thummel et al., 1996). Some variability may be due to the inducibility of the CYP3A4 gene by drugs and environmental chemicals. However, some of this variability is believed to be due to genetic factors. A previous study found a S222P allele in Finnish Caucasians and a M445T allele in Chinese (Sata et al., 2000). A very recent study identified seven new polymorphisms in European Caucasians (Eiselt et al., 2001). Two of these alleles, S222P and L373F, have been reported to affect catalytic activities toward certain substrates (Sata et al., 2000; Eiselt et al., 2001).

In the present study, we sequenced the coding regions and intron-exon junctions of CYP3A4 in DNAs from three different racial groups. Each racial group had been selected to represent ethnic diversity. A total of 28 SNPs in CYP3A4 were detected. Five SNPs produced amino acid substitutions including R162Q in exon 6; F189S in exon 7; L293P in exon 10; M445T in exon 12; and P467S in exon 12. Four of these are newly described alleles. Only the M445T allele had previously been reported (Sata et al., 2000; Eiselt et al., 2001). P189S and M445T were detected only in Caucasians in our study with frequencies of 2% and 4%, respectively. However, M445T has also been reported in Asians (Sata et al., 2000), indicating it is of ancient ancestry. The L293P and P467S alleles were found only in Asians, both at frequencies of 2% (L293 occurred in Chinese with a frequency of 10% while P467S occurred in Indo-Pakistani with a frequency of 12.5%). The coding change R162Q occurred only in African-Americans. In both individuals, this SNP was associated with an SNP in intron 10 (bp 169228) of the gene, in intron 7 (bp 164751), and in intron 11 (bp 172079). However, these intron SNPs were more frequent than the R162Q SNP in Africans. Interestingly, all alleles in African pygmies, and the majority (19/28) of alleles in African-Americans carried the SNP in intron 10 (bp 169228), which was not frequent in Caucasians. This SNP was also frequent in Asians (37.5%), indicating that it is associated with an ancient allele. The intron 7 SNP was also frequent in Africans (50% of the samples).

Testosterone and the OP insecticide chlorpyrifos were used as two examples of substrates for CYP3A4 to test effects of the coding mutations on function. The CYP3A4 active site is large (He et al., 1997), which explains its ability to metabolize a wide group of structurally diverse pharmacores (Ekins et al., 1999). Testosterone was selected as an example of a spatially large molecule that is metabolized by CYP3A4. Coding polymorphisms might potentially affect orientation of large substrates preferentially over their effects on binding of smaller substrates. Interestingly, the F189S and L293P mutations affected metabolism of both substrates in a similar fashion. The F189S allele exhibited significantly lower turnover numbers for both testosterone and chlorpyrifos than wild-type CYP3A4, while the L293P allele exhibited higher turnover numbers for both substrates. These results indicated that individuals might potentially have alterations in their ability to metabolize not only testosterone but potentially other pharmacores. Future studies will address metabolism of clinically important drugs.

Based on comparisons of the model of CYP3A4 with the crystal structure of CYP2C5, we would predict that the mutation at L293P is at the beginning of the I helix while residue F189S is at the end of helix E. These residues are not predicted to reside in the active-site cavity where they would

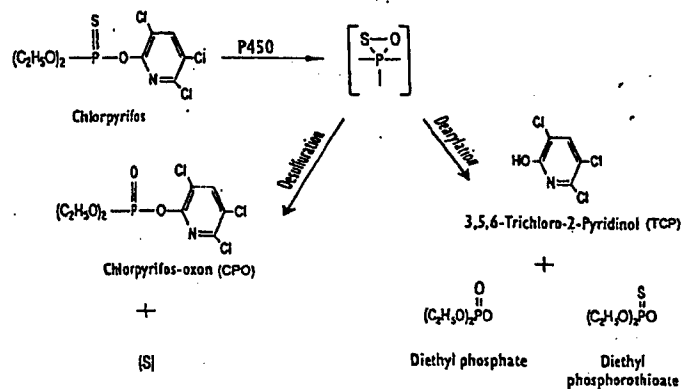


Fig. 4. Metabolic pathway of chlorpyrifos by P450.

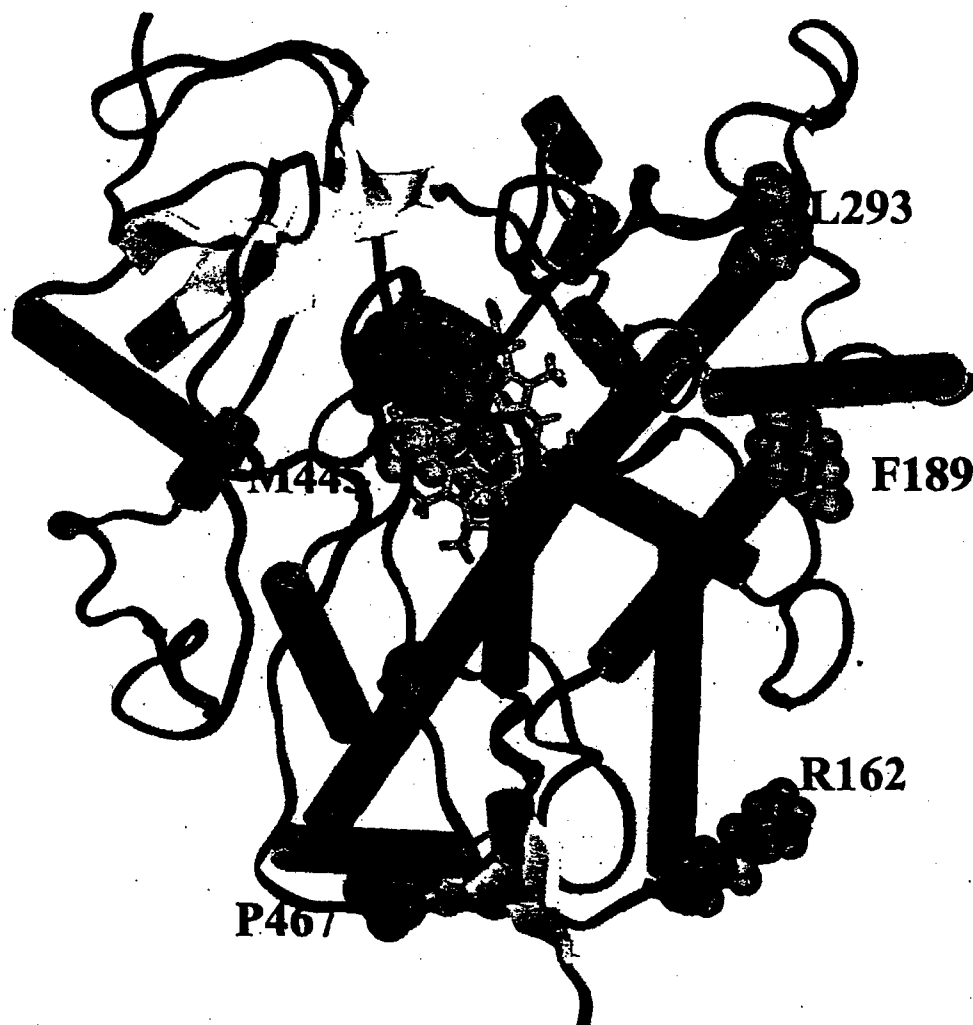


Fig. 5. Crystal structure of CYP3A4 showing locations of amino acid variants. Ribbon diagram of the molecular model of the human cytochrome P450 3A4 based on the solved x-ray crystal structure of the rabbit microsomal cytochrome P450 2C5 (protein database entry: 1DT6) (Williams et al., 2000). Complete side chains are shown in blue for the identified SNPs. The substrate testosterone is shown in gray docked in the enzyme active site. The heme is shown in purple.

directly interact with the substrate. However, the residues are nonconservative mutations in tightly packed regions, which could conceivably affect the conformation of the protein, substrate access, and/or catalytic activity. Our results indicating that the M445T mutation has no effect on testosterone or chlorpyrifos metabolism are consistent with the very recent report by Eiselt et al. (2001) using testosterone and progesterone as substrates. Modeling based on the crystal structure of CYP2C5 predicts that the M445T SNP is located in close proximity to the heme, but it is on the opposite side of the heme from the ligand binding site on helix I. This is consistent with the data indicating that this mutation may not affect catalytic activity.

CYP3A4 is important in the metabolism of environmental compounds as well as clinically important drugs. CYP3A4 is known to activate the OP insecticides parathion and chlorpyrifos into oxons that are neurotoxicants (Butler and Murray, 1997; Tang et al., 2001) (Fig. 5). CYP3A4 also inactivates chlorpyrifos into 2,3,5-trichloro-2-pyridinol. The relative rates of activation and inactivation are critical to the toxicity of the compound. The L293P allele could possibly increase

the toxicity of OP insecticides to individuals carrying this allele. Interestingly, the F189S allele decreased both activation and inactivation of chlorpyrifos and could also potentially affect toxicity after exposure to OP insecticides. In addition, CYP3A4 can activate aflatoxin B₁ into the reactive form, aflatoxin B₁-8, 9-epoxide, which is a mutagen (Gallagher et al., 1996; Chen et al., 1998). Thus polymorphisms of CYP3A4 could potentially influence the risk of different populations from various environmental compounds.

In summary, five coding polymorphisms in CYP3A4 were identified as M445T (CYP3A4*3), R162Q (CYP3A4*15), F189S (CYP3A4*16), L293P (CYP3A4*17), and P467S (CYP3A4*18) by resequencing 72 individuals from three diverse racial groups.¹ Four of these represent newly described CYP3A4 alleles. Two SNPs occurred in Caucasians (F189S

¹New CYP3A4 alleles were submitted to the CYP allele web page (www.imm.ki.se/CYPalleles). The names designated by the international allele nomenclature committee are CYP3A4*17 (F189S) and CYP3A4*18 (L292P and CYP23A4*19 (P467S). R162Q was submitted to the CYP3A4 allele web page by another laboratory while this work was in progress and was designated CYP3A4*15 but has not yet been published otherwise.

and M445T), while two occurred in Asians (L293P and P467S). M445T has also been reported previously in Asians. One coding SNP R162Q was detected only in African-Americans. Testosterone and the OP insecticide chlorpyrifos were selected to assess their catalytic activities of four new alleles. The F189L allele exhibited significantly a lower turnover number for both substrates than CYP3A4*1, while the L293P allele metabolized both substrates with a higher turnover number. Potentially, these alleles may contribute to the known variability in metabolism of clinically used drugs and environmental compounds that are CYP3A4 substrates. Future studies will examine a wider range of CYP3A4 substrates including clinically used drugs.

Acknowledgments

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IN VITRO HUMAN METABOLISM AND INTERACTIONS OF REPELLENT *N,N*-DIETHYL-*m*-TOLUAMIDE

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ABSTRACT:

Oxidative metabolism of the insect repellent *N,N*-diethyl-*m*-toluamide (DEET) by pooled human liver microsomes (HLM), rat liver microsomes (RLM), and mouse liver microsomes (MLM) was investigated. DEET is metabolized by cytochromes P450 (P450s) leading to the production of a ring methyl oxidation product, *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC), and an *N*-deethylated product, *N*-ethyl-*m*-toluamide (ET). Both the affinities and intrinsic clearance of HLM for ring hydroxylation are greater than those for *N*-deethylation. Pooled HLM show significantly lower affinities (K_m) than RLM for metabolism of DEET to either of the primary metabolites (BALC and ET). Among 15 cDNA-expressed P450 enzymes examined, CYP1A2, 2B6, 2D6*1 (Val₃₇₄), and 2E1 metabolized DEET to the BALC metabolite, whereas CYP3A4, 3A5, 2A6, and 2C19 produced the ET metabolite. CYP2B6 is the principal cytochrome

P450 involved in the metabolism of DEET to its major BALC metabolite, whereas CYP2C19 had the greatest activity for the formation of the ET metabolite. Use of phenotyped HLMs 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. Preincubation of human CYP2B6 with chlorpyrifos completely inhibited the metabolism of DEET. Preincubation of human or rodent microsomes with chlorpyrifos, permethrin, and pyridostigmine bromide alone or in combination can lead to either stimulation or inhibition of DEET metabolism.

N,N-Diethyl-*m*-toluamide, commonly known as DEET¹, is the principle active ingredient in most personal insect repellents worldwide and is highly effective against a broad spectrum of insect pests, including potential disease vectors such as mosquitoes, biting flies, and ticks (including ticks that may carry Lyme disease). DEET was first developed and patented in 1946 by the U.S. Army for use by military personnel and later registered for general public use in 1957 (Schoenig et al., 1999). Every year, approximately one-third of the U.S. population (75,000,000) uses DEET-containing insect repellent

products with DEET concentrations ranging from 10 to 100% in a variety of liquids, lotions, gels, sprays, sticks, and impregnated materials and more than 30 million packages of DEET-containing products are sold annually (Veltri et al., 1994). Approximately 230 products containing DEET are currently registered with the Environmental Protection Agency by about 70 different companies.

DEET is generally considered a benign chemical; however, isolated reports involving heavy and excessive exposure indicate a variety of toxic side effects, including toxic encephalopathy, seizure, acute manic psychosis, cardiovascular toxicity, and dermatitis (Robbins and Cherniack, 1986; Veltri et al., 1994). Because DEET was widely used during the Gulf War, it is one of several chemicals believed to have potential for possible detrimental interactions with other chemicals (Chaney et al., 1997, 1999; Haley and Kurt, 1997; McCain et al., 1997). For example, DEET has been shown to synergize seizures produced by pyridostigmine bromide and vice versa (Chaney et al., 1997, 1999). Subsequent investigations in rats have shown that DEET in combinations with pyridostigmine bromide or permethrin can lead to significant neurobehavioral deficits associated with significant inhibition of brainstem acetylcholinesterase activities (Abou-Donia et al., 2001). Mechanisms of these chemical interactions are currently unknown but may include the ability of one chemical to inhibit and/or induce the metabolism of another chemical. For example, the phosphorothioate organophosphate pesticide chlorpyrifos is capable of

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¹ Abbreviations used are: DEET, *N,N*-diethyl-*m*-toluamide; P450, cytochrome P450; BALC, *N,N*-diethyl-*m*-hydroxymethylbenzamide; ET, *N*-ethyl-*m*-toluamide; HPLC, high-performance liquid chromatography; RLM, rat liver microsomes; MLM, mouse liver microsomes; HLM, human liver microsomes; EROD, *O*-deethylation; MROD, methoxyresorufin *O*-demethylation (MROD); PROD, pentoxyresorufin *O*-dealkylation (PROD); BROD, benzyloxyresorufin *O*-dealkylation.

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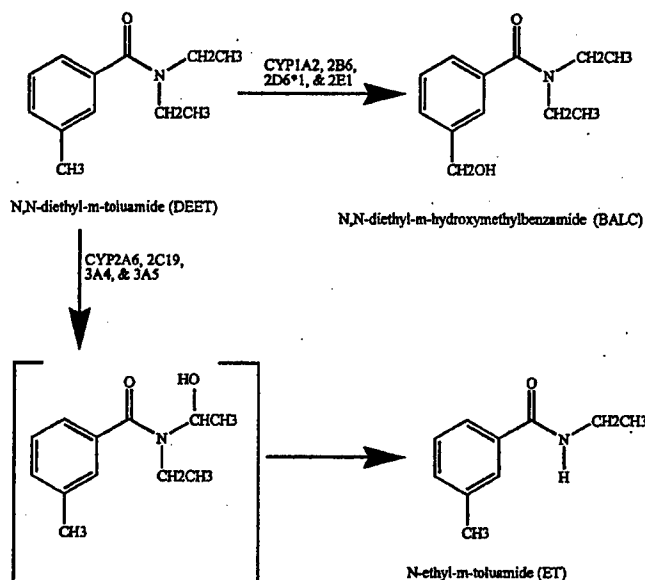


FIG. 1. Primary *in vitro* metabolites of DEET.

inhibiting metabolism of other chemicals due to its ability to irreversibly inhibit metabolizing enzymes such as cytochromes P450 (P450s) (Butler and Murray, 1997). Thus, an understanding of how DEET is metabolized in humans, the isoforms responsible for such metabolism, and the possible interactions of DEET in metabolism of other chemicals will aid in the evaluation of the possible role that DEET may play in deployment-related illnesses.

The available literature on the pharmacokinetics and metabolism of DEET has been reviewed (Qui et al., 1998). In rat, oxidative metabolism seems to account for most, if not all, of the metabolites derived from DEET. The major metabolites are *N,N*-diethyl-*m*-hydroxymethylbenzamide (BALC) and *N*-ethyl-*m*-toluamide (ET) (Fig. 1), indicating that either the *N*-ethyl or the ring methyl groups can be oxidized (Taylor, 1986; Yeung and Taylor, 1988; Taylor and Spooner, 1990; Schoenig et al., 1996; Constantino and Iley, 1999). These two major and several minor metabolites were characterized in liver microsomes from phenobarbital-pretreated rats (Taylor, 1986; Yeung and Taylor, 1988; Constantino and Iley, 1999). Studies of the metabolism of DEET by human enzymes are lacking. Studies of absorption and metabolism in humans have been conducted only at the level of determining urinary metabolites and suggest that 5 to 8% of topically applied DEET is rapidly absorbed and excreted. As many as six metabolites were recovered from the human urine samples (Selim et al., 1995).

The main objectives of the present study were to identify and quantify the oxidative metabolism of DEET by human liver microsomes and to compare the metabolism of DEET in human liver microsomes with that in rat and mouse liver microsomes. Further objectives were to identify the human P450 isoforms responsible for DEET metabolism, and to investigate potential interactions of DEET with other xenobiotics.

Materials and Methods

Chemicals. DEET, chlorpyrifos, chlorpyrifos-oxon, 3,5,6-trichloro-2-pyridinol, and permethrin (50:50 *cis-trans*) were purchased from ChemService (West Chester, PA). ET and BALC were synthesized as described previously (Taylor, 1986). Pyridostigmine bromide was purchased from Roche Molecular Biochemicals (Indianapolis, IN). HPLC grade acetonitrile and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA) and Mallinckrodt

Baker, Inc. (Paris, KY), respectively. All other chemicals were purchased, if not specified, from Sigma Chemical (St. Louis, MO).

Rodent Liver Microsome Preparation. Rat liver microsomes (RLM) and mouse liver microsomes (MLM) were prepared from adult male Long Evans rats and adult male CD-1 mice (Charles River Laboratories, Raleigh, NC), respectively, according to the method of Cook and Hodgson (1983). Briefly, immediately after sacrificing the animals, the fresh livers were excised, weighed, minced, and washed with ice-cold homogenized buffer (50 mM potassium phosphate buffer, pH 7.5; 0.1 mM EDTA; 1.15% potassium chloride). Samples were homogenized with a Polytron homogenizer in ice-cold homogenization buffer and centrifuged at 10,000g for 15 min. The supernatant was filtered through glass wool, centrifuged at 100,000g for 1 h, and the microsomal pellet was resuspended in storage buffer (50 mM potassium phosphate, pH 7.5; 0.1 mM EDTA; 0.25 M sucrose). All processes were performed at 0–4°C. The microsomal preparation was aliquoted and stored at –80°C until used.

Total cytochrome P450 content was determined by the CO-difference spectrum method of Omura and Sato (1964). Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976).

Human Liver Microsomes and Human Cytochrome P450 Isoforms. Pooled human liver microsomes (HLM) (pooled from 10 donors) and human P450 isoforms expressed in baculovirus-infected insect cells (SF9) (BTI-TN-5B1-4), CYP1A1, 1A2, 2A6, 1B1, 2B6, 2C8, 2C9*1 (Arg₁₁₄), 2C18, 2C19, 2D6*1 (Val₃₇₄), 2E1, 3A4, 3A5, 3A7, and 4A11 were purchased from GENTEST (Woburn, MA). Gender specific HLM (pooled from five male donors or five female donors, respectively) were purchased from GENTEST. Gender specific HLM (pooled from 10 male donors or 10 female donors, respectively) were also purchased from Xenotech (Kansas City, KS). Selected individual human liver microsomes (HG042, HG043, and HG095) were purchased from GENTEST.

In Vitro DEET Metabolism. Enzyme kinetic assays for microsomes were performed by incubation in 1.5-ml microcentrifuge tubes of serial concentrations of DEET (final concentrations 31.24–3000 μ M) with microsomes in 20 mM Tris-HCl buffer, pH 8.3 at 37°C, containing 5 mM MgCl₂ (final volume 1.0 ml) for 5 min. Preliminary studies demonstrated that a pH of 8.3 was optimal for the production of DEET metabolites as had previously been reported for metabolites of DEET by rat liver microsomes (Yeung and Taylor, 1988). The microsomal protein concentrations used in assays were 1.5 mg/ml for HLM, RLM, and MLM. After preincubation at 37°C for 5 min, reactions were initiated by the addition of ice-cold microsomes to prewarmed buffer/substrate/cofactors. The final concentration of the NADPH-generating system was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase. The controls were performed in the absence of the NADPH-generating system. Reactions were terminated by the addition of an equal volume of acetonitrile and vortexing. After 10-min centrifugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for BALC and ET concentrations by HPLC as described below.

The initial metabolic activity assays for human P450 isoforms were performed by incubation of DEET (final concentrations 1000 or 3000 μ M) with P450 isoforms (final P450 contents 100–200 pmol/ml) for 20 min in P450-specific buffers recommended by the supplier (GENTEST). The controls were performed in the absence of an NADPH-generating system. For CYP1A1, 1A2, 1B1, 2D6*1 (Val₃₇₄), 3A4, 3A5, and 3A7 a 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, was used. For CYP2B6, 2C8, 2C19, and 2E1 a 50 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, was used. For CYP2C9*1 (Arg₁₄₄), 2C18, and 4A11 a 100 mM Tris-HCl buffer with 3.3 mM MgCl₂, pH 7.5, was used. For CYP2A6 a 50 mM Tris-HCl buffer with 3.3 mM MgCl₂, pH 7.5, was used.

Enzyme kinetic assays for human CYP1A2, 2B6, and 2D6*1 (Val₃₇₄) were performed by incubations of serial concentrations of DEET (final concentrations 31.25–1000 μ M) (final P450 content 50 pmol) for 10 min in P450-specific buffers recommended by the supplier (GENTEST).

Assays of five pooled male and female HLM purchased from GENTEST and 10 pooled male and female HLM purchased from Xenotech were performed by incubations of DEET (final concentration 1000 μ M) with microsomes (final protein concentration 1.5 mg/ml) for 10 min. Similar conditions were used for assays of individual HLM.

TABLE 1

Kinetic parameters for the ring methyl oxidation and *N*-deethylation of DEET by pooled HLM, RLM, MLM, and DEET treated mouse liver microsomesMeans in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean $\pm$ S.E.M. ($n = 3$).

	BALC (Ring Methyl Oxidation)			ET (<i>N</i> -Deethylation)		
	K_m μM	V_{max} nmol/mg protein/min	CL_{int} $10^{-6}/mg$ protein/min	K_m μM	V_{max} nmol/mg protein/min	CL_{int} $10^{-6}/mg$ protein/min
HLM	67.6 $\pm$ 4.2 ^b	12.9 $\pm$ 1.6 ^{ab}	191.5 $\pm$ 15.4 ^b	842.5 $\pm$ 49.9 ^b	20.5 $\pm$ 3.4 ^a	24.4 $\pm$ 5.1 ^b
RLM	38.3 $\pm$ 0.2 ^a	17.6 $\pm$ 1.2 ^a	461.3 $\pm$ 23.5 ^a	214.3 $\pm$ 26.1 ^a	19.2 $\pm$ 2.8 ^a	89.5 $\pm$ 10.9 ^a
MLM	43.4 $\pm$ 0.6 ^{ab}	6.8 $\pm$ 1.4 ^b	156.8 $\pm$ 23.8 ^b	660.6 $\pm$ 59.5 ^b	14.5 $\pm$ 2.9 ^a	21.7 $\pm$ 3.9 ^b
MLM*	42.6 $\pm$ 13.6 ^{ab}	16.4 $\pm$ 3.4 ^a	385.1 $\pm$ 52.6 ^a	630.9 $\pm$ 128.0 ^b	22.9 $\pm$ 2.9 ^a	38.3 $\pm$ 5.1 ^b

* DEET treated animals LD₁₀ (200 mg/kg/day).

Induction. Adult male CD-1 mice, 28 to 30 g, were obtained from Charles River Laboratories and acclimated for 4 days. Low (2 mg/kg/day), medium (20 mg/kg/day), and high (200 mg/kg/day) doses of DEET in 100 μ l of corn oil were given intraperitoneally daily for 3 days. The dose range for DEET was selected to not exceed a dose known to produce a physiological effect (Chaney et al., 1999). Doses approximating LD₁₀ values for phenobarbital (80 mg/kg/day) in 100 μ l of water or 3-methylcholanthrene (20 mg/kg/day) in 100 μ l of corn oil were also administered intraperitoneally, to separate groups of mice, daily for 3 days. Controls were given corn oil only or water. Microsomes were prepared from livers of fed mice on the 4th day as described above.

The following substrates were used as indicators of the activities for the following isozymes: ethoxyresorufin *O*-deethylation (EROD) and methoxyresorufin *O*-demethylation (MROD) for CYP1A1/2 (Burke and Mayer, 1974; Nerurkar et al., 1993), pentoxyresorufin *O*-dealkylation (PROD) for CYP2B10 (Lubet et al., 1985), and benzyloxyresorufin *O*-dealkylation (BROD) for CYP2B (Nerurkar et al., 1993). Assays were conducted as described by Pohl and Fouts (1980). Briefly, assays were initiated by the addition of an NADPH-regenerating system and incubated for 5 min at 37°C. Product formation for EROD, MROD, PROD, and BROD activities were determined spectrofluorometrically (F-2000 fluorescence spectrophotometer; Hitachi, Tokyo, Japan) by comparison with a standard curve generated with resorufin.

Chlorpyrifos Metabolism. Chlorpyrifos metabolism activity assays were performed by incubation of 100 μ M chlorpyrifos with induced MLM for 5 min. The microsomal protein concentrations used in the assays were 1.0 mg/ml in 100 mM Tris-HCl buffer, pH 7.4 at 37°C, containing 5 mM MgCl₂ and 3 mM EDTA. Reactions were stopped by the addition of acetonitrile followed by centrifugation at 15,000 rpm. The supernatant was analyzed for chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol concentrations by HPLC as described by Tang et al. (2001).

Inhibition. The inhibition of human CYP2B6 by chlorpyrifos and chlorpyrifos-oxon were studied by incubating these two compounds (final concentration 100 μ M), CYP2B6 (50 pmol), NADPH-generating system, and 50 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, for 5 min at 37°C before adding DEET (final concentration 1000 μ M). The reaction was terminated after an additional 20 min by the addition of an equal volume of acetonitrile. After brief centrifugation the supernatant was analyzed for BALC concentration by HPLC.

To understand possible interactions produced by potential induction of metabolizing enzymes by DEET, effects on DEET metabolism were examined in HLM, MLM, and RLM after preincubations with chlorpyrifos, permethrin, and pyridostigmine bromide either alone or in combination (final concentration 100 μ M). In addition, the effects of these compounds were also examined in some mice that had been induced with phenobarbital (80 mg/kg/day), 3-methylcholanthrene (20 mg/kg/day), or the high dose of DEET (200 mg/kg/day). Microsomes (final protein concentrations 1.5 mg/ml), NADPH-generating system, and 20 mM Tris-HCl buffer with 5 mM MgCl₂, pH 8.3 at 37°C, were incubated for 5 min at 37°C before adding DEET (final concentration 1000 μ M). The reaction was terminated after 10 min by the addition of an equal volume of acetonitrile. After centrifugation at 15,000 rpm, the supernatant was analyzed for BALC and ET concentrations by HPLC.

All assays were conducted in triplicate. The protein concentrations and incubation times used in the assays were found to be in the linear range in

preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

Analysis of Metabolites by HPLC. Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan). The Shimadzu HPLC system used in this study consisted of two pumps (LC-10AT), a Shimadzu autoinjector (SIL-10AD VP), and a Waters 486 tunable absorbance detector (Waters, Milford, MA). For DEET metabolites, the mobile phase for pump A was 3.5% tetrahydrofuran, 96.5% water; for pump B 100% acetonitrile. A gradient system was used as described by Yeung and Taylor (1988) in the following manner: 0 to 3 min (10% B), 3 to 30 min (10–60% B), 30 to 32 min (60–10% B), and 32 to 35 min (10% B). The flow rate was 1 ml/min. For chlorpyrifos metabolites, the mobile phase for pump A was 10% acetonitrile, 89% H₂O and 1% phosphoric acid; for pump B 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min. Metabolites for DEET and chlorpyrifos were separated by a C₁₈ column (Luna 5 μ m, 150 $\times$ 3 mm; Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm. Under these conditions the retention times for BALC, ET, and DEET were 2.5, 5, and 11 min, respectively. The limit of detection for the two DEET metabolites was approximately 10 μ M. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. K_m and V_{max} were obtained from Lineweaver-Burk plots (1/velocity versus 1/substrate concentration).

Statistics. Significant differences between data sets were determined by one-way analysis of variance and multiple comparisons were performed with the Tukey-Kramer honestly significant different test by using a SAS program (SAS, 1989).

Results

Pooled HLM as well as RLM and MLM showed a much lower K_m (higher affinity) and higher intrinsic clearance for ring hydroxylation (BALC formation) than for *N*-deethylation (ET formation) from DEET (~10-fold differences) (Table 1). HLM also exhibited higher K_m values (lower affinities) than either RLM or MLM. HLM exhibited a lower intrinsic clearance rate [CL_{int} (V_{max}/K_m)] for both metabolites than RLM. However, the CL_{int} for MLM was similar to that of HLM. When mice were treated with the high dose (200 mg/kg/day) there was a significant increase in the V_{max} and intrinsic clearance of BALC and ET, indicating that DEET induced its own metabolism.

Among 15 different human P450 isoforms screened, only CYP1A2, 2B6, 2D6*1 (Val₃₇₄), and 2E1 displayed detectable BALC metabolite production (Table 2). The activity of CYP2E1 was significantly less than the activities of the other P450s producing the BALC metabolite. Production of the BALC metabolite was generally much higher than that of the ET metabolite. Isoforms producing detectable amounts of the ET metabolite included CYP3A4, 3A5, 2A6, and 2C19. These isoforms produced no detectable amounts of the BALC metabolite. CYP2C19 showed significantly higher activity than CYP3A4, 3A5, and 2A6. Isoforms CYP1A1, 1B1, 3A7, 2C8, 2C9*1 (Arg₁₄₄), 2C18, and 4A11 were inactive in the production of either metabolite.

Kinetic studies of CYP1A2, 2B6, and 2D6*1 (Val₃₇₄) with respect

TABLE 2

Ring methyl oxidation and *N*-deethylation activities toward DEET in human cytochrome P450 isoforms expressed in baculovirus-infected insect cells

Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean $\pm$ S.E.M. ($n = 3$).

	BALC (Ring Methyl Oxidation)	ET (<i>N</i> -Deethylation)
	nmol/nmol isoform/min	
1A2	68.94 $\pm$ 2.64 ^a	Not detected
2B6	69.51 $\pm$ 1.83 ^a	Not detected
2D6*1	56.56 $\pm$ 2.52 ^b	Not detected
2E1	3.34 $\pm$ 0.17 ^c	Not detected
2A6	Not detected	4.55 $\pm$ 0.30 ^a
2C19	Not detected	8.96 $\pm$ 0.82 ^b
3A4	Not detected	5.05 $\pm$ 0.20 ^a
3A5	Not detected	5.81 $\pm$ 0.24 ^a

to the production of the ring methyl oxidation product BALC are shown in Table 3. No significant differences in K_m , V_{max} , and CL_{int} (V_{max}/K_m) in production of the BALC metabolite were observed between CYP1A2 and 2B6. Activity of the CYP2D6 isoform was too low for accurate kinetic determinations. Comparisons of male and female differences in metabolism of DEET were performed using pooled liver microsomes from two different suppliers. Microsomes from GENTEST included five pooled males and females, whereas those from Xenotech included 10 individuals from each gender. In both cases, activity of females in the production of BALC and ET metabolites was greater than that of males; however, due to departure from randomness in the way these pooled samples were prepared the data are insufficient to demonstrate a definitive gender difference (data not shown).

To further determine the importance of CYP2B6 and 1A2 in ring methyl oxidation of DEET and, in addition, the importance of CYP3A4, 2C19, and 2A6 in *N*-deethylation of DEET, liver microsomes from three different individuals possessing varying levels of these isoforms were investigated with respect to their ability to metabolize DEET (Table 4). The individual with high levels of both CYP2B6 and 1A2 (HG042) had significantly greater ability to produce the BALC metabolite than the other two individuals. In contrast, individuals with high levels of CYP1A2 (HG043) or CYP2D6 (HG095) had significantly lower ability to metabolize DEET to the BALC metabolite, indicating the importance of CYP2B6 in formation of this metabolite. The individual with high levels of CYP3A4 and 2A6 but low level of CYP2C19 (HG042) had the highest activity for production of the ET metabolite. The individual (HG043) with the highest level of CYP2C19 but low levels of CYP3A4 and 2A6 had significantly greater ability to produce the ET metabolite than the individual (HG095) with very low levels of these isoforms.

Experiments were conducted to examine the potential of DEET to induce enzymes involved in metabolism. These experiments included the prototypical P450 inducers phenobarbital and 3-methylcholanthrene. Doses of DEET were low, medium, and high (2, 20, and 200 mg/kg/day). As expected, phenobarbital and 3-methylcholanthrene induced P450 content. Phenobarbital induced BROD (5.2-fold) and PROD (30-fold) activities, whereas 3-methylcholanthrene induced EROD (23-fold) and MROD (10.5-fold) activities. No significant levels of induction were observed for the two lower doses of DEET. In contrast, the high dose of DEET produced significant increases in BROD (3.5-fold) and in PROD activities (4.0-fold).

Studies were also conducted to examine the possible effect of DEET, phenobarbital, and 3-methylcholanthrene to induce metabolism of chlorpyrifos in mice. No significant differences were observed in the dearylation of chlorpyrifos with any treatment. However, sig-

TABLE 3

Kinetic parameters for ring methyl oxidation of DEET by human cytochrome P450 isoforms expressed in baculovirus infected insect cells

Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean $\pm$ S.E.M. ($n = 3$).

	BALC (Ring Methyl Oxidation)		
	K_m	V_{max}	CL_{int}
	μM	nmol/nmol isoform/min	10^{-6} /nmol isoform/min
CYP2B6	40.2 $\pm$ 1.2 ^a	22.3 $\pm$ 2.1 ^a	552.0 $\pm$ 40.4 ^a
CYP1A2	41.0 $\pm$ 2.0 ^a	24.5 $\pm$ 1.2 ^a	598.7 $\pm$ 39.6 ^a
CYP2D6	>1000		

TABLE 4

Ring methyl oxidation and *N*-deethylation activities toward DEET in individual human liver microsomes

BALC and ET activities are based on 5- and 20-min incubations, respectively. Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean $\pm$ S.E.M. ($n = 3$).

	BALC (Ring Methyl Oxidation)	ET (<i>N</i> -Deethylation)
	nmol/mg protein/min	
HG042*	3.30 $\pm$ 0.17 ^a	1.59 $\pm$ 0.03 ^a
HG043*	0.54 $\pm$ 0.03 ^b	1.14 $\pm$ 0.05 ^b
HG095*	0.52 $\pm$ 0.01 ^b	0.63 $\pm$ 0.03 ^c

* Individual human liver microsomes (protein concentration 20 mg/ml). CYP2B6, 2D6, 2E1, 1A2, 3A4, 2C19, and 2A6 activities (picomoles of product per milligram of protein per minute), represented by (*S*)-mephenytoin *N*-demethylase, bufuralol 1'-hydroxylase (amount of activity inhibited by 1 μM quinidine), chlorzoxazone 6-hydroxylase, phenacetin *O*-deethylase, testosterone 6 β -hydroxylase, (*S*)-mephenytoin 4'-hydroxylase, and coumarin 7-hydroxylase catalytic activities, respectively, are 270, 68, 1700, 640, 13,000, 8, and 2300 for HG042 (48-year-old female); 34, 20, 1100, 630, 5600, 700, and 850 for HG043 (23-year-old female); and 12, 160, 1200, 280, 760, 30, and 200 for HG095 (47-year-old female) (data were provided by GENTEST).

nificant increases in chlorpyrifos desulfuration activity were observed with phenobarbital (5.5-fold) and the high dose of DEET (2.8-fold).

The possibility that chlorpyrifos or chlorpyrifos-oxon may inhibit DEET metabolism by human CYP2B6 was investigated by incubating 100 μM concentration of each substrate for 5 min before addition of DEET as a substrate. Chlorpyrifos preincubation resulted in 100% inhibition of the production of the BALC metabolite, whereas for chlorpyrifos-oxon, 58% inhibition was observed.

The effects of chlorpyrifos, permethrin, and pyridostigmine bromide alone or in combination on DEET metabolism were investigated using human, rat, and mouse liver microsomes (Table 5). Preincubation of pooled HLM with permethrin, pyridostigmine bromide, and permethrin + pyridostigmine bromide significantly increased the production of BALC. Preincubation of pooled HLM with chlorpyrifos alone or in combination with any other compound significantly decreased the production of BALC. Preincubation of pooled HLM with chlorpyrifos, permethrin, pyridostigmine bromide alone or in combination showed no significant differences on the production of ET. Generally, preincubation of pooled HLM, RLM, MLM, DEET-treated MLM, phenobarbital-treated MLM, and 3-methylcholanthrene-treated MLM with chlorpyrifos alone or in combination of any other compound significantly inhibited the production of BALC and ET.

Discussion

Despite the extensive use of DEET as an insect repellent worldwide, no in vitro studies have been carried out using human enzymes and none of the rat studies have been at the level of individual isoforms. The mean metabolic intrinsic clearance rates, as estimated by V_{max}/K_m , indicate that RLM metabolize DEET more efficiently than pooled HLM or MLM and that the BALC metabolite is produced more readily than the ET metabolite.

TABLE 5

Effects of chlorpyrifos (CPS), permethrin (PMT), and pyridostigmine bromide (PYB) alone and in combination on DEET metabolism by pooled HLM, RLM, MLM, DEET-treated MLM, phenobarbital (PB)-treated MLM, and 3-methylcholanthrene (3-MC)-treated MLM

DEET High (DH) 200 mg/kg/day, PB 80 mg/kg/day, 3-MC 20 mg/kg/day. Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean $\pm$ S.E.M. ($n = 3$).

Treatment	HLM		RLM		MLM (Control)			MLM (DH)			MLM (PB)			MLM (3-MC)		
	BALC	ET	BALC	ET	BALC	ET	BALC	ET	BALC	ET	BALC	ET	BALC	ET		
nmol product formed/mg protein/min																
Control	1.1 ± 0.1 ^b	1.5 ± 0.1 ^a	4.3 ± 0.1 ^a	3.1 ± 0.2 ^a	1.3 ± 0.1 ^a	1.7 ± 0.3 ^a	2.0 ± 0.2 ^a	1.9 ± 0.1 ^a	4.8 ± 0.3 ^a	4.3 ± 0.3 ^a	1.3 ± 0.1 ^a	2.0 ± 0.1 ^a				
CPS	0.3 ± 0.0 ^c	1.4 ± 0.0 ^a	1.1 ± 0.1 ^c	1.6 ± 0.1 ^{bc}	0.2 ± 0.0 ^b	0.8 ± 0.2 ^b	0.3 ± 0.1 ^b	1.0 ± 0.0 ^b	1.0 ± 0.2 ^b	1.6 ± 0.3 ^b	0.6 ± 0.1 ^b	1.0 ± 0.2 ^b				
PMT	1.7 ± 0.1 ^a	1.6 ± 0.1 ^a	2.2 ± 0.0 ^b	2.3 ± 0.1 ^{ac}	0.9 ± 0.2 ^a	1.4 ± 0.2 ^a	1.8 ± 0.4 ^a	2.4 ± 0.5 ^a	2.5 ± 0.2 ^a	3.4 ± 0.3 ^a	0.8 ± 0.1 ^{ac}	1.7 ± 0.2 ^a				
PYB	1.8 ± 0.1 ^a	2.1 ± 0.1 ^a	3.6 ± 0.2 ^a	3.0 ± 0.1 ^a	1.2 ± 0.2 ^a	1.8 ± 0.2 ^a	2.2 ± 0.3 ^a	2.8 ± 0.5 ^a	2.9 ± 0.3 ^a	3.0 ± 0.2 ^a	1.2 ± 0.1 ^{ac}	1.8 ± 0.1 ^a				
CPS + PMT	0.6 ± 0.0 ^c	1.6 ± 0.3 ^a	1.1 ± 0.2 ^c	1.4 ± 0.0 ^{bc}	0.3 ± 0.0 ^b	0.8 ± 0.1 ^b	0.3 ± 0.0 ^b	0.9 ± 0.1 ^b	0.4 ± 0.1 ^b	1.1 ± 0.1 ^b	0.5 ± 0.1 ^b	0.7 ± 0.1 ^b				
CPS + PYB	0.4 ± 0.0 ^c	1.3 ± 0.1 ^a	1.5 ± 0.4 ^{bc}	2.0 ± 0.3 ^a	0.2 ± 0.0 ^b	0.8 ± 0.1 ^b	0.2 ± 0.1 ^b	0.8 ± 0.2 ^b	0.5 ± 0.0 ^b	1.3 ± 0.1 ^b	0.6 ± 0.0 ^b	0.9 ± 0.0 ^b				
PMT + PYB	1.6 ± 0.1 ^a	1.6 ± 0.1 ^a	2.5 ± 0.1 ^b	2.4 ± 0.2 ^{ac}	0.9 ± 0.0 ^a	1.7 ± 0.2 ^a	1.7 ± 0.5 ^a	2.4 ± 0.6 ^a	2.6 ± 0.2 ^a	3.2 ± 0.3 ^a	0.6 ± 0.1 ^b	1.1 ± 0.1 ^b				
CPS + PMT + PYB	0.6 ± 0.0 ^c	1.4 ± 0.1 ^a	0.9 ± 0.1 ^c	1.3 ± 0.0 ^{bc}	0.2 ± 0.1 ^b	0.8 ± 0.1 ^b	0.2 ± 0.0 ^b	1.0 ± 0.1 ^b	0.9 ± 0.2 ^b	1.5 ± 0.1 ^b	0.3 ± 0.0 ^a	0.9 ± 0.1 ^b				

nmol product formed/mg protein/min

A previous study using rat liver microsomes demonstrated a significant gender difference in the metabolism of DEET with males having nearly 3-fold greater metabolizing ability than females (Yeung and Taylor, 1988). Although some pooled female HLM showed significantly higher activities in BALC and ET production from DEET than pooled male HLM the results could not be regarded as definitive as noted above. Further research with large randomly selected pooled male and female HLM or microsomes from individuals is required to better understand possible gender differences in humans.

It is of interest that although eight different isoforms are capable of metabolizing DEET, each isoform produced only one metabolite. Results from individual incubations of DEET with various P450 isoforms suggested that CYP1A2 and 2B6 were highly active in production of the BALC metabolite, whereas CYP3A4, 2C19, and 2A6 were important in the formation of the ET metabolite. Inasmuch as different individuals have varying levels of each of these isoforms, we selected microsomes from individuals possessing widely varying activities of these isoforms to represent contrasting levels of predicted metabolic activity. As expected, the individual with high levels of CYP2B6 and 1A2 had the greatest ability to produce the BALC metabolite. In contrast, individuals possessing high levels of either CYP3A4 or 2C19 had the greatest levels of the ET metabolite production, whereas the individual with the lowest levels of these isoforms had the lowest ET metabolite production. Based on these results it seems reasonable to suggest that individuals, regardless of gender, with varying activities of these P450 isoforms, will be more or less efficient in the metabolism of DEET.

To determine which P450 isoforms were inducible by DEET, substrate-specific assays were conducted using microsomes from DEET-treated mice. DEET treatment did not induce CYP1A1/1A2 as determined from EROD and MROD activity measurement. In contrast, PROD and BROD activities, measures of CYP2B isoform activity, significantly increased in the animals treated with the highest dose of DEET. These induction studies corroborated our results, which indicated that CYP2B6, similar to phenobarbital-induced CYP2B isoforms in rodents (Levi et al., 1988; Fabrizio et al., 1999), is one of the most important P450 isoforms in ring methyl oxidation of DEET and, furthermore, that DEET induces its own metabolism.

Tang et al. (2001) reported that CYP2B6 has the highest activity for desulfuration of chlorpyrifos, an activation process. In this study, mice treated intraperitoneally with the highest dose of DEET had significant induction of CYP2B. This induction was demonstrated to result in increased chlorpyrifos desulfuration (2.8-fold), resulting in the production of the activation product, chlorpyrifos oxon. The lower doses of DEET were not effective inducers of CYP2B6. Although these results may imply that DEET could increase organophosphate toxicity, the high levels necessary to produce the effects, combined with the mode of administration, might suggest that risks through epidermal exposures to humans may be minimal. On the other hand, preincubation of chlorpyrifos with CYP2B6 resulted in complete inhibition of DEET metabolism to the BALC metabolite. Thus, chlorpyrifos exposure could inhibit the subsequent metabolism of DEET in humans by inhibiting the isoforms involved in DEET metabolism.

Serious side effects caused by drug-drug interactions have been reported (Guengerich, 1997). Either inhibition or induction can modulate the activity of an enzyme; P450s may exhibit stimulation (positive cooperativity) in the presence of certain xenobiotic compounds (Guengerich, 1997; Szklarz and Halpert, 1998). Our data show stimulation of pooled HLM, wherein conversion of DEET to BALC increased significantly in the presence of permethrin, pyridostigmine bromide, and permethrin + pyridostigmine bromide. However, no stimulation was observed with RLM, MLM, and MLM induced with

DEET, phenobarbital, or 3-methylcholanthrene in the presence of permethrin, pyridostigmine bromide, and permethrin + pyridostigmine bromide. Inhibition in a sense may be considered more serious than enzyme induction because inhibition happens quickly, not taking time to develop, as with induction (Guengerich, 1997). Our data show the inhibition of pooled HLM, RLM, MLM, and MLM induced with DEET, phenobarbital, or 3-methylcholanthrene, wherein conversion of DEET to its metabolites, BALC and ET, decreased significantly in the presence of chlorpyrifos alone or in combination of any other tested compound.

In summary, the present investigation has demonstrated that human liver microsomes appear to have generally lower activities for DEET metabolism than those from rodent livers. A screen of human P450 isoforms demonstrated that different sets of isoforms are responsible for the production of each metabolite (BALC and ET). Individuals with varying levels of activities of these human P450 isoforms, regardless of gender, are more or less active in their metabolism of DEET. DEET induction studies, conducted in mice, demonstrate that exposure could result in the induction of CYP2B_s, resulting in potential interactions with other chemicals such as chlorpyrifos. MLM from DEET-treated mice metabolized chlorpyrifos more readily to chlorpyrifos-oxon, a potent anticholinesterase, than control MLM. Preincubation of chlorpyrifos alone with CYP2B₆ completely inhibited the metabolism of DEET, whereas preincubation of microsomes with chlorpyrifos, permethrin, and pyridostigmine bromide alone or in combinations may lead to either stimulation or inhibition of DEET metabolism.

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In vitro metabolism of carbaryl by human cytochrome P450 and its inhibition by chlorpyrifos

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Abstract

Carbaryl is a widely used anticholinesterase carbamate insecticide. Although previous studies have demonstrated that carbaryl can be metabolized by cytochrome P450 (CYP), the identification and characterization of CYP isoforms involved in metabolism have not been described either in humans or in experimental animals. The *in vitro* metabolic activities of human liver microsomes (HLM) and human cytochrome P450 (CYP) isoforms toward carbaryl were investigated in this study. The three major metabolites, i.e. 5-hydroxycarbaryl, 4-hydroxycarbaryl and carbaryl methylol, were identified after incubation of carbaryl with HLM or individual CYP isoforms and analysis by HPLC. Most of the 16 human CYP isoforms studied showed some metabolic activity toward carbaryl. CYP1A1 and 1A2 had the greatest ability to form 5-hydroxycarbaryl, while CYP3A4 and CYP1A1 were the most active in generation of 4-hydroxycarbaryl. The production of carbaryl methylol was primarily the result of metabolism by CYP2B6. Differential activities toward carbaryl were observed among five selected individual HLM samples with the largest difference occurring in the production of carbaryl methylol. Co-incubations of carbaryl and chlorpyrifos in HLM greatly inhibited carbaryl metabolism. The ability of HLM to metabolize carbaryl was also reduced by pre-incubation of HLM with chlorpyrifos. Chlorpyrifos inhibited the generation of carbaryl methylol, catalyzed predominately by CYP2B6, more than other pathways, correlating with an earlier observation that chlorpyrifos is metabolized to its oxon primarily by CYP2B6. Therefore, carbaryl metabolism in humans and its interaction with other chemicals is reflected by the concentration of CYP isoforms in HLM and their activities in the metabolic pathways

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1. Introduction

Carbaryl (1-naphthol *N*-methylcarbamate, CAS No. 63-25-2) is a broad spectrum carbamate insecticide with a variety of agricultural and non-agricultural applications. Due to its wide use, humans may be exposed to its residues through food and other routes [1]. The mechanism underlying the toxicity of carbamate pesticides is its anticholinesterase activity [2], the inhibition of cholinesterase causing accumulation of acetylcholine in synapses, resulting in malfunction of the nervous system. 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 limitations of the analytical techniques then available [3]. However, the importance of oxidative pathways had been shown earlier with the demonstration of NADPH-dependent metabolic activity toward carbamates in rat liver microsomes [4,5]. 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, which are subject to further conjugation, such as sulfate and glucuronic acid conjugates of 1-naphthol and 4-hydroxycarbaryl [6]. The major hydroxylation products include 5-hydroxycarbaryl (5-hydroxy 1-naphthyl *N*-methylcarbamate, CAS No. 5721-72-2), 4-hydroxycarbaryl (4-hydroxy 1-naphthyl *N*-methylcarbamate, CAS No. 5266-97-7) and carbaryl methylol (1-naphthyl *N*-(hydroxymethyl)carbamate, CAS No. 5266-96-6) [7–9] (Fig. 1). 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 [10,11]. It has been shown [11] that chickens had higher clearance rate for carbaryl than rats although they have lower carboxylesterase and A-esterase activities than rats, suggesting that these esterases did not contribute to the difference between chickens and rats. An inhibition study in rats and humans showed that the CYP inhibitor cimetidine reduced the metabolism of carbaryl, again suggesting that CYP play a major role in carbaryl metabolism [10].

Although carbaryl itself is an anticholinesterase, some hydroxylation products have been shown to be more active than the parent compound. It has been shown [12] 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 [3,13].

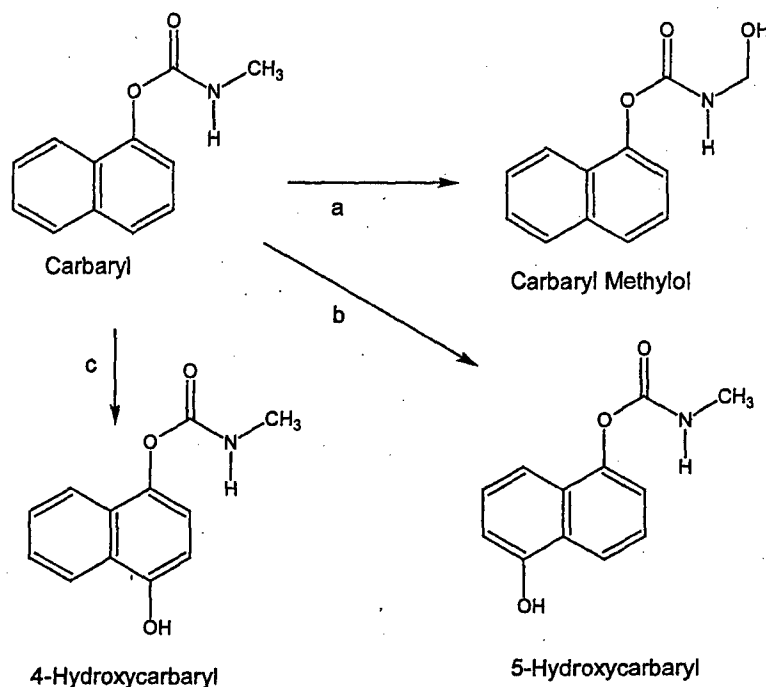


Fig. 1. Cytochrome P450-dependent metabolism of carbaryl: (a) methyl hydroxylation, (b) 5-hydroxylation, (c) 4-hydroxylation.

Both carbamate and organophosphorus insecticides are metabolized by CYP [6] and act as anticholinesterases [2]. It is possible, therefore, 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 [14], 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 [15].

Although the hepatic metabolism of carbaryl in humans has been previously investigated *in vitro* [9,16], contributions of CYP isoforms to the metabolic pathways have not been elucidated. Knowledge of the varying contributions of CYP isoforms to carbaryl metabolism will enable better understanding of differences in metabolism among individuals as well as among ethnic groups or populations and will provide important information relative to metabolic interactions of carbaryl with other chemicals. The present *in vitro* study was designed to examine: (1) oxidation activities toward carbaryl in pooled human liver microsomes (HLM); (2) human cytochrome P450 (CYP) isoforms responsible for metabolism of carbaryl; (3) potential differences in oxidation activities among individual HLMs; and (4) effects of pre- or co-incubation of chlorpyrifos on carbaryl metabolism.

2. Materials and methods

2.1. Chemicals

Carbaryl was purchased from ChemService (West Chester, PA). 5-Hydroxycarbaryl, 4-hydroxycarbaryl, carbaryl methylol and desmethylcarbaryl were obtained from the late Dr W.C. Dauterman. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO).

3. HLM and human CYP isoforms

Pooled and individual HLM and human CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C9*2, 2C9*3, 2C18, 2C19, 2D6*1, 2D6*10, 2E1, 3A4, 3A5, and 4A11 SupersomesTM were purchased from Gentest, a BD Biosciences Company (Woburn, MA). Individual HLM obtained from Gentest were from donors HG6, HG23, HG42, HG43 and HG112. CYP1A2, 2B6, 2C19, and 3A4 activities (pmol product/mg protein per min, represented by phenacetin *O*-deethylase, (*S*)-mephenytoin *N*-demethylase, (*S*)-mephenytoin 4'-hydroxylase, and testosterone 6 β -hydroxylase catalytic activities, respectively) in these individuals are 770, 3.1, 36, 2990 in HG6 (16-year-old male), 770, 12.2, 78.1, 4050 in HG23 (25-year-old male), 550, 140, 3.5, 14530 in HG42 (48-year-old female), 356, 7.4, 212, 3408 in HG43 (23-year-old female), 244, 59.1, 260, 17519 in HG112 (2-year-old female) (data were provided by GENTEST). Data for activity of CYP1A1 in these individuals were not available.

3.1. *In vitro* assay of carbaryl metabolism

Activity assays were performed by incubation of a single concentration of carbaryl (final concentration 500 μ M) with microsomal enzymes (1 mg protein for HLM, 18–50 pmol P450 for CYP isoforms) in the presence of an NADPH-generating system (final concentrations: 0.25 mM NADP, 2.5 mM glucose-6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) for 15 min. The buffer used for HLM was 100 mM Tris (pH 7.4) containing 5 mM MgCl₂. Buffers used for CYP isoforms were those recommended by Gentest, i.e. 100 mM potassium phosphate (pH 7.4) with 3.3 mM magnesium chloride for CYP1A1, 1A2, 2D6*1, 2D6*10, 3A4, 3A5; 50 mM Tris (pH 7.4) with 3.3 mM magnesium chloride for CYP2A6; 50 mM potassium phosphate (pH 7.4) with 3.3 mM magnesium chloride for CYP2B6, 2C8, 2C19, 2E1; 100 mM Tris (pH 7.5) with 3.3 mM magnesium chloride for CYP2C9*1, 2C9*2, 2C9*3, 2C18, 4A11.

Kinetic assays were performed by incubation of a series of concentrations of carbaryl (final concentrations 10–1000 μ M) with enzymes for 15 min under the same conditions described above. Preliminary experiments indicated that the 15 min was the most appropriate incubation time.

Reactions were initiated by the addition of the enzyme. Controls were performed in the absence of the NADPH-generating system. The reaction was terminated by the addition of ice-cold acetonitrile followed by vortexing. The supernatant after centrifugation (10 min at $21\,000 \times g$) was analyzed by HPLC.

To determine the potential role of esterase enzymes in carbaryl metabolism, incubations of carbaryl (final concentration $125\ \mu\text{M}$) were conducted in buffer only (0.1 M Tris-HCl/5 mM MgCl_2 , pH 7.4), pooled HLM (1 mg protein) in buffer, or pooled human liver cytosol (2 mg protein) in buffer for 15 min (assay volume $500\ \mu\text{l}$). No cofactors were added to these incubations.

3.2. Assay of carbaryl metabolism in the presence of chlorpyrifos

Varying concentrations of chlorpyrifos (0.25 – $125\ \mu\text{M}$) were added to one of two concentrations of carbaryl (10 and $500\ \mu\text{M}$) to assess the ability of chlorpyrifos to inhibit metabolism. Co-incubation was initiated by the addition of enzyme into a reaction mixture containing both chlorpyrifos and carbaryl in the presence of the NADPH-generating system and terminated after 15 min incubation.

Pre-incubation was initiated by the addition of enzyme into a reaction mixture containing chlorpyrifos in the presence of the NADPH-generating system. After 30 min pre-incubation, the reaction was started by the addition of carbaryl. The reaction was terminated after 15 min incubation.

3.3. Analysis of metabolites by HPLC

Carbaryl and its metabolites were separated by a Synergi Max C12 column (Phenomenex, Rancho Palos Verdes, CA) using a gradient HPLC system containing two pumps (Shimadzu LC-10AT). The mobile phase for pump A was 10% acetonitrile, 10% methanol and 80% water containing 3.5 mM tetrabutyl ammonium, for pump B 90% acetonitrile and 10% methanol. The flow rate was 1 ml/min. The gradient system was initiated at 10% of pump B and increased linearly as follow: 15% of pump B in 15 min, 35% of pump B through 25 min, and 35% of pump B till 35 min.

Using this gradient, carbaryl and its metabolites were well separated with baseline separation. Retention times for 5-hydroxycarbaryl, 4-hydroxycarbaryl, carbaryl methylol, desmethyl carbaryl, carbaryl, and 1-naphthol were 14, 16, 18, 22, 27, and 29.5 min, respectively. The limit of detections were 0.1, 0.6 and $0.9\ \text{pmol}$ for 5-hydroxycarbaryl, 3-hydroxycarbaryl and carbaryl methylol, respectively. The concentration ranges used for calibration were 0.039 – $5\ \mu\text{M}$ for 5-hydroxycarbaryl and 0.156 – $20\ \mu\text{M}$ for 3-hydroxycarbaryl, carbaryl methylol and 1-naphthol.

In incubations containing chlorpyrifos, the gradient was the same as described above before 35 min and then increased from 35% pump B at 35 min to 100% pump B at 36 min and maintained at 100% pump B until 50 min for elution of trichloropyridinal, chlorpyrifos oxon and chlorpyrifos at retention times of 30, 40 and 41 min.

Carbaryl, chlorpyrifos and their metabolites were detected by a Waters 486 Tunable Absorbance Detector at 290 nm (the maximal absorption for carbaryl and its metabolites produced) and identified by comparing retention times with standards. Concentrations of metabolites were obtained by extrapolation of peak area from a standard curve.

3.4. Enzyme kinetic calculations and statistics

Apparent K_m and V_{max} were determined by non-linear regression using a GRAPHPAD PRISM software and K_i was determined using a Dixon plot [17]. All values are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations). Significant differences between data sets were determined by one-way analysis of variance using an SAS program [18].

4. Results

Four metabolites were detected after incubation of carbaryl with pooled HLM in the presence of an NADPH regenerating system: 5-hydroxycarbaryl, 4-hydroxycarbaryl, carbaryl methylol, and 1-naphthol. The first three metabolites were generated only in the presence of an NADPH regenerating system and, therefore, were products of CYP-mediated reactions. The enzyme kinetics of CYP-mediated carbaryl metabolism indicates that 4-hydroxylation had the highest V_{max} , followed by methyl hydroxylation, while 5-hydroxylation had much lower V_{max} (Fig. 2 and Table 1). Methyl hydroxylation had the lowest K_m , while both ring hydroxylations had similar K_m , and, thus, methyl hydroxylation had the highest clearance rate (Table 1).

1-Naphthol, the fourth metabolite, was a product of hydrolysis because there were no significant differences in its generation in pooled HLM either in the presence or absence of the NADPH regenerating system. In the absence of the NADPH

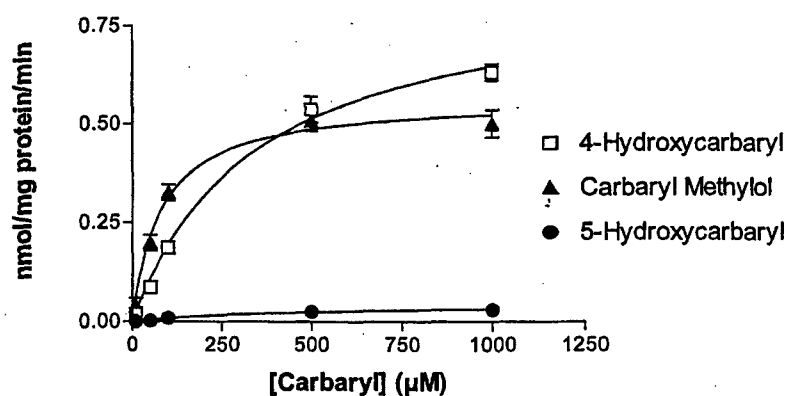


Fig. 2. Reaction velocities of carbaryl metabolism in HLM. Values are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations).

Table 1

Apparent K_m (μM) and V_{max} (nmol/mg protein per min) for carbaryl metabolism in pooled HLM (pooled from ten donors)

Metabolites	V_{max}	K_m	V_{max}/K_m
5-Hydroxycarbaryl	0.04 ± 0.01	349 ± 65	0.0001
4-Hydroxycarbaryl	0.87 ± 0.05	349 ± 57	0.0025
Carbaryl methylol	0.57 ± 0.03	81 ± 15	0.0070

Values are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations). V_{max}/K_m is expressed as ml/mg protein per min.

regenerating system, 1-naphthol was generated at a rate of 0.034 ± 0.006 nmol/mg protein per min after incubation of 125 μM carbaryl with pooled HLM. The potential hydrolysis of carbaryl by esterases in the cytosolic fraction was also examined. 1-Naphthol was the only carbaryl metabolite detected and was generated at a rate of 0.008 ± 0.001 nmol/mg protein per min at 125 μM carbaryl.

From five selected individual HLM, differences in generating 5-hydroxycarbaryl or 4-hydroxycarbaryl were only 2-fold among individuals and did not correlate with any single CYP isoform activity. The differences in generating carbaryl methylol were 5-fold between the lowest and the highest in these five individuals and were, apparently, correlated with the activity of CYP2B6 (Table 2).

Activities of CYP isoforms in metabolizing carbaryl are shown in Fig. 3. Of 16 CYP isoforms investigated (i.e. CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C9*2, 2C9*3, 2C18, 2C19, 2D6*1, 2D6*10, 2E1, 3A4, 3A5, 4A11), only CYP2D6*10 and 4A11 had no detectable activity toward carbaryl. All the other CYP isoforms were active in generating all three metabolites, although the extent of metabolism and the ratios of metabolites varied widely among isoforms. CYP1A1, 1A2, 2B6, 2C19, and 3A4 were among those most active in carbaryl metabolism. Their role toward carbaryl metabolism in microsomes may be estimated based on their clearance values (V_{max}/K_m) (Table 3) and their concentrations in liver microsomes.

The metabolism of carbaryl was inhibited by both co- and pre-incubation with chlorpyrifos (Fig. 4). Methyl hydroxylation (generating carbaryl methylol) was the pathway inhibited most significantly by chlorpyrifos while there was little effect of chlorpyrifos on 5-hydroxylation (generating 5-hydroxycarbaryl). Use of the Dixon

Table 2

Metabolic activity (nmol/mg protein per min) toward carbaryl in individual HLM

Individuals	5-Hydroxycarbaryl	4-Hydroxycarbaryl	Carbaryl methylol
HG6	0.02 ± 0.01	0.40 ± 0.02	0.17 ± 0.01
HG23	0.03 ± 0.01	0.48 ± 0.03	0.21 ± 0.02
HG42	0.04 ± 0.01	0.48 ± 0.12	0.93 ± 0.19
HG43	0.03 ± 0.01	0.31 ± 0.03	0.18 ± 0.02
HG112	0.05 ± 0.01	0.63 ± 0.05	0.72 ± 0.06

Activities are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations).

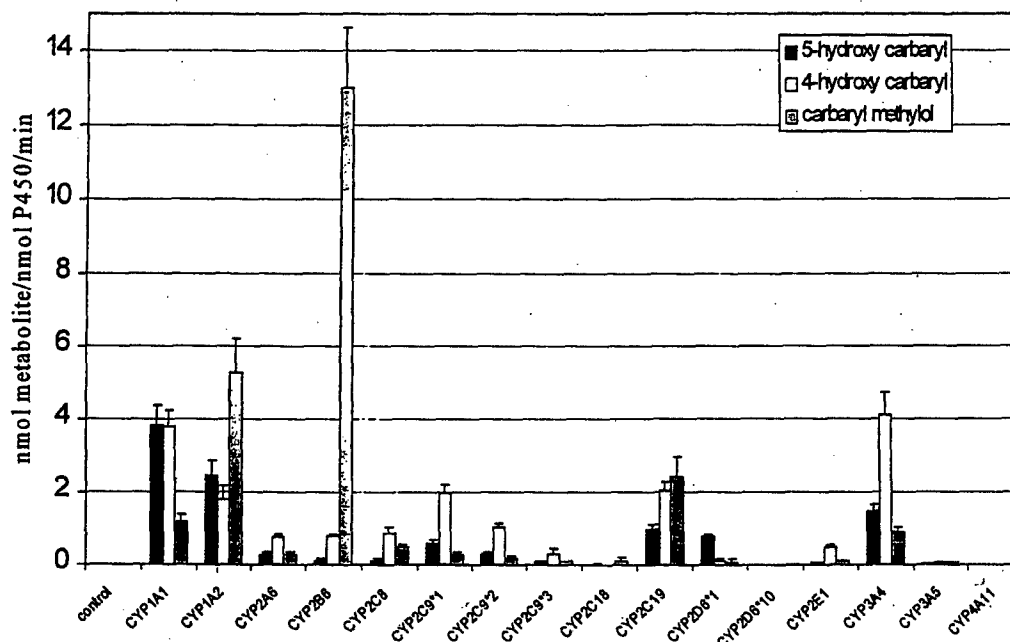


Fig. 3. Metabolic activities (nmol of product/nmol cytochrome P450/min $\pm$ S.E.M., $n = 3$ determinations) toward carbaryl in human cytochrome P450 isoform SupersomesTM.

plot produced a K_i of 149.9 μ M for chlorpyrifos inhibition of 4-hydroxylation of carbaryl and a K_i of 2.17 μ M for inhibition of methyl hydroxylation (Fig. 5).

5. Discussion

In this in vitro study, 5-hydroxy-, 4-hydroxycarbaryl and carbaryl methylol were identified as the major metabolites of carbaryl produced by HLM. 1-Naphthol was not, apparently, a major product of CYP-catalyzed reactions because there was no significant difference in the small amount of 1-naphthol production in the presence or absence of the NADPH-generating system. Other hydroxylation products of carbaryl have been reported previously in non-human species [19]. Presumably they represent minor products or are not formed in HLM since only the three metabolites discussed were found in the current study. Production of 1-naphthol in the absence of NADPH-generating system is minimal, suggesting hydrolysis of carbaryl in both microsomes and cytosol is not the major pathway of carbaryl metabolism. This result is consistent with the suggestion that hydrolysis is not favored in carbaryl metabolism because of generating a more lipophilic product (1-naphthol) [6].

Our data show that CYP isoforms are differentially active in metabolic pathways of carbaryl, CYP1A1, 1A2, 2B6, 2C19, and 3A4 being the most active isoforms in human metabolism of carbaryl. CYP1A1 and 1A2 had the greatest ability to form 5-hydroxy carbaryl, while CYP3A4 and CYP1A1 were the most active in generation of 4-hydroxy carbaryl. The production of carbaryl methylol was primarily the result of

Table 3
Apparent K_m (μM) and V_{max} (nmol/nmol P450/min) for carbaryl metabolism in several human CYP isoforms

CYP isoforms	5-Hydroxycarbaryl		4-Hydroxycarbaryl		Carbaryl methylol	
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
1A1	4.81 ± 0.33	15 ± 5	0.32	3.89 ± 0.19	20 ± 5	0.19
1A2	2.62 ± 0.13	89 ± 15	0.03	1.72 ± 0.08	58 ± 11	0.03
2B6	0.29 ± 0.05	110 ± 47	0.01	0.80 ± 0.05	11 ± 3	0.07
2C19	0.99 ± 0.09	62 ± 22	0.02	2.21 ± 0.17	44 ± 16	0.05
3A4	2.34 ± 0.34	281 ± 98	0.01	5.81 ± 0.50	235 ± 57	0.02
				1.19 ± 0.20	51 ± 31	0.02
				4.80 ± 0.20	36 ± 7	0.13
				15.54 ± 0.94	45 ± 10	0.35
				3.46 ± 0.24	15 ± 5	0.23
				1.47 ± 0.28	156 ± 91	0.01

Values are expressed as mean $\pm$ S.E.M. ($n = 3$ determinations). V_{max}/K_m is expressed as ml/mg protein per min.

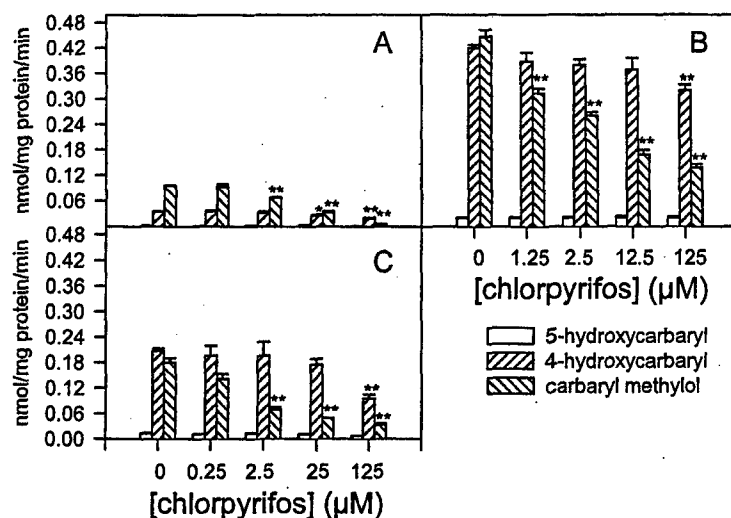


Fig. 4. Activities of carbaryl metabolism in the presence of chlorpyrifos: (A) co-incubation of chlorpyrifos with 10 μ M carbaryl, (B) co-incubation of chlorpyrifos with 500 μ M carbaryl, (C) pre-incubation of chlorpyrifos before incubation with 500 μ M carbaryl. Activities were expressed as nmol product/mg microsomal protein per min $\pm$ S.E.M. ($n = 3$ determinations). Significant differences from correspondent control (i.e. 0 μ M chlorpyrifos) were indicated by *, $P < 0.05$; or **, $P < 0.01$.

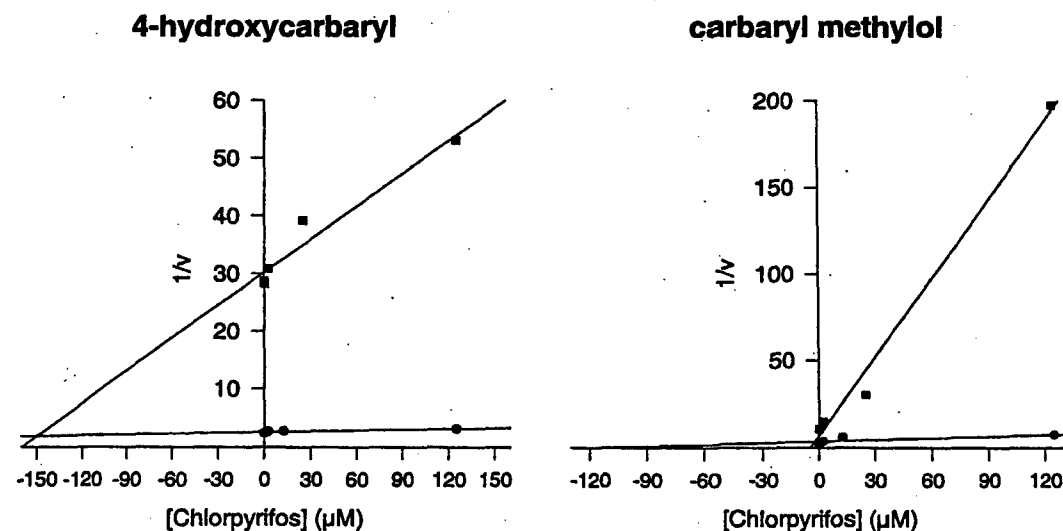


Fig. 5. Dixon plot of co-incubation of variant concentrations of chlorpyrifos with 10 μ M carbaryl (■) and 500 μ M carbaryl (●).

metabolism by CYP2B6. The kinetic parameters as well as concentrations of these isoforms in HLM may explain the relative activities in microsomes of these carbaryl metabolic pathways, i.e. the low activity of 5-hydroxylation in HLM may result from low concentrations of CYP1A1, while high activities of 4-hydroxylation and methyl hydroxylation may result from high concentrations of CYP3A4 in HLM and high

activity of CYP2B6 in methyl hydroxylation, respectively. However, the relatively low activity of 5-hydroxylation may be enhanced after a period of chronic exposure to carbaryl because previous studies have shown that carbaryl can induce CYP1A1 activity [20–22].

Differential metabolic activities toward carbaryl by variants of CYP2C9 and 2D6 were observed in this study. CYP2C9*1 (Arg₁₄₄, Ile₃₅₉), the most common form of CYP2C9, is more active in carbaryl metabolism than CYP2C9*2 (Cys₁₄₄) and CYP2C9*3 (Leu₃₅₉). CYP2D6*10 did not show any detectable activity toward carbaryl while CYP2D6*1 displayed some activity toward carbaryl, especially in the generation of 5-hydroxycarbaryl.

Variation in carbaryl metabolism among individual HLMs was observed in this study. Differences in 5- and 4-hydroxylations among 5 selected individuals were only 2-fold, while the difference in methyl hydroxylation was about 5-fold. Two or more CYP isoforms are involved in these ring hydroxylations with high activity. For example, both CYP1A1 and 1A2 are very active in 5-hydroxylation and CYP3A4, 1A1 and 2C19 are all involved in 4-hydroxylation with high activity. Thus, a change in a single isoform may not greatly affect the total activity of microsomes. Greater differences of ring hydroxylation activity may only be observed between individuals with low or high activities for all CYP1A1, 1A2, 2C19 and 3A4.

Methyl hydroxylation is a pathway that is predominately catalyzed by a single isoform, CYP2B6. Thus, changes in CYP2B6 activity are not thoroughly compensated for by other isoforms in the methyl hydroxylation pathway, and greater differences in methyl hydroxylation occur between individuals with low and high CYP2B6 (HG6 and HG42, respectively).

The effect of chlorpyrifos on carbaryl metabolism was investigated. Chlorpyrifos, a phosphorothionate insecticide, is activated to chlorpyrifos-oxon through a CYP-catalyzed desulfuration reaction [2]. The sulfur atom released from chlorpyrifos in this reaction is highly reactive and is believed to bind immediately to the heme iron of CYP and inhibit its activity [14]. CYP2B6 has been shown to have the highest chlorpyrifos desulfuration activity among all CYP isoforms [23]. A decrease in CYP2B6 activity after incubation with chlorpyrifos should, therefore, occur because of immediate binding to CYP2B6 of the sulfur atom released in the desulfuration reaction. CYP2B6 was also found in this study to have a predominant role in the generation of carbaryl methylol, thus carbaryl would also compete with chlorpyrifos for the active site of CYP2B6. Therefore, it is not surprising that the methyl hydroxylation pathway is the pathway of carbaryl metabolism most affected by chlorpyrifos, either co-incubation or pre-incubation.

In summary, carbaryl can be metabolized by CYP oxidation in HLM to form 5-hydroxycarbaryl, 4-hydroxycarbaryl, and carbaryl methylol. Most CYP isoforms responsible for xenobiotic metabolism have some activity toward carbaryl. The highest activity was observed in CYP1A1 for 5-hydroxylation, CYP3A4 for 4-hydroxylation, and CYP2B6 for methyl hydroxylation. Differential activities among carbaryl metabolic pathways in HLM may be attributed to different concentrations of these most active CYP isoforms in HLM. Likewise, the varying concentrations of CYP isoforms among individuals may contribute to the differences in carbaryl

metabolism among human populations. Metabolism of carbaryl by CYP oxidation is inhibited by chlorpyrifos. The most affected pathway is methyl hydroxylation, catalyzed predominately by CYP2B6, which is also the most active isoform for chlorpyrifos desulfuration.

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In vitro human metabolism of permethrin: the role of human alcohol and aldehyde dehydrogenases

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Abstract

Permethrin is a pyrethroid insecticide widely used in agriculture and public health. It has been suggested that permethrin may interact with other chemicals used during military deployments and, as a result, be a potential cause of Gulf War Related Illness. To determine the causal relationship between permethrin and human health effects, the basic enzymatic pathway of permethrin metabolism in humans should be understood. In the present study we report that *trans*-permethrin is metabolized in human liver fractions, producing phenoxybenzyl alcohol (PBOH) and phenoxybenzoic acid (PBCOOH). We identified human alcohol (ADH) and aldehyde dehydrogenases (ALDH) as the enzymes involved in the oxidation of phenoxybenzyl alcohol, the permethrin hydrolysis product, to phenoxybenzoic acid by way of phenoxybenzaldehyde (PBCHO). *Cis*-permethrin was not significantly metabolized in human liver fractions. Cytochrome P450 isoforms were not involved either in the hydrolysis of *trans*-permethrin or in the oxidation of PBOH to PBCOOH. Purified ADH isozymes oxidized PBOH to PBCHO and PBOH was a preferred substrate to ethyl alcohol. Purified ALDH was responsible for PBCHO oxidation to PBCOOH with similar substrate affinity to a previously known substrate, benzyl alcohol. Based on these observations, it appears that PBOH is oxidized to PBCHO by ADH and subsequently to PBCOOH by ALDH, although PBCHO does not accumulate during microsomal incubation. In order to analyze permethrin and its metabolites, previous HPLC-UV methods had to be re-validated and modified. The resulting refined HPLC-UV method is described in detail.

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Keywords: Permethrin; Permethrin metabolism; Human alcohol dehydrogenase; Human aldehyde dehydrogenase

1. Introduction

Permethrin (3-phenoxy-benzyl ($\pm$) *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylate) is a widely used photo-stable

synthetic pyrethroid insecticide acting as a neuropoison [1]. Permethrin is not acutely toxic to mammals since it is rapidly hydrolyzed [2,3]. However it is known that permethrin causes some toxic symptoms at high oral doses [4].

Recent controversies over the nature and the potential causes of Gulf War Related Illnesses have indicated potential roles for various chemicals used for military personnel [5,6]. In addition

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to permethrin these chemicals include pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide and chlorpyrifos. Interaction of permethrin with pyridostigmine bromide and *N,N*-diethyl-*m*-toluamide has been suggested as a potential cause of Gulf War Related Illnesses [6,7]. In a recent study on human metabolism of *N,N*-diethyl-*m*-toluamide, it was found that there are potential chemical interactions among deployment related chemicals through enzymatic induction and inhibition [8]. Also, there have been indications that permethrin metabolites (phenoxybenzyl alcohol, PBOH and phenoxybenzaldehyde, PBCHO) can be more cytotoxic [9] and have more potential as estrogenic agents [10] than the parent compound. To determine the causal relationship between potential chemical exposure and human health effects, it is important to define basic metabolic pathways and to identify the enzymes involved in humans.

Even though the importance of the liver in xenobiotic metabolism is well recognized, no permethrin metabolism studies based on human liver tissue or human liver microsomes have been reported. Previous studies of human permethrin metabolism have mainly focused on the detection of primary metabolites such as *cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylate (DCCA), PBOH, and phenoxybenzoic acid (PBCOOH) in either human blood or urine samples [11–13] and no reports are available on the identification of the enzymes involved in permethrin metabolism in humans.

Permethrin metabolites found in rodents indicate that the parent permethrin is hydrolyzed to DCCA and PBOH with further oxidation of PBOH to PBCOOH [14]. These basic metabolites are subject to further oxidative hydroxylation and conjugation. We are interested in delineating human enzymatic pathways involved in the metabolic transformation of permethrin and its hydrolysis products and here we report that, based on human liver fractions and purified human dehydrogenases, PBOH is oxidized to PBCHO by alcohol dehydrogenase (ADH) and then to PBCOOH by aldehyde dehydrogenase (ALDH). The further metabolism of the other hydrolysis product, DCCA, will be the subject of subsequent studies. Regarding the HPLC analysis method for permethrin and its metabolites, we revalidated and modified previously used methods. We report, as a part of human permethrin metabolism study, an efficient HPLC-UV method that can separate five compounds (two permethrin

isomers, PBOH, PBCHO, and PBCOOH) with emphasis on the optimal detection wavelength and a proper mobile phase pH range for an ionizable metabolite (PBCOOH).

2. Methods

2.1. Chemicals, human liver fractions, and human CYPs

Cis-permethrin (98% purity), PBOH (98% purity), and PBCOOH (99% purity) were purchased from Chem Service (West Chester, PA). *Trans*-permethrin (98.1% purity) and PBCHO (98% purity) were purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals, if not specified, were purchased from Sigma (St. Louis, MO). Pooled human liver microsomes and cytosol were purchased from Gentest Biosciences (Woburn, MA). Microsomal and cytosolic fractions were derived from the same batch of 13 pooled human liver samples. Human CYP 1A1, 1A2, 1B1, 2A6, 2C8, 2C9, 2C18, 2C19, 2E1, 3A4, 3A5, 3A7, and 4A11 Supersomes expressed in a baculovirus expression system were purchased from Gentest Biosciences (Woburn, MA).

2.2. HPLC method development

Permethrin isomers (*trans* and *cis*) and three major metabolites (PBOH, PBCHO, and PBCOOH) were analyzed using a Shimadzu HPLC system (Kyoto, Japan) consisting of a pump (LC-10AT VP), a solvent proportioning valve (FCV-10AL VP), a degasser (DGU-14A), a controller (SCL-10A VP), and an auto injector (SIL-10AD VP). Permethrin isomers and metabolites were separated on Phenomenex (Torrance, CA) Luna⁺ column (reverse phase C18, 5 μ m, 150 $\times$ 3.0 mm) and detected using a Shimadzu SPD-10AV VP UV-VIS detector. Two solvents (solvent A: 90% acetonitrile and 10% H₂O, solvent B: 100% H₂O adjusted to pH 1.7 with 85% phosphoric acid) were used for gradient elution (flow rate: 1 ml/min). The gradient system (linear increase) was initiated with 50% of solvent A and 50% of solvent B reaching 75% of solvent A at 6 min and 100% of solvent A at 7 min. One hundred percent of solvent A was maintained for 4 min and then reduced to 50% at 12 min, which was kept for 4 min to prepare the column for

initial conditions. The chromatographic analysis was conducted at ambient temperature.

A series of dilutions prepared in Tris (0.1 M, pH 7.5)/acetonitrile (1:1, v/v) from a concentrated mixture of five compounds were used to construct a standard curve based on peak areas. In order to find the optimal detection wavelength, standard curves for permethrin isomers and metabolites were constructed at four previously used wavelengths (210, 230, 254, and 270 nm). *Trans*-permethrin and two metabolites were identified by comparing retention times with analytical standards. For each compound, the lowest detectable concentration was determined as the detection limit assuming a signal-to-noise ratio of 3:1. The lowest concentration of each compound generating reproducible and predictable peak area was determined as the limit of quantitation. For this study, the solvent B pH was set at 1.7.

Among the compounds analyzed, PBCOOH is ionized at neutral pH. For consistent peak identification and quantification, ionization should be suppressed. In order to characterize the effect of mobile phase pH on the protonation of PBCOOH, the metabolites were analyzed at several different mobile phase pH levels. The mobile phase pH was adjusted by increasing the hydrogen ion concentration in solvent B (pH levels of 1.7, 2.3, 3.2, and 8.0) with 85% phosphoric acid. The eluted compounds were detected at 230 nm.

2.3. Permethrin metabolism in human liver fractions and CYPs

Trans-permethrin was incubated with human liver fractions. A reaction mixture of 500 μ l in 0.1 M Tris buffer (pH 7.5) contained 0.5 mg of cytosolic or microsomal protein, 10 μ l of *trans*-permethrin stock solution (10 mM in acetonitrile), 25 μ l of NADPH regenerating system solution A, and 10 μ l of NADPH generation system solution B. For CYP isoform incubations 25 pmol in 500 μ l of reaction buffer (as recommended by Gentest) was used. Solution A of the NADPH regenerating system contained 26.0 mM NADP⁺ and 66 mM of glucose-6-phosphate in phosphate buffer (pH 7.4). Solution B of the NADPH regenerating system contained 40 U/ml of glucose-6-phosphate dehydrogenase in phosphate buffer (pH 7.4). All components except the liver fractions or CYP isoforms were mixed and pre-incubated for 5 min at 37°C. One hundred minute incubations were started when the liver fractions were added to the pre-warmed reaction mixture. After the incuba-

tion was completed, 500 μ l of cold acetonitrile was added to each reaction mixture to stop enzymatic reactions. The reaction mixtures were centrifuged at 20,000g for 5 min using a microcentrifuge. The supernatant was sampled and stored at 4°C until HPLC analysis. The concentration of each compound was calculated based on the standard curve constructed at 230 nm with solvent B, pH 1.7.

2.4. Protein purification

Human ADH isozymes were expressed and purified from *Escherichia coli* (JM105 strain) containing pKK223-3 expression vector (Pharmacia Biotech, Uppsala, Sweden) inserted with human ADH cDNA. The *E. coli* strain was provided by Dr. Brimfield (US Army Medical Research Institute of Chemical Defense). *E. coli* cells were grown in Terrific Broth media (with 50 mg ampicillin/L) and harvested as described previously [15] with some modifications. After inoculation of *E. coli* cells, cells were grown at 37°C until the absorbance reached 0.6–0.8 at 595 nm. Then, isopropylthiogalactoside (1.0 ml of 0.1 M stock per liter media) and ZnSO₄ (0.7 ml of 100 mM stock per liter media) were added to the media and incubation temperature was reduced to room temperature. The cells were cultured for 12–24 h before collection and collected by centrifugation at 4000g for 10 min. Collected cells were disrupted using a sonicator (550 sonic dismembrator, Fisher Scientific, Fair Lawn, NJ) in cell lysis buffer (pH 8.0, 10 mM Tris, 2 mM dithiothreitol (DTT), 0.1 mM ZnSO₄, and 1 mM benzamidine). The cytosolic ADHs enzymes were contained in the supernatant after ultracentrifugation (100,000g for 35 min at 4°C).

ADHs were purified using modifications of methods involving three chromatography columns in sequence as previously described [16]: DEAE-Sephacrose CL-6B (Pharmacia Biotech, Uppsala, Sweden), SP-Sephacrose Fast Flow (Pharmacia Biotech, Uppsala, Sweden), and Affi-Gel Blue Gel (Bio-Rad, Hercules, CA) columns. For β I and β II purifications, the supernatant was directly applied to the DEAE-Sephacrose column. Cell lysis buffer described above was used as an elution buffer at a flow rate of 1.7 ml/min. Active eluent fractions were collected and buffer-exchanged to SP-Sephacrose buffer (pH 8.0, 10 mM Hepes, 2 mM DTT, 0.1 mM ZnSO₄, and 1 mM benzamidine) using ultrafiltration tubes (30,000 Da molecular weight cutoff, Centricon, Millipore, Bedford, MA). ADH activity was measured in 100 mM glycine buffer (pH 10.0)

with 2.5 mM NAD⁺ and 33 mM ethanol at 25 °C. ADH activity was measured using the absorbance change at 340 nm with a molar extinction coefficient of 6.22 mM/min/cm for NADH. After binding to SP-Sepharose and rinsed with the column buffer, ADH proteins were eluted using a NaCl gradient from 0 to 250 mM prepared in the column buffer. Collected active fractions were buffer-exchanged to Affi-Gel Blue Gel column buffer (pH 7.4, 10 mM sodium phosphate, 0.5 mM DTT, and 0.1 mM ZnSO₄). β ADH proteins strongly bind to Affi-Gel Blue columns and 0.8 M NaCl solution prepared in the column buffer was used to elute bound proteins after a thorough rinsing step. Pooled active fractions were buffer-exchanged to Affi-Gel Blue Gel column buffer and stored at -70 °C with 50% glycerol. Flow rate for Affi-Gel Blue Gel column was 1.0 ml/min. Protein concentration was measured based on the bicinchoninic acid reaction [17].

For α ADH, which has a lower isoelectric point than β ADH isozymes, the pH of the SP-Sepharose elution buffer was lowered to 7.2. All other chromatography procedures were the same as described for β ADH isozymes. For δ ADH, the pH of the SP-Sepharose elution buffer was 6.4 with 77 mM sodium phosphate, 1 mM DTT, 0.1 mM ZnSO₄, and 1 mM benzimidazole. δ ADH from the SP-Sepharose column was eluted using a linear gradient elution starting with 7 mM and ending with 65 mM sodium phosphate buffer (pH 6.4). The Affi-Gel Blue Gel column buffer for δ ADH had the same composition as other isozymes with a lower pH (6.4). δ ADH was eluted from the Affi-Gel Blue Gel column using a linear gradient elution starting with column buffer and ending with 100 mM Tris buffer (pH 8.8). The storage conditions for α and δ isozymes were the same as for the β ADHs. The purity of ADH enzymes was evaluated based on SDS-PAGE with Coomassie blue staining.

Purified human ALDH3A1 was provided by Dr. Alan Townsend (Wake Forest University, School of Medicine). The ALDH3A1 provided had been purified from Chinese hamster lung fibroblast cell line (V79/SD1) stably transfected with ALDH3A1 cDNA containing Δ pCEP4 Δ mammalian expression vector [18].

2.5. Enzyme kinetics

Purified ADH isozymes were assayed for PBOH oxidation to PBCHO and K_m/V_{max} values were calculated based on Michaelis-Menten steady-state kinetics. A series of different concen-

trations of PBOH and ethyl alcohol were prepared in 100 mM glycine buffer (pH 10.0) and mixed with NAD⁺ (final concentration 2.5 mM). The reaction mixtures were pre-warmed to 25 °C and the enzymatic reaction was started by adding purified ADH to the reaction mixture. The reaction rate was calculated by spectrophotometric measurement of NADH formation (UV-2101PC, Shimadzu, Kyoto, Japan) at 340 nm (molar extinction coefficient of 6.22 mM/min/cm).

For the ALDH assay a series of different concentrations of PBCHO were prepared in 33 mM sodium pyrophosphate buffer (pH 8.2) and mixed with 1 mM EDTA, 0.1 mM pyrazole (ADH inhibitor) and NAD⁺ (final concentration 2.5 mM). The reaction mixtures were pre-warmed to 37 °C and the enzymatic reaction was started by adding purified ALDH to the reaction mixture. As in ADH assay, the reaction rate of PBCHO oxidation to PBCOOH was determined by NADH formation.

In order to determine whether PBOH or PBCHO are oxidized by CYPs, 13 isoforms were incubated, separately, in the presence of NADPH regeneration system with the two compounds. Other conditions were the same as in the *trans*-permethrin incubation. GraphPad [19] program was used for Michaelis-Menten model fitting to estimate K_m and V_{max} values.

2.6. Statistical analysis

The concentrations of each compound were presented as means $\pm$ 1 standard error of mean (SEM). The number of replicates per treatment was 3. Metabolite production data were analyzed using an analysis of variance (ANOVA) with a subsequent multiple comparison test (all pair-wise differences) using the Tukey method [20]. Statistical tests were performed using S-Plus statistical software [21]. The level of statistical significance for all tests was $p < 0.05$.

3. Results

3.1. Permethrin HPLC analysis

The HPLC analysis method described in Section 2 reliably resolved and detected permethrin isomers and three major metabolites. Using this HPLC method PBOH, PBCHO, PBCOOH, *trans*-permethrin, and *cis*-permethrin were eluted at 3.7, 4.1, 5.9, 10.2, and 10.5 min, respectively.

As shown in Fig. 1, permethrin and its metabolites showed the highest levels of absorption at 210 nm among the four different detection wavelengths previously used in permethrin metabolism studies. The second highest absorption was observed at 230 nm and the compounds showed significantly lower absorption at 254 and 270 nm. While higher absorption was observed at 210 nm, lower detection and quantitation limits were obtained at 230 nm as shown in Table 1. This was due to relatively high levels of background noise at 210 nm.

Mobile phase hydrogen ion concentration clearly affected the resolution of ionizable metabolite (PBCOOH). As shown in Fig. 2, PBCOOH did not form a distinguishable peak at

the solvent B, pH 8.0. As the protonation of PBCOOH proceeded with increasing hydrogen ion concentration in the mobile phase, a separate peak of PBCOOH gradually became distinguishable, forming a clearly identifiable peak at the lowest solvent B, pH 1.7, among the four tested solvent B pHs (Fig. 2). A pH 1.7 for solvent B and a detection wavelength of 230 nm were used for all HPLC analyses in this study.

3.2. *In vitro* human liver metabolism of permethrin

Using the HPLC analysis method developed in this study, the metabolic products of *trans*-permethrin metabolism were profiled after incubating with pooled human liver microsomes and cytosol.

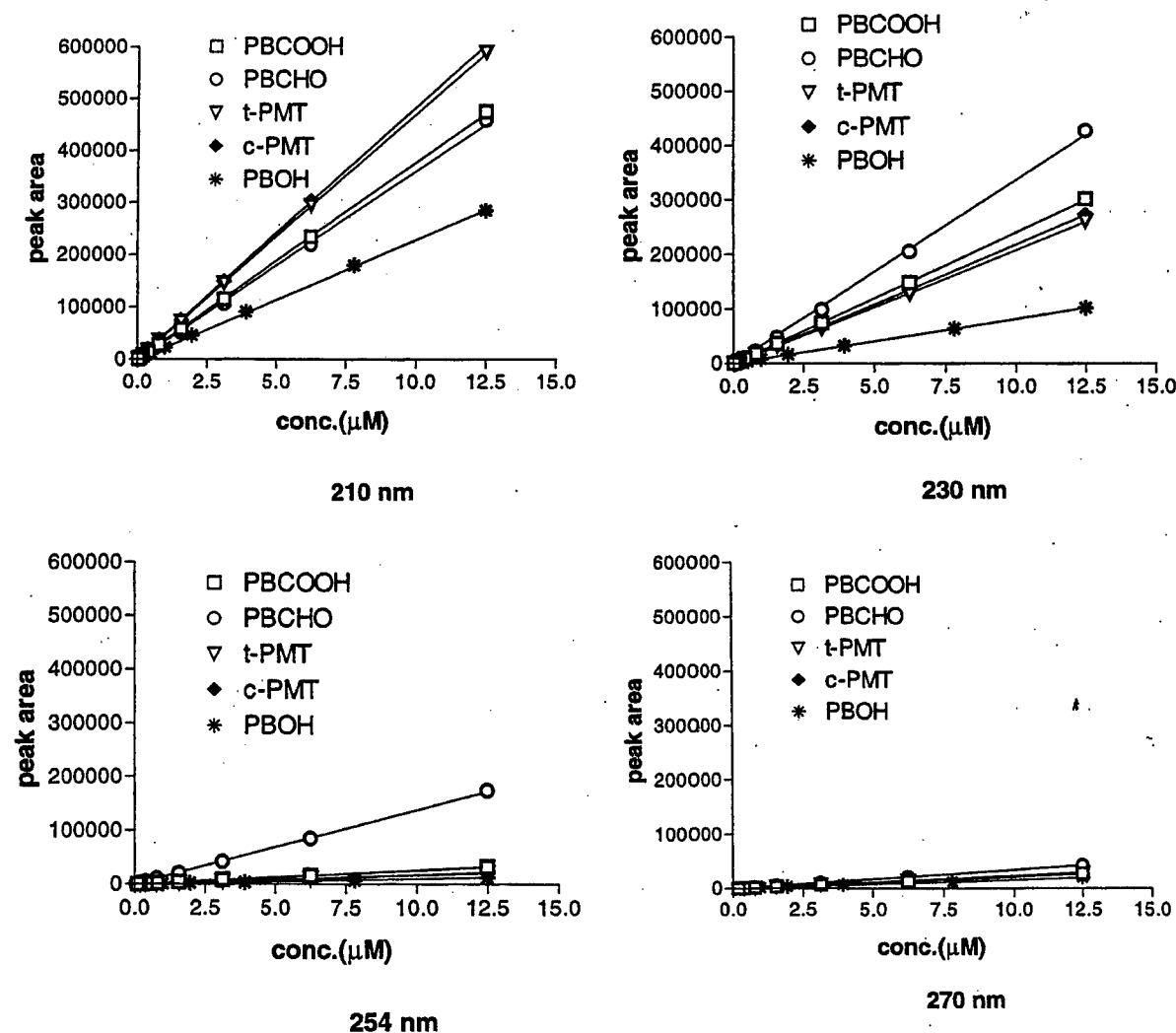


Fig. 1. Standard calibration curves for permethrin isomers and their metabolites at four different wavelengths. t-PMT, *trans*-permethrin; c-PMT, *cis*-permethrin; PBOH, phenoxybenzyl alcohol; PBCHO, phenoxybenzaldehyde; and PBCOOH, phenoxybenzoic acid. Solvent B, pH 1.7.

Table 1

Limits of detection and quantitation for permethrin isomers and their metabolites at different detection wavelengths based on the HPLC method described in Section 2

Wavelength (nm)	Compound	Limit of detection (nM)	Limit of quantitation (nM)
210	PBOH	120	120
	PBCOOH	24	49
	PBCHO	49	98
	t-PMT	24	49
	c-PMT	24	97
230	PBOH	120	120
	PBCOOH	12	12
	PBCHO	12	24
	t-PMT	24	49
	c-PMT	24	49
254	PBOH	490	980
	PBCOOH	98	390
	PBCHO	49	49
	t-PMT	195	390
	c-PMT	195	390
270	PBOH	240	490
	PBCOOH	195	390
	PBCHO	195	195
	t-PMT	390	390
	c-PMT	390	390

t-PMT, *trans*-permethrin; c-PMT, *cis*-permethrin; PBOH, phenoxybenzyl alcohol; PBCHO, phenoxybenzaldehyde; and PBCOOH, phenoxybenzoic acid.

Since no significant *cis*-permethrin metabolism was observed in either cytosolic or microsomal fractions, subsequent metabolism studies focused on *trans*-permethrin metabolism. Fig. 3 shows a typical metabolite profile of human liver microsomal metabolism of *trans*-permethrin. While PBOH and PBCOOH were detected, PBCHO was rarely detected in microsomal incubations. No *trans*-permethrin metabolites were produced in the absence of human liver fractions.

On incubation with the microsomal fraction without an NADPH regenerating system, the sum of the concentration of unmetabolized *trans*-permethrin and the two metabolites equaled 100 μmol (initial *trans*-permethrin concentration) in 500 μl indicating no metabolites other than PBOH and PBCOOH were produced in the incubation. In the remainder of the treatments, the total concentrations were significantly less than 100 μmol (Fig. 4). In these treatments the difference between 100 μmol and the values in Fig. 4 indicated the likelihood that metabolites other than PBOH and PBCOOH were produced. The amount of these unaccounted metabolites was greater in microsomes and cytosol containing the NADPH regenerating system. Fig. 5 shows that

the highest level of overall *trans*-permethrin metabolism occurred in the microsomal fraction with NADPH and NADPH enhanced *trans*-permethrin metabolism in both fractions.

Potential involvement of human CYP isoforms in the hydrolysis of *trans*-permethrin was explored by incubating *trans*-permethrin with CYP isoforms. None of these incubations resulted in detectable products nor did they result in observable loss of the parent *trans*-permethrin (data not shown).

3.3. Phenoxybenzyl alcohol oxidation to phenoxybenzoic acid by purified human alcohol and aldehyde dehydrogenases

All four purified human ADHs readily oxidized PBOH to PBCHO with the lowest K_m observed with β -I ADH and the highest K_m with δ ADH (Table 2). The range of K_m values for PBOH oxidation was two orders of magnitude smaller than that for ethyl alcohol oxidation to acetaldehyde indicating a much higher substrate affinity for PBOH. V_{max} values were relatively close for both substrates. As a result, for all isozymes the catalytic efficiencies (V_{max}/K_m) for PBOH are

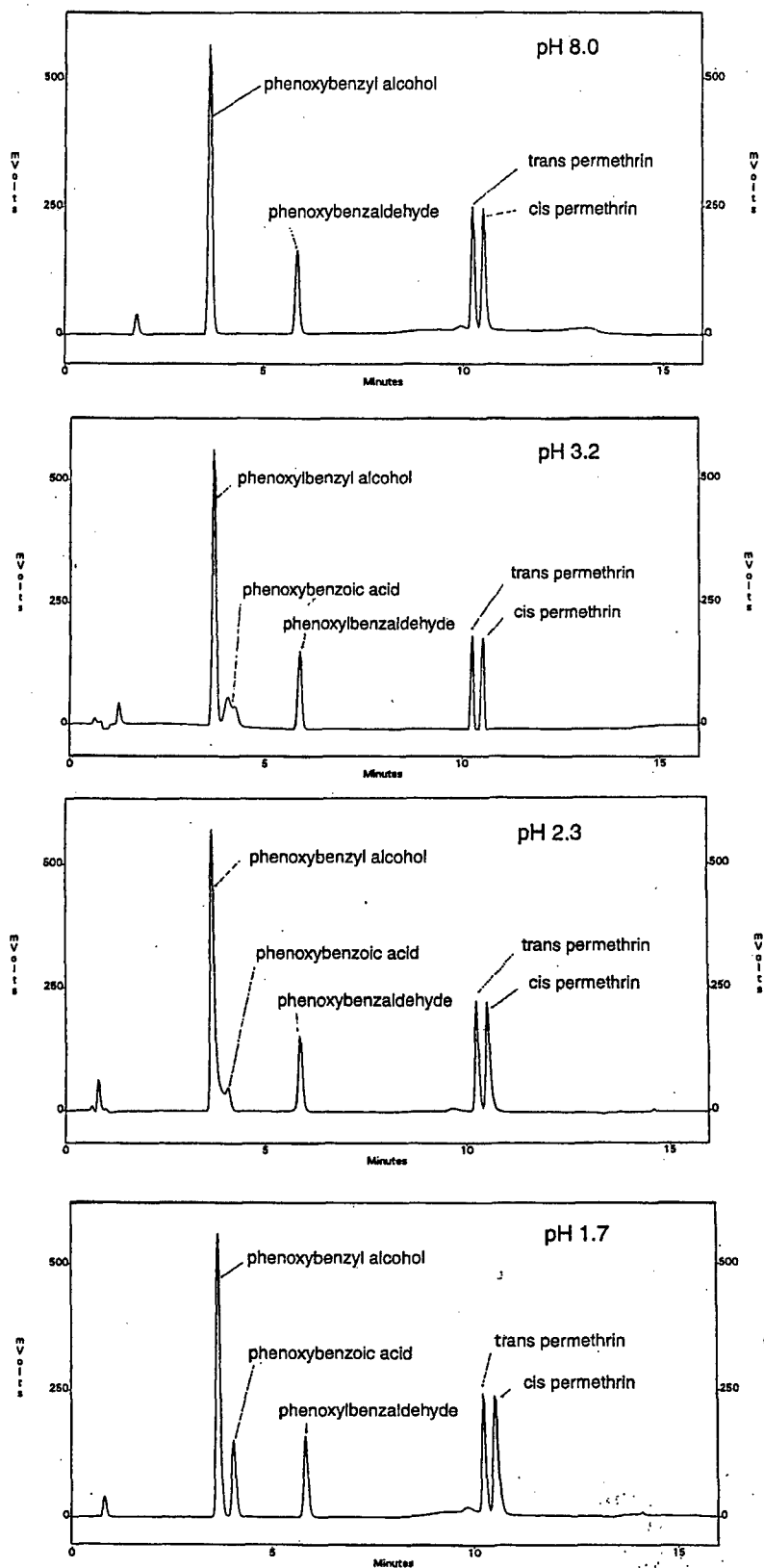


Fig. 2. The effect of pH change in the mobile phase solvent B on the resolution of permethrin isomers and their metabolites. pH was adjusted with 85% phosphoric acid. The compounds were detected at 230 nm.

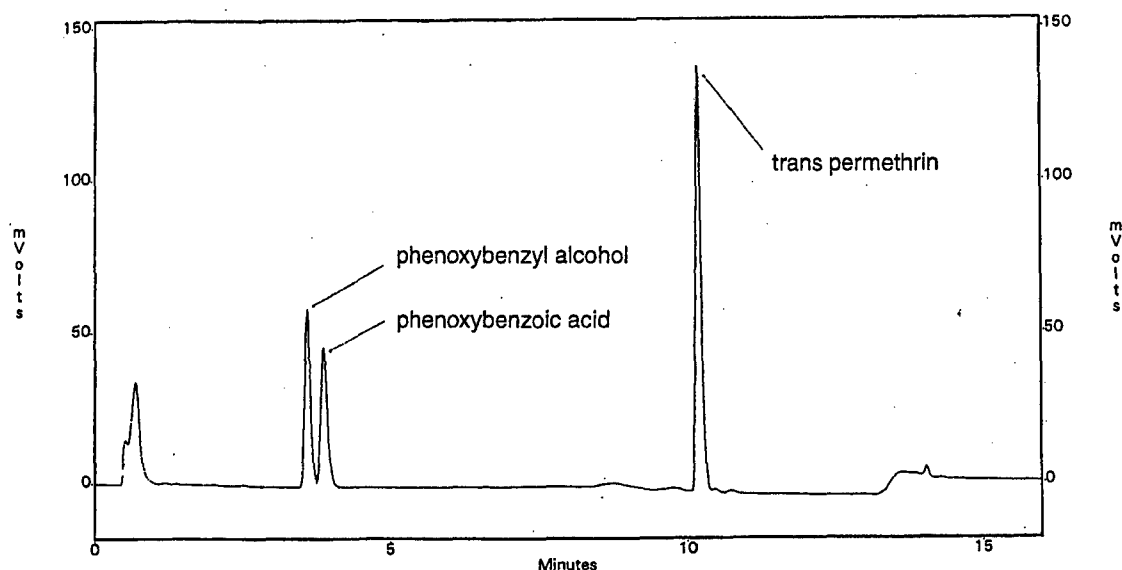


Fig. 3. Metabolite profile of *trans*-permethrin (100 μ M) incubation with pooled human liver microsomal fraction (0.5 mg protein/500 μ l of 0.1 M Tris (pH 7.5) for 100 min at 37 $^{\circ}$ C). Solvent B, pH 1.7, and the compounds were detected at 230 nm.

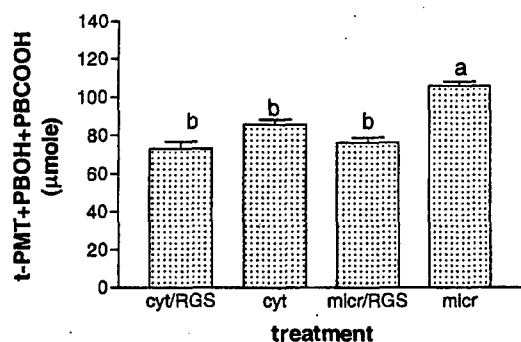


Fig. 4. Sum of *trans*-permethrin and two major metabolites (PBOH and PBCOOH) after incubation with human liver cytosolic and microsomal fractions (500 μ l). cyt/RGS: incubated in human liver cytosol with NADPH regenerating system. cyt: incubated in human liver cytosol without NADPH regenerating system. micr/RGS: incubated in human liver microsomes with NADPH regenerating system. micr: incubated in human liver microsomes without NADPH regenerating system. Values are presented as means $\pm$ SEM ($n = 3$). A different letter indicates a significant difference ($p < 0.05$) among treatments in ANOVA multiple comparison analysis.

significantly higher than those for ethyl alcohol. In contrast, kinetic parameters and catalytic efficiency for ALDH3A1 toward PBCHO were relatively close to a known ALDH3A1 substrate, benzaldehyde (Table 3). NAD^+ was required in both ADH and ALDH reactions.

HPLC analysis indicated that PBOH was not oxidized by ALDH3A1 and no PBCOOH was

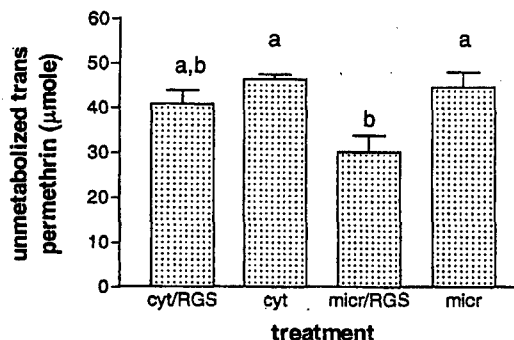


Fig. 5. Amount of unmetabolized *trans*-permethrin after incubation with human liver cytosolic and microsomal fractions (500 μ l). cyt/RGS: incubated in human liver cytosol with NADPH regenerating system. cyt: incubated in human liver cytosol without NADPH regenerating system. micr: incubated in human liver microsomes with NADPH regenerating system. micr: incubated in human liver microsomes without NADPH regenerating system. Values are presented as means $\pm$ SEM ($n = 3$). A different letter indicates a significant difference ($p < 0.05$) among treatments in ANOVA multiple comparison analysis.

produced from PBOH by ADH isozymes (data not shown). Also, ADHs and ALDH3A1 incubation with *trans/cis*-permethrin did not produce any metabolites indicating ADH/ALDH are not involved in permethrin hydrolysis. Human CYP isoforms are not involved in PBOH oxidation to PBCOOH. Incubation of PBOH and PBCHO with human CYP isoforms with a NADPH

Table 2

Kinetic constants of human alcohol dehydrogenase isoforms for phenoxybenzyl alcohol (PBOH) oxidation to phenoxybenzaldehyde (PBCHO) and ethyl alcohol (EtOH) oxidation

Isozyme	Substrate	K_m (μ M) (SE)	V_{max} (nmol/mg/min) (SE)	V_{max}/K_m (min^{-1})
α	PBOH	34 (3)	2765 (68)	82.15
β -I	PBOH	4 (1)	389 (23)	103.46
β -II	PBOH	29 (4)	266 (10)	9.19
δ	PBOH	48 (6)	6452 (203)	134.81
α	EtOH	1570 (180)	3987 (122)	2.54
β -I	EtOH	1120 (160)	541 (21)	0.48
β -II	EtOH	4750 (510)	657 (18)	0.14
δ	EtOH	4060 (330)	7752 (158)	1.91

Table 3

Kinetic constants of human aldehyde dehydrogenase isoform ALDH3A1 for phenoxybenzaldehyde (PBCHO) oxidation to phenoxybenzoic acid (PBCOOH)

Substrate	K_m (μ M) (SE)	V_{max} (nmol/mg/min) (SE)	V_{max}/K_m (min^{-1})
Benzaldehyde	148 (58)	2878 (283)	19.45
Phenoxybenzaldehyde	230 (49)	2111 (107)	9.18

regeneration system did not result in detectable products (data not shown).

4. Discussion

The present study is the first report on the characterization of the permethrin metabolic pathway in humans. This study demonstrated that *trans*-permethrin is hydrolyzed and oxidized in human liver cytosol and liver microsomes producing PBOH and PBCOOH. We provide evidence that in human metabolism, PBOH is oxidized to PBCOOH via PBCHO by ADH and ALDH.

Previously described HPLC-UV methods for analysis of permethrin and its metabolites used a variety of wavelength and mobile phase conditions [22–24]. The only previous article reporting a separation method that included all five compounds analyzed in this study was that by Bast et al. [23]. Improvements in mobile phase in this study resulted in decreasing elution time for *cis*-permethrin (the last peak to be eluted) from 25 to 11 min without compromising peak resolution. Mobile phase acidification to protonate PBCOOH (pK_a value of 3.95), which is ionized at neutral pH, had also not been described. As shown in Fig. 2, insufficient protonation leads to a broad or missing peak, confounding interpretation of HPLC results. In our gradient scheme, a pH level

below 2 was required for solvent B to achieve reliable protonation of PBCOOH for accurate qualitative and quantitative analysis (Fig. 2).

The central ester linkage in permethrin has been shown to be cleaved in experimental animals by esterases producing PBOH and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid [14,25]. It was assumed that PBOH is further oxidized to PBCHO and PBCOOH with further hydroxylations and conjugations on these metabolites before excretion. Even though these modifications were presumed to be carried out by Phase I and II enzymes [26], neither the pathways beyond hydrolysis of the ester linkage nor the enzymes involved were identified.

Incubation of *trans*-permethrin with either human liver cytosol or microsomal fractions resulted in the formation of two primary metabolites, PBOH and PBCOOH, indicating that esterases in either cell fraction can cleave the ester bond (Figs. 3 and 4). To date the number of esterases involved in permethrin hydrolysis is not known, and their substrate specificities remain poorly characterized. Soderlund et al. [25] suggested that, based on selective inhibition, *trans*/*cis*-permethrin hydrolysis is mediated by several esterases with differing substrate specificities and inhibitor sensitivities. In this study *cis*-permethrin was barely hydrolyzed in human liver fractions. Even though previous animal studies have shown that *cis*-permethrin is less prone to hydrolysis

compared to *trans* isomer [14,27], the almost total lack of hydrolysis in human liver fractions implies that human liver esterase specificity or composition is significantly different from animals.

Even though the addition of an NADPH regenerating system facilitated *trans*-permethrin metabolism in both the cytosolic and microsomal fractions, it appears that CYP isozymes are not involved in *trans*-permethrin hydrolysis and subsequent oxidation of PBOH to PBCOOH. This assumption is deduced from the observation that incubation of *trans*-permethrin with 13 individual human CYP isoforms expressed in insect supersomes did not produce PBOH/PBCOOH and the same incubation with PBOH did not produce PBCHO/PBCOOH. In similar studies conducted in our laboratory [8,28,29], the combination of CYP isoforms and the NADPH regeneration system used in this study have demonstrated varying levels of oxidative reactions mediated by CYP isoforms.

It is assumed that in conjunction with conjugation enzymes, CYP isoforms might be involved in hydroxylation and conjugation of permethrin and its metabolites, and likely account for the production of the unaccounted metabolites, which ranges from 0 to 50% of metabolized *trans*-permethrin depending on the presence of the NADPH regenerating system. The products of secondary hydroxylation and subsequent conjugation are believed to be more polar and undetected with the HPLC method used in this study.

Replacement of the NADPH regenerating system with NAD^+ in the liver fractions significantly increased the production of PBCOOH, suggesting that PBOH oxidation is mediated by NAD^+ requiring enzymes and not by CYP isoforms. Also from occasional resolution of small PBCHO peaks on incubation with liver fractions, it was deduced that in the liver fractions PBOH is oxidized to PBCOOH via PBCHO. This postulation was confirmed by the observation that incubating PBOH with purified human ADH isozymes produced PBCHO. In a similar manner, incubation of PBCHO with purified human ALDH produced a PBCOOH peak (data not shown). Incubation of PBOH with ALDH did not produce PBCOOH. These results provide direct evidence that ADH and ALDH are involved in the sequential oxidation of PBOH to PBCOOH via PBCHO.

PBOH is a better substrate than ethyl alcohol (Table 2) for human ADH isoforms. This is in

agreement with previous observations that human ADH isozymes showed higher affinities for alcohols with larger side chains [30,31]. All tested human ADHs were very specific for PBOH oxidation to PBCOOH with no significant amount of PBCOOH produced. Also ADHs did not show any enzymatic activities with either *trans*-permethrin or PBCHO.

Human ADH isoforms are a family of dimeric NAD^+ - and zinc-dependent enzymes with nine known different subunits [32]. Human ADH isoforms are involved in the oxidation of ethyl alcohol and various kinds of endogenous alcoholic compounds such as steroid and retinoid metabolites [33,34]. α , β -I, and β -II ADHs are hepatic enzymes while δ ADH is found in the stomach [32]. As implied by the symbolic designations, β isozymes are polymorphic. Most Caucasian populations have β -I ADH, which has a higher affinity for ethyl alcohol, whereas the less efficient β -II ADH is found in 50% of the Asian population [15]. Our results showed that differential enzymatic activity of the β isoforms was more pronounced with PBOH implying that genetically dependent differential permethrin metabolism may have significant physiological ramifications. In this study we have not investigated the interaction between ethyl alcohol and permethrin, but it is plausible that ethyl alcohol consumption might affect permethrin metabolism by ADHs as ethyl alcohol inhibited retinoic acid formation by ADHs [35].

Human ALDHs are a group of enzymes catalyzing the conversion of various aldehydes to the corresponding acids by means of an NAD(P)^+ -dependent reaction. ALDH3A1 is viewed as a cellular protective mechanism and showed high enzymatic activity to aromatic aldehydes such as benzaldehyde [36]. Also ALDH3A1 detoxifies endogenous aldehydes such as the byproducts of lipid peroxidation as well as exogenous aldehyde species such as antineoplastic prodrugs (cyclophosphamide and ifosfamide) [36]. ALDH3A1 is found in stomach tissue (mucosa), and is not constitutively expressed in liver. However, in a rat study it was found that many agents such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene could induce this isozyme in liver [37]. ALDH3A1 showed slightly higher substrate affinity and higher V_{max} values for benzaldehyde resulting in higher enzymatic efficiency for the compound over PBCHO. ALDH3A1 did not show any enzymatic activities with either *trans*-permethrin or PBOH.

In this study, PBCHO was actively oxidized to PBCOOH in pooled human liver microsomes, which may not contain sufficiently high levels of ALDH3A1. This result implies that ALDH1A1 and ALDH2, which are found mainly in liver tissue and have been demonstrated to possess benzaldehyde metabolic capability [36], were involved in the oxidation. Another possibility is that there are other enzymes with a substrate range overlapping with that of ALDH.

In conclusion we report that *trans*-permethrin is actively hydrolyzed in human liver fractions and that the hydrolysis product of *trans*-permethrin, PBOH, is oxidized to PBCOOH via PBCHO by ADHs and ALDH. In future studies, the effects of active metabolites of other deployment related chemicals (e.g., chlorpyrifos, pyridostigmine bromide, *N,N*-diethyl-*m*-toluamide) on *trans*-permethrin hydrolysis and subsequent metabolite oxidation need to be studied. Also it will be important to assess how the active participation of ADH and ALDH in metabolizing exogenous substrates affects the oxidation of endogenous substrates whose transformation has important physiological functions.

Acknowledgments

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INHIBITION AND ACTIVATION OF THE HUMAN LIVER MICROSOMAL AND HUMAN CYTOCHROME P450 3A4 METABOLISM OF TESTOSTERONE BY DEPLOYMENT-RELATED CHEMICALS

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ABSTRACT:

Cytochrome P450 (P450) enzymes are major catalysts involved in the metabolism of xenobiotics and endogenous substrates such as testosterone (TST). Major TST metabolites formed by human liver microsomes include 6 β -hydroxytestosterone (6 β -OHTST), 2 β -hydroxytestosterone (2 β -OHTST), and 15 β -hydroxytestosterone (15 β -OHTST). A screen of 16 cDNA-expressed human P450 isoforms demonstrated that 94% of all TST metabolites are produced by members of the CYP3A subfamily with 6 β -OHTST accounting for 86% of all TST metabolites. Similar K_m values were observed for production of 6 β -, 2 β -, and 15 β -OHTST with human liver microsomes (HLM) and CYP3A4. However, V_{max} and CL_{int} were significantly higher for 6 β -OHTST than 2 β -OHTST (~18-fold) and 15 β -OHTST (~40-fold). Preincubation of HLM with a variety of ligands, including chemicals used in military deployments, resulted in varying levels of inhibition or activation of TST metabolism. The great-

est inhibition of TST metabolism in HLM was following preincubation with organophosphorus compounds, including chlorpyrifos, phorate, and fonofos, with up to 80% inhibition noticed for several metabolites including 6 β -OHTST. Preincubation of CYP3A4 with chlorpyrifos, but not chlorpyrifos-oxon, resulted in 98% inhibition of TST metabolism. Phorate and fonofos also inhibited the production of most primary metabolites of CYP3A4. Kinetic analysis indicated that chlorpyrifos was one of the most potent inhibitors of major TST metabolites followed by fonofos and phorate. Chlorpyrifos, fonofos, and phorate inhibited major TST metabolites non-competitively and irreversibly. Conversely, preincubation of CYP3A4 with pyridostigmine bromide increased metabolite levels of 6 β -OHTST and 2 β -OHTST. Preincubation of human aromatase (CYP19) with the test chemicals had no effect on the production of the endogenous estrogen, 17 β -estradiol.

The cytochrome P450 (P450¹) monooxygenase system is comprised of a superfamily of heme-containing enzymes, expressed in many mammalian tissues with the highest levels found in liver, and capable of catalyzing the metabolism of a wide range of both endogenous and exogenous substrates (Nelson et al., 1996). Human CYP3A4 is one of the most important and most abundant drug-metabolizing P450 isoforms in human liver microsomes and accounts for approximately 40% of the total P450 in human liver microsomes (Lehmann et al., 1998). CYP3A4 not only metabolizes xenobiotics but is also responsible for the metabolism of endogenous compounds, such as steroid hormones. Human CYP3A4 plays an important role in the metabolism of testosterone (TST), androstenedione (AD), and progesterone (Waxman et al., 1988). Direct and indirect approaches

have been employed to show that isoforms belonging to the CYP3A subfamily are the major contributors to 6 β -hydroxylation of testosterone as well as the production of several minor metabolites (Waxman et al., 1988, 1991; Yamazaki and Shimada, 1997).

In the human male, TST is the major circulating androgen. TST is essential for the development and maintenance of specific reproductive tissues as well as for other characteristic male properties such as control of spermatogenesis, retention of nitrogen, promotion of muscle strength, hair growth, bone density, and many aspects of sexually dimorphic behavior (Nieschlag and Behre, 1998; Wilson et al., 1998). Maintaining hormonal balance relies upon a number of variables including rate of hormone synthesis, interactions among hormones, and rates of secretion, transport, and metabolism. P450s are a major controlling element in the maintenance of proper steroid hormone levels in mammalian systems. Exposure to foreign compounds can exert changes in endocrine function both directly (hormone agonists or antagonists) or indirectly (altering circulating levels of hormones by influencing rates of hormone synthesis or metabolism) that can severely affect steroid hormone action (Wilson and LeBlanc, 1998). Steroids such as TST are hydroxylated by P450 in a regioselective and stereoselective manner (Waxman et al., 1988). It follows that perturbation of the P450 system by xenobiotics may in turn affect the subsequent metabolism and disposition of TST. Perturbations in TST metabolism may affect levels of circulating TST with possible reproductive and other consequences, including further modulation of the expression of some P450 proteins.

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¹ Abbreviations used are: P450, cytochrome P450; TST, testosterone; AD, androstenedione; DEET, *N,N*-diethyl-*m*-toluamide; KTST, ketotestosterone; OHAD, hydroxyandrostenedione; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; OHTST, hydroxytestosterone; K_i , inhibition constant; b_5 , cytochrome b_5 .

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Following the Gulf War some veterans reported illnesses which may have been the result of chemical exposures. Some studies of these veterans have concluded that significant correlations between perceived illnesses and chemical use exist (Haley and Kurt, 1997). The reported chemical exposures included the insect repellent *N,N*-diethyl-*m*-toluamide (DEET), insecticides such as permethrin and chlorpyrifos to protect against insect borne diseases and the neuroprotective agent pyridostigmine bromide to protect against possible nerve gas attack. It has been reported that chlorpyrifos and DEET are metabolized by human P450s (Tang et al., 2001; Usmani et al., 2002) and that interactions of Gulf War related chemicals can inhibit or induce the P450s involved in their metabolism (Usmani et al., 2002). Other studies have reported that interaction of Gulf War related chemicals could produce greater than additive toxicity in rats and mice (Chaney et al., 1997; McCain et al., 1997), increased neurotoxicity in hens associated with increased inhibition of brain acetylcholinesterase and Neurotoxicity Target Esterase (Abou-Donia et al., 1996a,b), and neurobehavioral deficit associated with significant inhibition of brainstem acetylcholinesterase activities in rats (Abou-Donia et al., 2001). However, no studies have been carried out to examine the induction or inhibition potential of these or related compounds on human P450-mediated metabolism of steroid hormones, such as TST. An understanding of how Gulf War related chemicals affects the metabolism of TST could aid in the evaluation of the possible role that these chemicals may play in deployment-related illnesses.

The main objectives of present study were to identify human liver P450 isoforms responsible for TST metabolism and the products of their activity using an improved HPLC method, to study the effects of various deployment-related chemicals on the metabolism of TST using HLM and CYP3A4, and to study the effects of the test compounds on human aromatase (CYP19).

Materials and Methods

Chemicals. DEET, chlorpyrifos, chlorpyrifos-oxon, phorate, fonofos, deltamethrin, fipronil, imidacloprid, and permethrin (isomeric mix 78% *trans*-20% *cis*) were purchased from Chem Service (West Chester, PA). Pyridostigmine bromide was purchased from Roche Diagnostics (Indianapolis, IN). 6 α -, 15 β -, 15 α -, 7 α -, 6 β -, 16 α -, 16 β -, 2 α -, 2 β -, 11 β -OHTST, 11-ketotestosterone (11-KTST), 11 β -hydroxyandrostenedione (11 β -OHAD), AD, and 4-hydroxyandrostenedione (4-OHAD) were purchased from Steraloids (Newport, RI). HPLC grade water, methanol, acetonitrile, and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA). TST, 17 β -estradiol, and all other chemicals were purchased, if not specified, from Sigma-Aldrich (St. Louis, MO).

Human Liver Microsomes and Human P450 Isoforms. Pooled human liver microsomes (HLM) (pooled from 21 donors) and human P450 isoforms expressed in baculovirus infected insect cells (Sf9) (BTI-TN-5B1-4), CYP1A1, 1A2, 2B6, 3A4, 3A5, 3A7, 4A11, 2B6, 2C8, 2A6, 2C9*1 (Arg₁₁₄), 2C9*2 (Cys₁₄₄), 2C9*3 (Leu₃₅₉), 2C18, 2C19, 2D6*1 (Val₃₇₄), 2E1, and human aromatase (CYP19) were purchased from BD Gentest Corporation.

In Vitro TST Metabolism. Metabolic activity assays for human P450 isoforms were performed by incubation of TST (final concentrations, 250 μ M) with an NADPH-regenerating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) in specific buffers recommended by the supplier (BD Gentest Corporation). For CYP1A1, 1A2, 2E1, 2C8, 2D6*1 (Val₃₇₄), 3A4, 3A5, 3A7, 2B6, 2C18, 2C19, and an insect cell control, a 100 mM potassium phosphate buffer with 3.3 mM MgCl₂ (pH 7.4) was used. For 2C9*1 (Arg₁₁₄), 2C9*2 (Cys₁₄₄), 2C9*3 (Leu₃₅₉), 4A11, and 2A6, a 100 mM Tris-HCl buffer with 3.3 mM MgCl₂ (pH 7.5) was used. After preincubation at 37°C for 5 min, the reactions were initiated by the addition of ice-cold P450 isoforms (final P450 contents 50 pmol/ml) for 30 min at 37°C. The controls were performed under identical conditions with the insect cell control.

Enzyme kinetic assays for HLM and CYP3A4 were performed by incubation of serial concentrations of TST (final concentrations, 9.375–500 μ M) with

HLM (final protein concentration, 1 mg/ml) or CYP3A4 (final concentration, 50 pmol/ml) in 100 mM potassium phosphate buffer (pH 7.4 at 37°C) containing 3.3 mM MgCl₂. After preincubation at 37°C for 5 min, the reactions were initiated by the addition of ice-cold HLM or CYP3A4 for 10 min.

The effects of test chemicals on TST metabolism were examined in HLM and CYP3A4 after preincubation with test compounds. The HLM (final protein concentration, 1 mg/ml) or CYP3A4 (final concentration, 50 pmol/ml) were incubated with individual test compounds (final concentration, 100 μ M), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 250 μ M).

Range finding assays were conducted for chlorpyrifos, fonofos, and phorate inhibition of TST major metabolites. Varying concentrations of chlorpyrifos, fonofos, and phorate (0.5–100 μ M) were incubated with CYP3A4 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 100 μ M). Reactions were terminated and analyzed as described above. With selected concentration levels based on the range finding assay, the mode of chlorpyrifos, fonofos, and phorate inhibition on TST major metabolites was investigated. For Michaelis-Menten plots, chlorpyrifos (2 μ M), fonofos (5 μ M), and phorate (30 μ M) were incubated with CYP3A4 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 9.375–500 μ M).

To demonstrate whether chlorpyrifos inhibition is reversible or irreversible, incubations with and without chlorpyrifos (2 μ M) were conducted with varying concentrations of CYP3A4 (0.78–6.25 pmol), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 100 μ M).

To determine (inhibition constant) K_i values, chlorpyrifos (1–8 μ M), fonofos (1–25 μ M), and phorate (10–100 μ M) were incubated for 5 min at 37°C with CYP3A4 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, prior to adding TST (final concentrations, 50, 100, or 200 μ M). K_i values were calculated from Dixon plots.

Since cytochrome *b₅* (*b₅*) is not coexpressed with CYP3A5 as supplied by BD Biosciences (San Jose, CA), a comparison of CYP3A5 metabolism of TST was made using 10 pmol 3A5 with and without addition of 20 pmol *b₅*.

Human aromatase (CYP19) catalyzes the conversion of TST to estradiol. To study the effects of the test chemicals on this conversion, test compounds (final concentration, 200 μ M) or a well known competitive inhibitor, 4-OHAD (final concentration, 200 μ M) were incubated with CYP19 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 100 μ M). The reaction was terminated after an additional 10 min, and supernatant was analyzed for 17 β -estradiol concentration by HPLC.

All assays were conducted in triplicate. All reactions were terminated by the addition of an equal volume of methanol and vortexing. After 10-min centrif-

TABLE 1
HPLC retention times for testosterone and hydroxylated testosterone metabolites

Common Name	Chemical Name	Retention Time min
6 α -Hydroxytestosterone	4-Androsten-6 α ,17 β -diol-3-one	14.38
15 β -Hydroxytestosterone	4-Androsten-15 β ,17 β -diol-3-one	15.11
15 α -Hydroxytestosterone	4-Androsten-15 α ,17 β -diol-3-one	15.53
7 α -Hydroxytestosterone	4-Androsten-7 α ,17 β -diol-3-one	15.81
6 β -Hydroxytestosterone	4-Androsten-6 β ,17 β -diol-3-one	16.25
16 α -Hydroxytestosterone	4-Androsten-16 α ,17 β -diol-3-one	17.43
11-Ketotestosterone	4-Androsten-17 β -ol-3,11-dione	18.24
16 β -Hydroxytestosterone	4-Androsten-16 β ,17 β -diol-3-one	19.34
11 β -Hydroxyandrostenedione	4-Androsten-11 β -ol-3,17-dione	19.68
2 α -Hydroxytestosterone	4-Androsten-2 α ,17 β -diol-3-one	20.68
2 β -Hydroxytestosterone	4-Androsten-2 β ,17 β -diol-3-one	21.55
11 β -Hydroxytestosterone	4-Androsten-11 β ,17 β -diol-3-one	21.86
Androstenedione	4-Androsten-3,17-dione	24.92
4-Hydroxyandrostenedione	4-Androsten-4-ol-3,17-dione	27.20
Testosterone	4-Androsten-17 β -ol-3-one	28.90

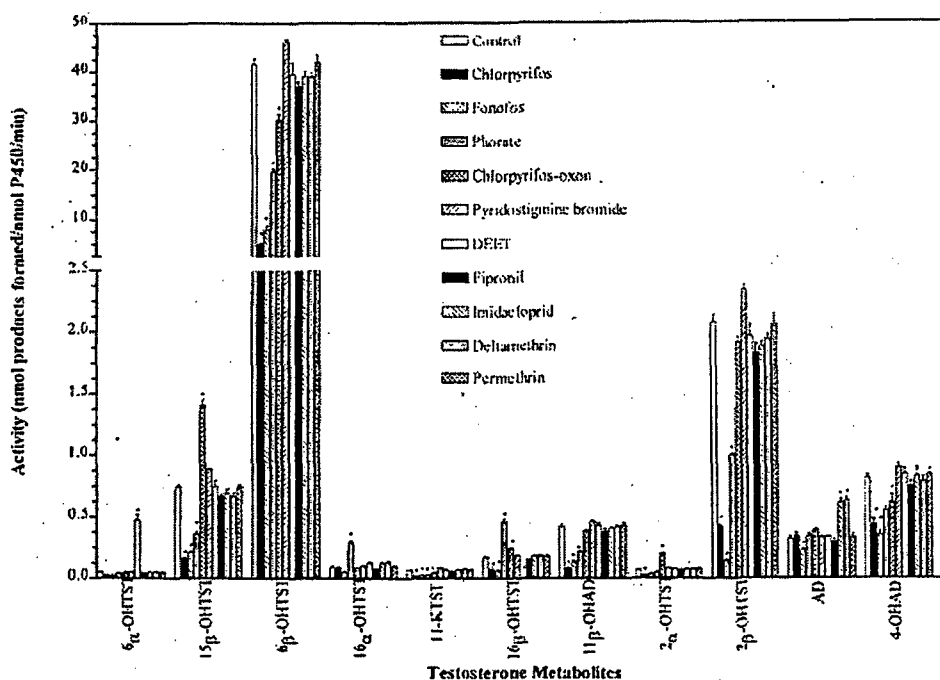


FIG. 1. Effects of deployment-related chemicals on the rate of testosterone metabolism by pooled human liver microsomes.

Specific activities were expressed as nanomole products formed per nanomole P450 per minute. *, statistically significantly different when compared with respective control ($P < 0.01$).

ugation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for TST metabolite concentrations by HPLC. The protein concentrations and incubation times used in the assays were found to be in the linear range in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of a NADPH-generating system.

Analysis of Metabolites by HPLC. Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan). The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of one pump (LC-10AT VP), a four-position solvent selection proportioning valve (FCV-10AL VP), a degasser (DUG-14A), a Shimadzu autoinjector (SIL-10AD VP), and a Shimadzu UV/VIS detector (SPD-10AV VP). All system components were controlled through the Shimadzu powerline firmware. Data were collected via a Shimadzu system controller (SCL-10A VP) and analyzed using CLASS-VP 4.3 software. A reverse phase HPLC method was modified based on the HPLC method of Purdon and Lehman-McKeeman (1997), for the separation of TST and its potential metabolites. The mobile phase for pump A was 5% tetrahydrofuran, 95% water, for pump B 100% methanol. A gradient system was employed in the following manner: 0 to 1 min (30% B), 1 to 10 min (30–60%

B), 10 to 22 min (60–65% B), 22 to 28 min (65–80% B), 28 to 30 min (80–90% B), 30 to 32 min (90% B), 32 to 34 min (90–30% B), and 34 to 36 min (30% B). The flow rate was 0.5 ml/min. Metabolites were separated by a Prodigy column [Prodigy 3 μ , 150 $\times$ 4.6 mm, ODS (3), 100A; Phenomenex, Rancho Palos Verdes, CA] and detected at 247 nm. A summary of the retention times of TST and 14 TST metabolites are presented in Table 1. The limits of detection for most of TST metabolites were approximately 0.04 μ M except for 6 β -OHTST (0.15 μ M) and 4-OHAD (0.30 μ M). Standards of TST metabolites were made in methanol and 50- μ l standard or sample injected on HPLC. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. Percentages of individual metabolites are expressed on the basis of the total metabolites produced by the isoform or preparation in question.

For 17 β -estradiol, the mobile phase was 60% H₂O and 40% acetonitrile. TST and 17 β -estradiol were eluted isocratically at a flow rate of 1.0 ml/min for 15 min, separated by a Prodigy column [Prodigy 3 μ , 150 $\times$ 4.6 mm, ODS (3), 100A, Phenomenex, Rancho Palos Verdes, CA] and detected at 200 nm. The retention time of 17 β -estradiol and TST was 10.4 and 11.3 min, respectively.

TABLE 2

Testosterone hydroxylation by human cytochrome P450 isoforms expressed in baculovirus-infected insect cells (nanomoles per nanomole isoforms per minute)

Isoforms	6 α -OHTST	15 β -OHTST	6 β -OHTST	16 α -OHTST	11-KT	16 β -OHTST	11 β -OHAD	2 α -OHTST	2 β -OHTST	AD	4-OHAD
1A1	NDA	NDA	3.01 $\pm$ 0.14	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
1A2	NDA	NDA	0.64 $\pm$ 0.02	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
2A6	0.05 $\pm$ 0.00	0.14 $\pm$ 0.01	NDA	NDA	NDA	NDA	NDA	NDA	NDA	0.53 $\pm$ 0.05	NDA
3A4	0.22 $\pm$ 0.02	3.18 $\pm$ 0.11	157.7 $\pm$ 6.00	NDA	1.04 $\pm$ 0.04	0.44 $\pm$ 0.01	1.70 $\pm$ 0.02	0.19 $\pm$ 0.01	7.05 $\pm$ 0.23	0.27 $\pm$ 0.01	2.23 $\pm$ 0.24
3A5	0.11 $\pm$ 0.01	NDA	12.4 $\pm$ 1.57	NDA	NDA	0.08 $\pm$ 0.01	0.14 $\pm$ 0.02	NDA	0.74 $\pm$ 0.10	NDA	NDA
3A7	0.09 $\pm$ 0.01	0.15 $\pm$ 0.02	3.89 $\pm$ 0.34	NDA	0.15 $\pm$ 0.02	0.27 $\pm$ 0.02	0.79 $\pm$ 0.05	3.05 $\pm$ 0.24	0.61 $\pm$ 0.06	0.13 $\pm$ 0.00	NDA
4A11	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	0.62 $\pm$ 0.01	NDA
2B6	0.05 $\pm$ 0.01	NDA	0.23 $\pm$ 0.01	0.17 $\pm$ 0.00	0.13 $\pm$ 0.03	0.61 $\pm$ 0.12	NDA	NDA	0.03 $\pm$ 0.00	NDA	NDA
2C8	NDA	NDA	NDA	0.38 $\pm$ 0.03	0.14 $\pm$ 0.03	NDA	NDA	NDA	NDA	NDA	NDA
2C9*1	NDA	NDA	NDA	NDA	NDA	0.18 $\pm$ 0.01	NDA	NDA	NDA	NDA	NDA
2C9*2	NDA	NDA	NDA	NDA	NDA	0.11 $\pm$ 0.02	NDA	NDA	NDA	NDA	NDA
2C18	0.05 $\pm$ 0.01	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
2C19	0.05 $\pm$ 0.00	NDA	0.43 $\pm$ 0.04	0.15 $\pm$ 0.01	NDA	0.32 $\pm$ 0.05	NDA	NDA	0.04 $\pm$ 0.01	2.53 $\pm$ 0.28	NDA
2E1	NDA	NDA	NDA	NDA	0.15 $\pm$ 0.03	NDA	NDA	NDA	NDA	NDA	NDA
2D6*1	NDA	NDA	1.49 $\pm$ 0.02	NDA	NDA	NDA	NDA	NDA	0.06 $\pm$ 0.00	1.40 $\pm$ 0.08	NDA

NDA, no detectable activity; no metabolite was formed with 2C9*3.

TABLE 3

Kinetic parameters for the production of major testosterone metabolites by human liver microsomes and CYP3A4

Means in the same column followed by the same letter are not significantly different ($P < 0.01$). Values are the mean $\pm$ S.E.M. ($n = 3$).

	Human Liver Microsomes				CYP3A4			
	K_m	V_{max}	CL_{int}	R^2	K_m	V_{max}	CL_{int}	R^2
	μM	nmol/mg protein/min	$\mu l/mg$ protein/min		μM	nmol/nmol 3A4/min	$\mu l/nmol$ 3A4/min	
6 β -OHTST	120.4 $\pm$ 19.4 ^a	36.3 $\pm$ 2.3 ^a	300.0 ^a	0.94	107.7 $\pm$ 12.5 ^a	284.8 $\pm$ 12.5 ^a	2600.0 ^a	0.97
2 β -OHTST	119.2 $\pm$ 18.0 ^a	2.0 $\pm$ 0.1 ^b	20.0 ^b	0.95	122.8 $\pm$ 14.1 ^a	15.7 $\pm$ 0.7 ^b	130.0 ^b	0.98
15 β -OHTST	138.9 $\pm$ 28.2 ^a	0.8 $\pm$ 0.1 ^c	6.0 ^c	0.91	108.7 $\pm$ 16.4 ^a	7.1 $\pm$ 0.4 ^c	70.0 ^c	0.96

The limit of detection for 17 β -estradiol was approximately 0.10 μM . Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve.

Data Analysis and Statistics. The apparent K_m and V_{max} parameters were calculated using nonlinear regression analysis program (Prism, GraphPad software Inc., San Diego, CA), and the K_i values were estimated by nonlinear regression analysis from the Dixon plot (Segel, 1975) using SigmaPlot Enzyme Kinetics Module (Chicago, IL). Significant differences between data sets were determined by one-way analysis of variance, and multiple comparisons were performed with the Dunnett's method using a JMP 4.0.2, SAS program (SAS, 1989).

Results

Four major metabolites were formed after incubation of TST with pooled HLM: 6 β -, 2 β -, 15 β OHTST, and 4-OHAD as well as seven minor metabolites (Fig. 1). Among 16 different human P450 isoforms screened, only 2C9*3 (Leu₃₅₉) had no detectable activity toward TST (Table 2). All other P450 isoforms were active in generating one or more than one TST metabolites, although the extent of metabolism and the ratios of metabolites varied widely among isoforms. In this comparison of metabolite production by equal quantities of each isoform, CYP3A4, 3A5, and 3A7 were most active in TST metabo-

lism among all the P450 isoforms tested (93.5% of the metabolites produced by all isoforms). Among members of the CYP3A subfamily, CYP3A4 produced the highest amount of total TST metabolites (88.5%) compared with 3A5 (6.9%) and 3A7 (4.6%). 6 β -OHTST, the most prominent TST metabolite, mainly produced by the CYP3A subfamily, accounts for 86% of all TST metabolites. Among the CYP3A subfamily, CYP3A4 produced the highest amount of 6 β -OHTST (90.6%) compared with 3A5 (7.1%) and 3A7 (2.2%). Other major TST metabolites formed by CYP3A4 were 15 β -, 2 β -OHTST, and 4-OHAD, whereas 6 α -, 16 β -, 11 β -, 2 α -OHTST, 11-KTST, and AD were minor metabolites. Among the P450 isoforms tested, CYP3A5 and 3A7 were significantly more important in forming the major TST metabolites than most of the others, but their activity was 10- to 20-fold less than that of CYP3A4. Interestingly, CYP3A7 produced 16 times more 2 α -OHTST than CYP3A4. CYP1A1 is involved in the oxidation of TST at the 6 β -position (3.0 nmol/nmol isoform/min), whereas CYP1A2 oxidized TST poorly at the 6 β -position (0.6 nmol/nmol isoform/min). As can be observed in Table 2, the other P450 isoforms tested generally produced small amounts of one or more TST metabolites. CYP2C19 metabolized TST to AD more actively

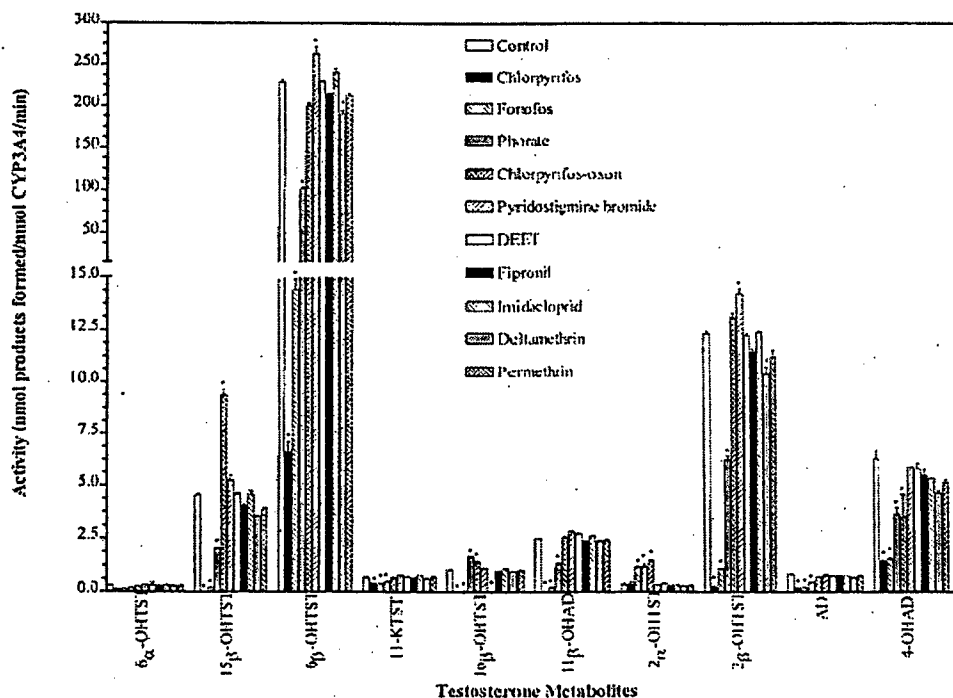


FIG. 2. Effects of deployment-related chemicals on the rate of testosterone metabolism by CYP3A4.

Specific activities were expressed as nanomole products formed per nanomole CYP3A4 per minute. *, statistically significantly different when compared with respective control ($P < 0.01$).

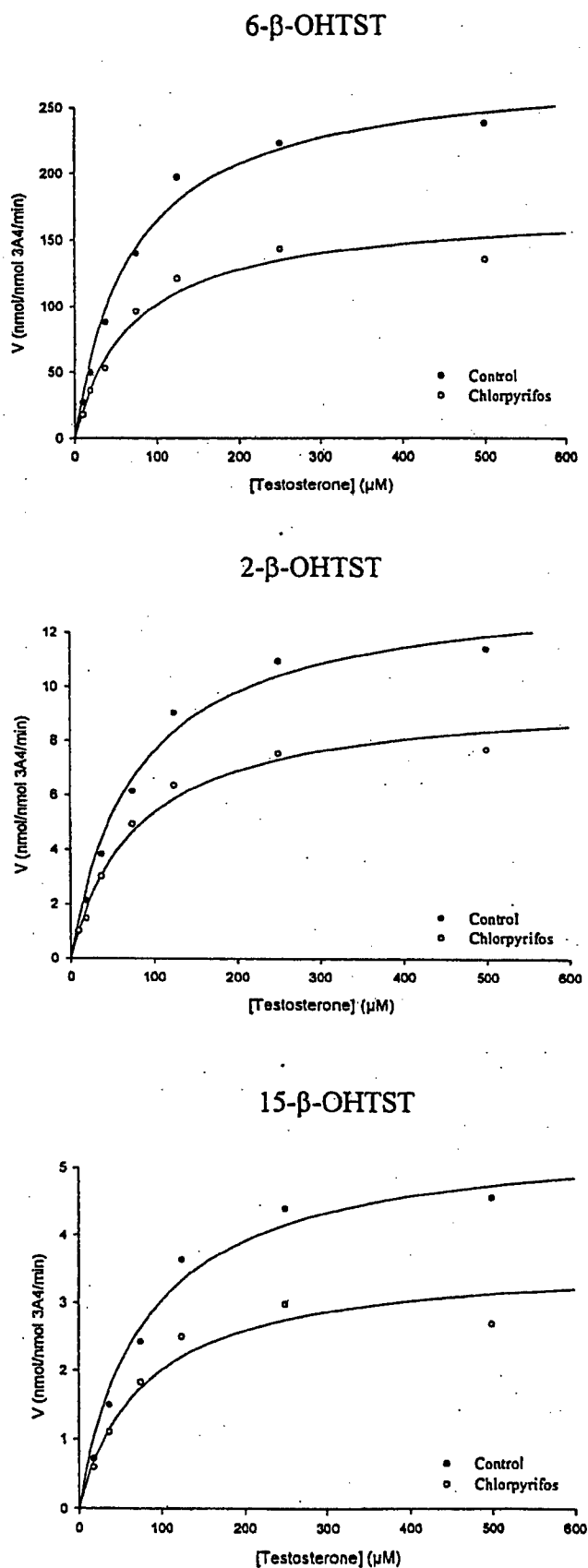


FIG. 3. Representative Michaelis-Menten plots for the inhibition of CYP3A4-mediated testosterone hydroxylation by chlorpyrifos (2 μ M).

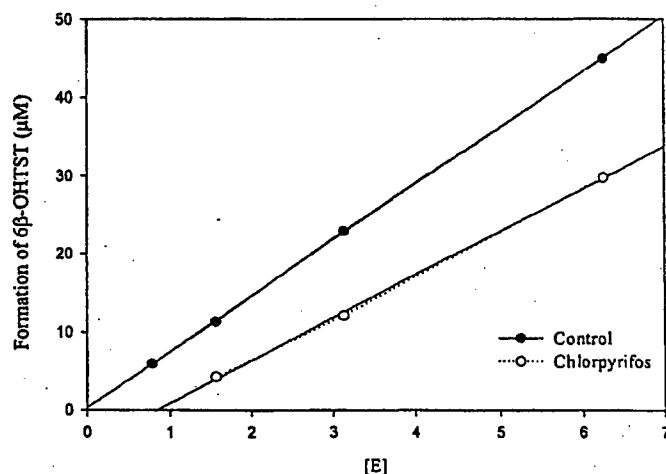


FIG. 4. Representative plot of V_{max} versus amount of enzyme (CYP3A4) added to distinguish between a reversible and an irreversible noncompetitive inhibitor chlorpyrifos (2 μ M).

than any other isoform tested, whereas it catalyzed the formation of 6 α -, 6 β -, 16 α -, 16 β -, and 2 β -OHTST poorly.

HLM and CYP3A4 displayed similar K_m values for 6 β -, 2 β -, and 15 β -OHTST (Table 3). V_{max} and intrinsic clearance rate [Cl_{int} (V_{max}/K_m)] for 6 β -OHTST was significantly higher than 2 β -OHTST (~18-fold) and 15 β -OHTST (~40-fold), respectively.

The effects of various deployment-related chemicals on TST metabolism were investigated by preincubating them with pooled HLM (Fig. 1). Preincubation of pooled HLM with chlorpyrifos, phorate, and fonofos resulted in significant inhibition of 6 β -, 2 β -, 15 β -OHTST, 11-KTST, 11 β -OHAD, and 4-OHAD. Preincubation of pooled HLM with DEET, chlorpyrifos-oxon, phorate, imidacloprid, and deltamethrin in some cases caused small but significant increases in the production of some TST metabolites by HLM.

Preincubation of CYP3A4 with a variety of chemicals resulted in varying levels of activation and inhibition of TST metabolism (Fig. 2). The greatest inhibition of TST metabolism was observed for the organophosphorus compound chlorpyrifos with up to 98% inhibition of major (6 β -, 2 β -, 15 β -OHTST, and 4-OHAD) and several minor (11-KTST, 16 β -OHTST, 11 β -OHAD, and AD) TST metabolites. However, chlorpyrifos-oxon, an active metabolite of chlorpyrifos, has no inhibitory effect on the major TST metabolites. Two other organophosphorus compounds, phorate and fonofos, also significantly inhibited formation of several TST metabolites including 6 β -, 2 β -, 15 β -OHTST, 11-KTST, 11 β -OHAD, AD, and 4-OHAD. In contrast, preincubation of CYP3A4 with pyridostigmine bromide resulted in the production of small but significantly greater levels of the 6 β - and 2 β -OHTST metabolites. Some other TST metabolites were also significantly increased by preincubation of CYP3A4 with chlorpyrifos-oxon, phorate, and fonofos.

To investigate the type of inhibition of CYP3A4 by chlorpyrifos, fonofos, and phorate on major TST metabolites, chlorpyrifos (2 μ M), fonofos (5 μ M), and phorate (30 μ M) were preincubated for 5 min before adding the varying concentrations of TST. Michaelis-Menten plots showed that the V_{max} values were significantly reduced without affecting K_m values, indicative of a noncompetitive inhibition of major TST metabolites by chlorpyrifos (Fig. 3). Similar results were obtained with fonofos and phorate (data were not shown). Further investigation of noncompetitive reversible or nonreversible inhibition data revealed that the inhibition is nonreversible (Fig. 4).

The K_i , an indicator of inhibitor affinity to target enzyme, was calculated by Dixon plot (Table 4; Fig. 5). Chlorpyrifos was the most

TABLE 4

Kinetics parameters for the inhibition of CYP3A4-mediated production of major testosterone metabolites by chlorpyrifos, fonofos, and phorate

Inhibitors	6 β -OHTST		2 β -OHTST		15 β -OHTST	
	K_i	R^2	K_i	R^2	K_i	R^2
Chlorpyrifos	2.0 $\pm$ 0.2	0.99	3.6 $\pm$ 0.3	0.98	3.7 $\pm$ 0.4	0.97
Fonofos	5.8 $\pm$ 0.6	0.98	10.1 $\pm$ 0.7	0.98	6.3 $\pm$ 0.6	0.97
Phorate	34.1 $\pm$ 2.7	0.98	42.9 $\pm$ 4.1	0.98	33.8 $\pm$ 3.6	0.97

potent inhibitor of major TST metabolites with K_i values ranges from 2.0, 3.6, and 3.7 μ M for 6 β -, 2 β -, 15 β -OHTST, respectively. Fonofos was the second best inhibitor with K_i values ranging from 5.8, 10.1, and 6.3 μ M for 6 β -, 2 β -, 15 β -OHTST, respectively. Phorate K_i values ranged from 34.1, 42.9, and 33.8 μ M for 6 β -, 2 β -, 15 β -OHTST, respectively.

We investigated the possibility that b_2 may stimulate CYP3A5 catalytic activity by incubating b_2 (20 pmol) and CYP3A5 (10 pmol), which, in the preparations used, does not have b_2 coexpressed, with 250 μ M of TST for 10 min. Addition of b_2 resulted in a more than 2-fold increase in TST 6 β - and 2 β -OHTST activity.

The possibility that conversion of TST to estradiol, which is catalyzed by aromatase (CYP19), could be inhibited by the test compounds was also investigated. Preincubation of human aromatase (CYP19) with various chemicals (chlorpyrifos, chlorpyrifos-oxon, permethrin, pyridostigmine bromide, DEET, phorate, fonofos, fipronil, imidacloprid, and deltamethrin) had no significant effect on the production of estradiol (data not shown). However, incubation with 4-OHAD, a well known competitive aromatase inhibitor, resulted in 90% inhibition of the aromatase enzyme activity.

Discussion

P450-dependent hydroxylation appears to be a major pathway of oxidative metabolism of TST in mammalian liver. Studies carried out using human P450 isoforms provide further insight into the range of TST hydroxylation reactions that can be catalyzed by human P450 enzymes. Our isoform data corroborates earlier findings (Waxman et al., 1988, 1991; Yamazaki and Shimada, 1997) that CYP3A4 is one of the major isoforms responsible for TST metabolism, and 6 β -OHTST is the major TST metabolite. Greater than 82% of the TST metabolites are formed by CYP3A4, and 87% of the major 6 β -OHTST metabolite is formed by CYP3A4. The mean metabolic intrinsic clearance rates, as estimated by V_{max}/K_m , also indicated that 6 β -OHTST is the major metabolite of TST. Interestingly, CYP3A4 also metabolized TST to 4-OHAD, a potent inhibitor of extrahepatic aromatase (CYP19). It has been reported that 4-OHAD was able to inhibit 90% of the aromatase activity at a concentration of 1 μ M (Mak et al., 1999). The physiological significance or consequence of this reaction is unclear and will require further investigation. Our results indicate that CYP1A1 and 1A2 were able to metabolize TST to 6 β -OHTST, however, activity of CYP1A1 was much higher (4.7-fold) than CYP1A2. Consistent with a previous report (Yamazaki and Shimada, 1997), our data also indicated that CYP2C19 catalyzed oxidation of TST to form AD as a major TST metabolite. However, CYP2C18, which has 81% amino acid sequence identity to CYP2C19, exhibited distinctly poor hydroxylation activity in comparison with CYP2C19. Furthermore, our data indicated that CYP2D6*1, 4A11, and 2A6 metabolized TST to form AD but not as actively as CYP2C19. Guengerich et al. (2002) characterized the affinity of CYP2D6 for testosterone.

Endogenous steroids, such as TST, always exist in vivo, and considerable amounts of these steroids are metabolized by the P450s expressed in the human liver, where foreign compounds are mainly

metabolized. If xenobiotics substantially affect TST metabolism, it may alter the rate of TST metabolism, which may ultimately disrupt TST homeostasis. Preincubation of pooled HLM with organophosphorus compounds, such as chlorpyrifos, phorate, and fonofos, resulted in the extensive inhibition of major and some minor TST

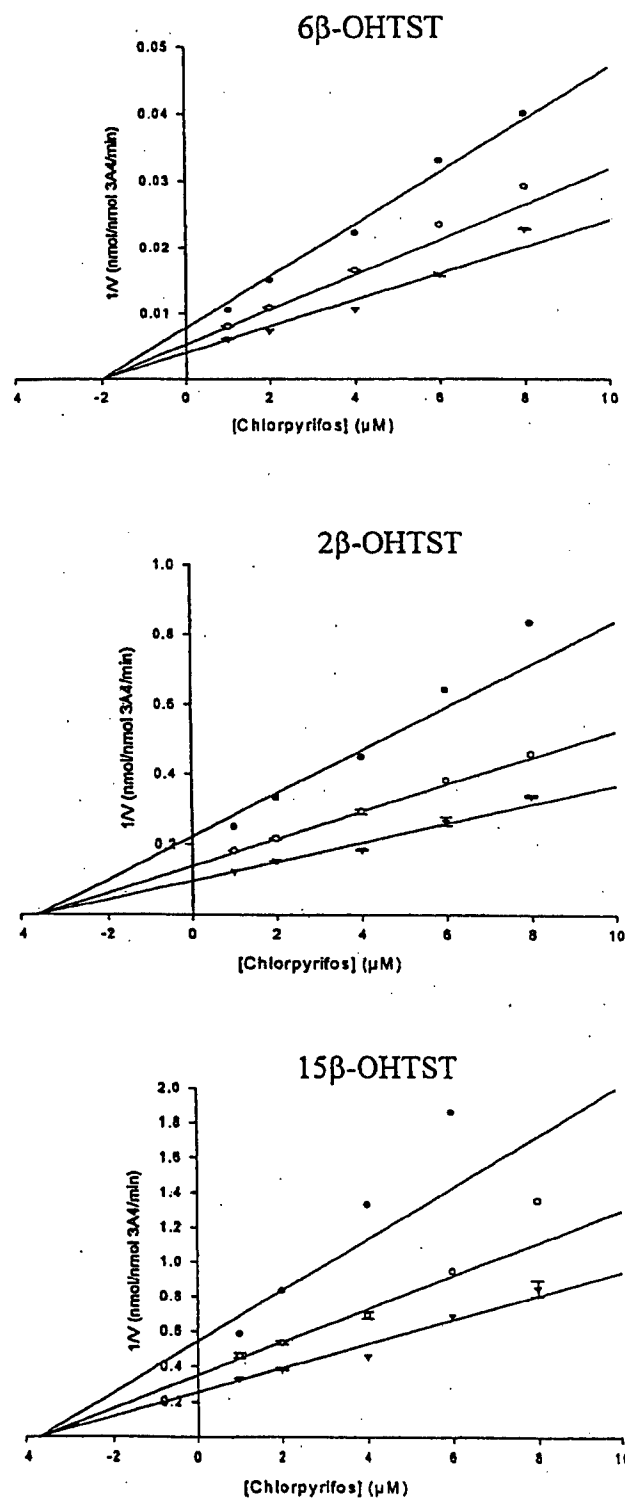


FIG. 5. Representative Dixon plots for the inhibition of CYP3A4-mediated testosterone hydroxylation by chlorpyrifos.

Testosterone concentrations were 50 μ M (●), 100 μ M (○), and 200 μ M (▼).

metabolites. Chlorpyrifos, fonofos, and phorate inhibited major TST metabolites noncompetitively and irreversibly, and it is clear that organophosphorus compounds are some of the most potent inhibitors of the CYP3A4-dependent oxidation of TST yet described. Organophosphorus pesticides, such as chlorpyrifos, phorate, and fonofos are activated by a P450-catalyzed desulfuration reaction (Fukuto, 1990). The sulfur atom released from these pesticides in this reaction is highly reactive and is believed to bind immediately to the heme iron of P450 and inhibit its activity (Neal, 1980). On the other hand, enzyme stimulation is a process by which direct addition of one chemical to an enzyme stimulates the rate of reaction of the substrate (Guengerich, 1997). Our data indicated that some compounds, such as pyridostigmine bromide, DEET, chlorpyrifos-oxon, phorate, imidacloprid, and deltamethrin may stimulate the production of some of the TST metabolites.

Several studies, including this, have shown that CYP3A4 is the major P450 involved in the metabolism of TST in human liver microsomes (Waxman et al., 1988, 1991; Yamazaki and Shimada, 1997). Either inhibition or induction can modulate the activity of an enzyme; P450s may exhibit stimulation or inhibition in the presence of certain xenobiotic compounds (Guengerich, 1997; Szklarz and Halpert, 1998). It has been suggested that CYP3A4 is an allosteric enzyme, even though the identity of the allosteric site is not known (Shimada and Guengerich 1989; Lee et al., 1995). In addition, little is known about the active site topology of CYP3A4, although it is generally recognized that the active site of this enzyme has the capacity to accommodate large molecules and even more than one substrate (Shou et al., 1994). Inhibition may, in some interactions, be more serious than enzyme induction since inhibition happens more rapidly, not taking time to develop, as with induction (Guengerich, 1997). Preincubation of CYP3A4 with chlorpyrifos resulted in almost complete inhibition of major TST metabolites. The K_i value indicated that chlorpyrifos is one of the most potent inhibitors yet shown for the production of major TST metabolites. This inhibition was not due to inhibition by the metabolite, chlorpyrifos-oxon, since the latter had no inhibitory effect on the production of the major TST metabolites. Phorate and fonofos also inhibited the production of major and some minor metabolites of TST. The K_i value indicated that fonofos was a much better inhibitor of major TST metabolites than phorate. The possibility exists that inhibition of CYP3A4 may lead to higher levels of TST and may alter hormonal properties. However, in vivo studies are necessary to understand the impact of these changes. Preincubation with pyridostigmine bromide resulted in higher production of 6 β - and 2 β -OHTST, suggesting stimulation of CYP3A4. Preincubation with chlorpyrifos-oxon, phorate, and fonofos with CYP3A4 also resulted in activation of the production of some TST metabolites. A number of in vivo studies in rodents have shown that organochlorine pesticides increased the overall rate of TST metabolism (Cassidy et al., 1994; Wilson and LeBlanc, 1998; Dai et al., 2001).

Several studies have demonstrated that simultaneous expression of CYP3A4 and P450 reductase in bacterial or baculovirus-based insect cell membranes can produce high catalytic activity for TST 6 β -OHTST in the absence of b_5 (Guengerich and Johnson 1997; Shaw et al., 1997), although addition of b_5 to the system can enhance the reaction rates (Yamazaki et al., 1999). In contrast to the CYP3A4 used in these experiments, cytochrome b_5 was not coexpressed in CYP3A5. A comparison of CYP3A5 with and without the addition of exogenous b_5 demonstrated a 2-fold increase in the activity of 6 β - and 2 β -OHTST in the presence of b_5 .

Human aromatase (CYP19), an extrahepatic P450, catalyzes the conversion of TST via three hydroxylation steps to estradiol. Inhibitors of aromatase currently in use have received considerable attention

as treatments for postmenopausal breast cancer and other estrogen-dependent diseases (Bordie et al., 1999). Endocrine disruptors are hormone mimics that modify hormonal action in humans. Currently, inhibitors of human aromatase have been identified as potential endocrine disruptors or environmental toxicants (Mak et al., 1999). The chemicals used in this study have no significant effect on the activity of aromatase.

In conclusion, the hydroxylation of TST by P450 isoforms indicates important functions for these enzymes other than detoxification of xenobiotics. The present study provided further insight into the range of TST hydroxylation reactions that can be catalyzed by different human P450 isoforms. The deployment-related chemicals used in this study, including pesticides, caused a marked modification of P450-mediated TST metabolism in vitro. Organophosphorus pesticides were very potent inhibitors of the production of the primary metabolites of CYP3A4 and inhibited major TST metabolites noncompetitively and irreversibly. Addition of b_5 to CYP3A5 increased the catalytic activity of this enzyme. Preincubation of the test chemicals had no effect on the production of estradiol from TST.

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Chloropyrifos Oxon and Carbaryl Inhibition of *trans*-Permethrin Hydrolysis in Human Liver Fractions

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ABSTRACT

Permethrin is a pyrethroid insecticide that was used in the Gulf War along with other deployment-related chemicals such as chlorpyrifos, pyridostigmine bromide and N,N-diethyl-*m*-toluamide. In this study the inhibitory effects of chlorpyrifos on *trans*-permethrin hydrolysis in humans was investigated using human liver fractions. Chlorpyrifos oxon (CPO), a major reactive metabolite of chlorpyrifos, strongly inhibits *trans*-permethrin hydrolysis in human liver fractions while pyridostigmine bromide and N,N-diethyl-*m*-toluamide has no inhibitory effect on *trans*-permethrin hydrolysis. Complete inhibition by CPO indicates that the esterases involved in *trans*-permethrin hydrolysis are B-esterases, which are inhibited by organophosphorus anticholinesterase agents. CPO shows irreversible inhibition reflecting the known mechanism of organophosphorus anticholinesterase agents, namely; covalent modification of the active site of the esterases in question. Carbaryl, another known anticholinesterase agent showed non-competitive inhibition kinetics with a K_i two orders of magnitude higher than that for CPO. This agrees with previous reports that, compared to organophosphorus compounds, carbaryl is a reversible inhibitor and, as a result, the inhibition is less persistent. In contrast to CPO inhibition, *trans*-permethrin hydrolysis was not completely inhibited by carbaryl even at high concentrations, leading to the assumption that at least two B esterases are involved in *trans*-permethrin hydrolysis in human liver fractions, one with high sensitivity to carbaryl inhibition and the other(s) with lower or no sensitivity to carbaryl inhibition. This study suggests that potential interactions with organophosphorus chemicals should be considered when permethrin toxicity is being assessed in regard to occupational or deployment-related exposure.