MALATHION BIOACTIVATION IN THE HUMAN LIVER: THE CONTRIBUTION OF DIFFERENT CYTOCHROME P450 ISOFORMS

Franca M. Buratti, Alessandra D’Aniello, MariaTeresa Volpe, Annarita Meneguz, and Emanuela Testai

Environment and Primary Prevention Department (F.M.B., A.D.A., E.T.) and Drug Research and Evaluation Department (M.T.V., A.M.), Istituto Superiore di Sanità, Rome, Italy

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ABSTRACT:

Among organophosphorothioate (OPT) pesticides, malathion is considered relatively safe for use in mammals. Its rapid degradation by carboxylesterases competes with the cytochrome P450 (P450)-catalyzed formation of malaoxon, the toxic metabolite. However, impurities in commercial formulations are potent inhibitors of carboxylesterase, allowing a dramatic increase in malaoxon formation. Malathion desulfuration has been characterized in human liver microsomes (HLMs) with a method based on acetylcholinesterase inhibition that is able to detect nanomolar levels of oxon. The active P450 isoforms have been identified by means of a multifaceted strategy, including the use of cDNA-expressed human P450s and correlation, immunoinhibition, and chemical inhibition studies in a panel of phenotyped HLMs. HLMs catalyzed malaoxon formation with a high level of variability (>200-fold). One or two components (Kmapp1 = 53–67 μM; Kmapp2 = 427–1721 μM) were evidenced, depending on the relative specific P450 content. Results from different approaches indicated that, at low malathion concentration, malaoxon formation is catalyzed by CYP1A2 and, to a lesser extent, 2B6, whereas the role of 3A4 is relevant only at high malathion levels. These results are in line with those found with chlorpyrifos, diazinon, azinphos-methyl, and parathion, characterized by the presence of an aromatic ring in the molecule. Since malathion has linear chains as substituents at the thiocarbonyl sulfur, it can be hypothesized that, independently from the chemical structure, OPTs are bioactivated by the same P450s. These results also suggest that CYP1A2 and 2B6 can be considered as possible metabolic biomarkers of susceptibility to OPT-induced toxic effects at actual human exposure levels.

Malathion is one of the more frequently used organophosphoro-thioate (OPT) insecticides in the world, both in agriculture and in residential settings; in addition, it has been used in malaria eradication programs in Africa and Central America or in wide-scale pest control, including the Mediterranean fruit fly in the United States, through aerial applications. The reason for such widespread use lies in its relatively low toxicity to mammals and high selectivity toward insects, paralleled by a moderate persistence in the environment, when compared with other OPTs (Wauchope et al., 1992). Indeed, mammalian carboxylesterases catalyze rapid degradation of malathion to monoacid and diacid derivatives (Kutz et al., 1992; USEPA, 2000); the reaction efficiently competes with the cytochrome P450-catalyzed formation of malathion phosphate triester malaoxon, which in turn can be degraded by carboxylesterase, and dimethylphosphates, a detoxification product (Fig. 1).

Malathion toxicity, in a manner similar to all OPTs, depends on its bioactivation to the toxic metabolite malaoxon (Thompson et al., 1979a,b); in these conditions, the major pathway of malathion metabolism is the cytochrome P450-catalyzed bioactivation, resulting in a dramatic increase in the production of malaoxon.

In recent years a number of papers have been published on the activity of human P450s involved in the biotransformation of some members of the OPT class (Butler and Murray, 1997; Mutch et al., 1999; Kappers et al., 2001; Tang et al., 2001; Vitozzi et al., 2001; Buratti et al., 2002, 2003). It has been demonstrated with human liver microsomes and recombinant human P450s that at low pesticide concentrations (<10 μM), azinphos-methyl, chlorpyrifos, diazinon, and parathion are mainly desulfurated by CYP1A2 (Buratti et al., 1989), causing the accumulation of acetylcholine within synapses and the consequent overstimulation of postsynaptic receptors. Impurities, such as isomalathion and various trimethylphosphorothioate esters, present in the technical grade malathion or formed during storage, can potentiate malathion-induced toxicity up to 10-fold (Pellegrini and Santi 1972; Aldridge et al., 1979) and have been considered responsible for other effects, including DNA lesions (Flessel et al., 1993; Blasiak et al., 1999). The presence of isomalathion in commercial formulations was implicated in the 1976 epidemic malathion poisoning of 2800 spraymen (including 5 deaths) among 7500 field workers, during the Pakistan malaria control program (Baker et al., 1978). The effect has been attributed to the potent inhibition exerted by isomalathion on hepatic and serum carboxylesterase activity (Aldridge et al., 1979; Talcott et al., 1979a,b); in these conditions, the major pathway of malathion metabolism is the cytochrome P450-catalyzed bioactivation, resulting in a dramatic increase in the production of malaoxon.

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ABBREVIATIONS: OPT, organophosphorothioate; P450, cytochrome P450 isoform; HLM, human liver microsome; CLi, intrinsic clearance; AChE, acetylcholinesterase; iso-OMPA, tetraisopropyl-pyrophosphoramide; Ab, antibody.
Malathion (purity 99%) and malaoxon (purity 88%) were obtained from Chem Service Inc. (West Chester, PA). Roche Diagnostics (Mannheim, Germany) supplied NADPH, NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase; Triton X-100, dithiobisnitrobenzoic acid, and acetylthiocholine were purchased from Fluka (Buchs, Switzerland). Tetra-raisopropyl-pyrophosphoramide (iso-OMPA) and isoform-selective cytochrome P450 inhibitors furafylline, troleandomycin, orphenadrine, caffeine, and paraxantine were supplied by Sigma-Aldrich (St. Louis, MO). Defatted bovine serum albumin was obtained from Serva (Heidelberg, Germany). Dextromethorphan, 3-methoxymorphinan, S-mephenytoin, nirvanol, S-4-hydroxymephenytoin, and anti-human CYP1A2, 1A1, 2A6, 2B6, 2D6, 2E1, and 3A4 antibodies (Abs) for immunoinhibition studies were purchased from BD-Gentest (Woburn, MA); according to the supplier, Abs show no cross-activities to one another. All other analytical grade chemicals were obtained from commercially available sources.

Animals, Recombinant Human P450, and Human Liver Microsomes (HLMs). Sprague-Dawley rats weighing 150 to 200 g were purchased from Charles River Italica (Calco, Italy). The animals were maintained in an air-conditioned room and were allowed free access to standard chow and tap water for a week before starting the experiment. Rats were killed by decapitation, and the whole brain was immediately taken. The European Community Directive (86/609/EEC) and Italian National rules on animal care and use have been followed.

Supersomes, that is, microsomal fractions prepared from baculovirus-infected insect cells expressing single cDNA-derived human CYP (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4), were purchased from BD-Gentest (Woburn, MA). Control Supersomes were prepared from uninfected cells. The protein content of different preparations was in the range of 3.8 to 12 mg/ml; cytochrome P450 content, determined by the supplier, was 1 nmol P450/ml.

HLMs were prepared from a single liver sample (HLM-), a kind gift of the University of Washington (Seattle, WA) (Buratti et al., 2003). Protein concentration (23.2 mg/ml) and P450 content (0.56 nmol/mg protein) were measured according to the methods of Oyama and Eagle (1956) and Omura and Sato (1964), respectively.

Alternatively, HLMs from hepatic biopsies, either individual (HLM-), HLM-pooled or pooled from five donors (HLM-), were purchased from Human Biologics International Corp. (Scottsdale, AZ). Protein concentration was 20 mg/ml; cytochrome P450 content ranged between 0.14 and 0.82 nmol/mg protein. HLMs were fully characterized by the supplier for monooxygenase activities by using selective model substrates for each single P450. The range and mean value of monooxygenase activities of all tested HLMs are reported in Table 2. The same marker activities for CYP1A2, 1A1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 were measured according to the methods of Oyama and Eagle (1956) and Omura and Sato (1964), respectively.

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Preparation of Soluble AChE. Extraction of the enzyme was carried out as described by Meneguz et al. (1989) with minor modification. Briefly, brain was
homogenized at 4°C in 0.03 M Tris-HCl buffer, pH 8.5, (tissue/buffer ratio 1:10). The homogenate was centrifuged at 100,000g for 60 min at 4°C to obtain a cytosolic supernatant containing the AChE-soluble form. AChE activity was determined in each preparation by the method of Ellman et al. (1961) and was in the range 0.5 to 1 mmol acetylcholine hydrolyzed - min^-1 g tissue^-1.

Malathion Production from Malathion. Enzymatic Incubation. The standard incubation mixture (0.5 ml) contained 2 mM glucose 6-phosphate, 2 mM MgCl₂, 2 to 4 U/ml glucose-6-phosphate dehydrogenase, 1 mM NADP, in 50 mM Tris-HCl, 1 mM EDTA (pH 7.4). When incubations with cDNA-expressed P450s were carried out, 15 to 20 µl of AChE (corresponding to 1 to 1.5 µmol of acetylcholine hydrolyzed/min) were added, followed by the addition of malathion as 10 µM of melathion solution at different concentrations, to obtain a substrate final concentration in the range 0.01 to 50 µM malathion. The mixture was preincubated at 37°C for 3 min before starting the reaction by adding Supersomes (25–50 pmol of P450/ml). CYP2E1 could not be tested, due to the strong inhibitory effect exerted on this recombinant isoform by very low levels of organic solvents, including methanol, in which malathion was dissolved. Different P450 concentrations (10, 25, 40, 50, or 100 pmol of P450/ml) and incubation times (1, 2, 3, 5, or 10 min) were used in preliminary studies to verify the linearity of the reaction versus enzyme concentration and time. When incubations with HLMs were carried out, the standard incubation mixture contained 0.3 to 1 mg of protein/ml and 50 µM iso-OMPA, added to inhibit nonspecific esterases, the presence of which was assessed in standard incubations carried out in the absence of both AChE and pesticide. The optimal inhibiting conditions to be used (i.e., iso-OMPA concentration and time of preincubation) were previously determined (Buratti et al., 2003). The mixture was preincubated at 37°C for 15 min; then, 15 to 20 µl of AChE were added, and the reaction was started with 10 µl of malathion methanolic solution (10–500 µM malathion as final concentration). Different protein concentrations (0.2, 0.4, 0.5, 0.7, and 1 mg/ml) and incubation times (1, 2, 5, 10, or 20 min) were used in preliminary studies to verify the linearity of the reaction versus enzyme concentration and time.

The enzymatic incubations both with Supersomes and HLMs were carried out at 37°C under shaking (80 cycles/min) and stopped 2 min after by adding 0.5 ml of cold incubation buffer, containing 8% Triton X-100. Controls and blanks were carried out in parallel with corresponding samples. Controls differed from samples for the presence of Supersomes from uninfected cells, or for the absence of NAPD when HLMs were tested. They allow an estimate of AChE total activity in the absence of inhibition (set as 100% cholinergic activity). Blanks differed from controls for the absence of acetylchlolethine. Determination of Malaoxon Formation. Once incubation was stopped, samples containing Supersomes were kept at 37°C under shaking for 2 h to complete AChE inhibition reaction. This step was avoided with HLMs. Then samples were kept in ice for 3 min and subsequently at 25°C for 20 min. Assay of residual AChE activity was carried out with the spectrophotometric method of Ellman et al. (1961), by adding 1 mM dithiobisnitrobenzoic acid in 0.038 M Tris-HCl buffer, pH 7.2 (300 µM of acetylthiocholine in 0.038 M Tris-HCl 100 µl) to start the reaction. After 30 min at room temperature, the absorbance was measured at λ = 412 nm.

The amount of malaoxon formed was determined by being referred to a calibration standard curve, obtained with known amounts of oxon, and plotting the percentage inhibition versus the logaritum of the oxon concentration. The ranges of six malathion concentrations with Supersomes and HLMs were 15 to 200 nM malaoxon (average correlation coefficient R² = 0.987) and 200 to 1100 nM malathion (average correlation coefficient R² = 0.965), respectively.

Correlation Studies. Oxon formation from malathion was determined in standard incubations with 16 individual HLMs, fully characterized for single P450 activities. They were selected to have a broad range of activity for all the different cytochrome P450 isoforms. Malathion was tested at two pesticide concentrations (50 µM and 400 µM). Correlation coefficients were calculated by plotting the rate of malathion formation versus the marker activity of each P450.

Immuno inhibition of Malathion Formation. The immuno inhibition studies were carried out at 50 µM and 400 µM malathion with three different human samples (HLM1, HLM2, and HLM3). HLMs (1 mg of protein) suspended in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4 were mixed with different amounts of Abs and preincubated at 4°C for 20 min. Then, the standard incubation conditions were followed. In a separate set of experiments, the anti-CYP1A2, 3A4, and 2B6 Abs were simultaneously added to the incubation mixture, to evaluate the additivity of their inhibitory effects. The amounts of Abs used were expected to produce maximal inhibition (>80%) of the specific enzymatic activity, as indicated by the supplier: 3 µl/100 µg protein anti-CYP1A2 Ab, anti-CYP2B6 Ab, and anti-CYP2D6 Ab; 5 µl/100 µg protein anti-CYP2A6 Ab and anti-CYP2E1 Ab; 10 µl/100 µg protein anti-CYP3A4 Ab; and 30 µl/100 µg protein anti-CYP1A1 Ab. Two kind of controls (A and B) and blanks were carried out in parallel with corresponding samples. In control A, the presence of Abs was replaced by Tris buffer and was set as 100% metabolic activity. Control B differed from control A for the absence of NAPD and was set as 100% cholinergic activity. Blanks differed from controls and samples for the absence of acetylchlolethine.

Chemical Inhibition of Malaoxon Formation. Isoform-selective cytochrome P450 inhibitors were added to the standard incubation mixture at the following final concentrations: 5 µM orphenadrine, 20 µM furafylline, and 100 µM tolazolymycin, as specific inhibitors of CYP2B6, 1A2 and 3A4, respectively. These inhibitors were chosen on the basis of results obtained in the immunoinhibition studies, to evaluate the impact of their inhibitory effects on the kinetics of malaoxon formation. Incubations were carried out with three individual HLMs (HLM1–3) in a range of different malathion concentrations (10–500 µM). Inhibitors were diluted in Tris-HCl buffer except for tolazolymycin, which was dissolved in acetonitrile (final acetonitrile concentration, 0.5%). The presence of acetonitrile caused ≥5% decrease with respect to activity measured in the absence of the solvent. Inhibitor concentrations were chosen to theoretically suppress >80% of P450 activity, based on published Kᵢ values. Before starting the reaction of malathion bioactivation, standard incubation mixtures were preincubated with the three mechanism-based inhibitor control A, the presence of Abs was replaced by Tris buffer and was set as 100% metabolic activity. Control B contained the inhibitor but not NAPD and was set as 100% cholinergic activity. Blanks differed from controls and samples for the absence of acetylchlolethine.

Data Analysis. The kinetic parameters Kᵢ and V_max of recombinant human P450 were obtained from a nonlinear regression fit curve with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The kinetic parameters Kᵢ,app and V_max,app of HLMs were obtained from the Eadie-Hofstee plot. The intrinsic clearance (CLᵢ) was calculated as the V_max,app/Kᵢ,app ratio or obtained as the slope of the linear tract of the Michaelis-Menten curve, when the enzymatic activity was linear over the range of tested substrate concentrations. In correlation studies, P450 activities measured in 16 HLMs tested toward malathion and model substrates, with ≥3 S.D. of the mean being considered outliers that were not included in the calculation of Pearson’s regression coefficient (r). The significance of results obtained from correlation and inhibition studies was analyzed by ANOVA, with the Bonferroni post hoc test.

Results

In Vitro Malathion Bioactivation by cDNA-Expressed Human P450s. Preliminary Studies. The absence of nonspecific esterase in Supersome preparations that could interfere with AChE activity determination has been previously shown (Buratti et al., 2002). The direct inhibition of AChE activity, due to unmetabolized malathion or impurities, was measured in standard incubations of control Supersomes in the absence of NAPD. Malathion did not affect AChE activity up to 50 µM, the highest pesticide concentration tested. When malathion formation was tested at different incubation times and P450 concentrations, the standard experimental condition used resulted in the linear range of the reaction; indeed, the reaction was linear up to 5 min and 100 pmol of P450/ml (data not shown).

Kinetic Parameters of Malaoxon Formation. CYP2C19, 1A2, and 2B6 were very efficient in catalyzing malathion sulfoxidation. Indeed, in standard incubations containing 50 pmol of P450/ml, the levels of malaoxon produced overwhelmed the levels of AChE, reaching 100% inhibi-
tion. Therefore, to test malaoxon formation in the range 0.01 to 2.5 μM malathion, it was necessary to reduce to 1/2 the P450 content in the standard incubation. The dependence of CYP2C19, 1A2, and 2B6 activity on malathion concentration was represented by typical saturation curves (data not shown). CYP2C19 showed the highest affinity toward malathion (corresponding to the lowest $K_m$) and the highest $V_{max}$ for malaoxon production (Table 1). The ranking of CL$_r$ values was 2C19 > 1A2 > 2B6 (Table 1). CYP1A1 activity deviated from linearity only at the highest malathion concentration used (50 μM), whereas CYP3A4, 2C8, and 2C9 were still linear up to this concentration (data not shown); they all were endowed with very low CL$_r$ values (Table 1). CYP2D6 and 2A6 were not able to catalyze any malathion desulfuration even at the highest pesticide concentration tested.

To preliminarily estimate the relative contribution of different P450s to malathion formation, a tentative extrapolation has been carried out, taking into account CL$_r$ values and the average P450 content in human livers (Rendic and Di Carlo, 1997). It appeared that CYP1A2 accounted for >80% of the reaction, whereas CYP2C19 and 2B6, due to their lower hepatic content, were responsible for 14 and 1.6%, respectively (Table 1). When $V_{max}$ or V data obtained at 50 μM malathion were used, it appeared that the contribution of CYP3A4 was greatly increased and similar to CYP1A2 (43% and 40%, respectively) (data not shown), suggesting that the catalytic activity of 3A4 may become relevant with increasing pesticide concentrations.

**In Vitro Malathion Bioactivation by HLMs. Preliminary Studies.** The presence of esterase activity in all the tested HLMs, measured as acetylthiocholine hydrolysis, varied in the panel of sample used (range 0.92–3.84 μmol acetylthiocholine hydrolyzed/min). The addition of 50 μM iso-OMPA to the incubation mixture (Buratti et al., 2003) completely inhibited nonspecific esterase activity, preventing the interference with AChE activity determination. The direct inhibition of AChE activity (Table 1) produced malaoxon levels similar to those of HLM2 and 2A4 may become relevant with increasing pesticide concentrations.

**Characterization of Malathion Formation by HLMs.** HLMs from single human livers (HLM$_1$, HLM$_2$, and HLM$_3$) or pooled from five donors (HLM$_4$) were used to characterize the malathion desulfuration reaction and to show the dependence of malathion formation on malathion concentration. The samples were selected on the basis of their relative content of those P450s able to catalyze malathion desulfuration at significant levels, when tested as single cDNA-expressed enzymes. The P450 activities for the individual HLMs used are reported in Table 2. The marker activities corresponding to the other P450s were in the average range expressed within the panel of individual HLMs tested during the study. Results showed that all HLM samples can bioactivate malathion to malaoxon in the range of pesticide concentrations tested (10–500 μM), although they showed a different kinetic behavior and variable efficiencies, expressed as intrinsic clearance, depending on their relative P450 content.

With both HLM$_1$ and HLM$_2$, endowed with 2- to 3-fold higher levels of CYP1A2-related activity with respect to the other two samples (Table 2), malathion desulfuration was described by saturation curves (Fig. 2A). Data of malathion production by both HLM$_1$ and HLM$_2$ fitted two straight lines in the Eadie-Hofstee plot, indicating that two distinct components with different affinities, each of them catalyzed by one or more isoforms, are involved in the desulfuration reaction (Fig. 2A), giving rise to different kinetic parameters (Table 3). The high-affinity phase appeared to deviate from linearity at about 100 μM malathion, and it was characterized by similar $K_{m,app1}$ in the two samples (53–67 μM). However, the efficiency (CL$_{1}$) of the reaction differed between the two samples, depending on the 10-fold higher $V_{max,app}$ expressed by HLM$_2$ (Table 3). The low-affinity phase was not yet saturated at 500 μM malathion in both HLMs and the kinetic parameters $K_{m,app2}$ and CL$_{2}$ were highly variable. The two HLMs, characterized by similar scant levels of CYP2C19 (CYP1A2-to-CYP2C19 was 10 and 18.5, respectively) and by comparable CYP3A4-related activity, differ only in CYP2B6 content, which was higher in HLM$_2$ (Table 2). In HLM$_1$ and HLM$_2$, which had low CYP1A2 content, only one component was evident: malathion desulfuration activity was linear up to 500 μM malathion (Fig. 2B). HLM$_2$ endowed with efficient CYP2B6-, 3A4-, and 2C19-marker activity (Table 2) produced malaoxon levels similar to those of HLM$_2$ (Fig. 2), whereas in HLM$_1$ characterized by quite low levels of each single P450, malathion bioactivation was less efficient. The CL$_{1}$ values for HLM$_1$ and HLM$_2$ (8.0 ± 0.3 and 1.23 ± 0.02 μl min$^{-1}$ mg$^{-1}$, respectively) were comparable to the one corresponding to the low-affinity component of desulfuration evidenced with HLM$_2$ and HLM$_1$, respectively (Table 3).

**Correlation Studies.** Correlation studies were carried out to measure malathion formation in incubations with HLMs from 16 individual donors. Malathion concentrations (50 and 400 μM) were selected, on the basis of the calculated $K_{m,app}$ values (Table 4), as representative of the high- and the low-affinity components of the desulfuration reaction, producing at the same time significant levels of malaoxon. Results showed a relatively high level of

<table>
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<tr>
<th>TABLE 1</th>
<th>Kinetic parameters of malathion desulfuration by recombinant human P450s</th>
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<tr>
<td>P450</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>2C19</td>
<td>5.00 ± 0.01</td>
</tr>
<tr>
<td>1A2</td>
<td>3.67 ± 0.03</td>
</tr>
<tr>
<td>2B6</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>1A1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>3A4</td>
<td>N.D.</td>
</tr>
<tr>
<td>2C9</td>
<td>N.D.</td>
</tr>
<tr>
<td>2C8</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

$^a$ Values of the relative contributions (percentage) to total malathion desulfuration were calculated from CL$_{1}$ values of the active P450s, taking into account the average human hepatic content of each isoform (1A2, 12%; 3A4, 40%; 2C19, 1.1%; 2B6, 0.2%; 2C8, 5.4%; 2C9, 9.4%; 1A1, traces) as reported in Rendic and Di Carlo (1997).

$^b$ CL$_{1}$ values represent the slope of the linear enzymatic activity.
was the only activity correlating with malaoxon formation at 50 μM malathion, whereas the low-affinity phase showed significant correlation (Table 4). Caffeine N3-demethylation (CYP1A2) indicated that anti-CYP1A2, 3A4, and 2B6 Abs were responsible for significant inhibition in malaoxon formation (Table 5).

A different pattern of inhibition was evident when the two malathion concentrations were tested, indicating that different P450s are involved in the low- and the high-affinity phase of the desulfuration reaction. Indeed, at 50 μM malathion, the addition of anti-CYP1A2 Ab caused a strong inhibition of malaoxon formation when the three individual HLMs were tested (less than 30% residual activity). In HLM2, although the CYP1A2-to-CYP2B6 ratio (0.68) was similar to the ones in HLM3 and HLM1 (0.53 and 0.34, respectively), addition of anti-CYP2B6 Ab to the mixture also had a significant inhibitory effect on malathion desulfuration (about 30% residual activity) (Table 5), probably due to the higher S-mephenytoin N-demethylation activity in this sample (Table 2). When 400 μM malathion was incubated in the presence of each single Ab, either anti-CYP3A4 Abs or anti-CYP2B6 Abs were responsible for a substantial inhibition of malathion desulfuration (50–70%) with the three HLMs (Table 5), whereas anti-CYP1A2 Abs did not affect malaoxon formation.

When the three Abs, singularly active in preventing malaoxon formation, were simultaneously added to the incubation mixture, the range of residual activity was 16 to 27% at 50 μM and 7 to 12% at 400 μM malathion (Table 5). These results suggested a moderate to negligible contribution to the reaction by P450s other than the inhibited ones.

**Chemical Inhibition Studies.** Single chemical-specific P450 inhibitors were incubated with three individual HLMs used for the characterization of malathion bioactivation at different pesticide concentrations (range 10–500 μM malathion) (Fig. 3).

When orphenadrine was used with HLM1 and HLM2, no inhibition was measured up to 100 μM malathion, whereas the low-affinity phase disappeared (Fig. 3, A and B). Indeed, the Eadie-Hofstee plots of the curves obtained for HLM1 and HLM2 in the presence of the inhibitor fitted a straight line with high coefficients ($r = 0.902$ and $r = 0.828$, respectively) (Table 4).

**Immunoinhibition Studies.** Results from immunoinhibition studies with three different HLM samples (HLM1, HLM2, and HLM3) indicated that anti-CYP1A2, 3A4, and 2B6 Abs were responsible for significant inhibition in malaoxon formation (Table 5).

When furafylline was used, no malaoxon formation was detectable up to 50 μM malathion (data not shown), whereas it was quite an efficient inhibitor (about 70% residual activity) in HLM3 (Fig. 3C) at malathion concentrations higher than 200 μM.

The simultaneous presence of the three inhibitors, specific for...
CYP1A2, 2B6, and 3A4, caused an almost complete inhibition of malaoxon formation both at 50 and 400 μM malathion, with a residual activity of 4.8 and 2.6%, respectively, suggesting a negligible contribution to the reaction by other P450s.

**Discussion**

In this paper, we reported the characterization of malathion desulfuration in HLMs within a broad range of concentrations (0.1–500 μM). The study has been carried out with a multifaceted integrated experimental strategy: the enzyme specificity obtained by using recombinant P450s has been paralleled by the kinetics characterization of the reaction in HLMs. These data, corroborated by results from correlation, chemical inhibition, and immunoinhibition studies, have allowed the identification of P450s involved in malathion bioactivation in the human liver.

The use of in vitro concentrations close to the actual human exposure levels is crucial to obtain data relevant to the in vivo situation. It has been suggested that in vitro concentrations of OPTs ≤10 μM are the most appropriate to mimic in vivo conditions (Buratti et al., 2002, 2003), based on chlorpyrifos plasma concentrations (90 nM) measured in human volunteers (Nolan et al., 1984) and on the estimation of environmental exposure in U.S. adults and children (about 1.4–1.61 μg/kg/day) (Schurdt et al., 1998; Rigas et al., 2001), obtained by applying pharmacokinetic models to biomonitoring data. On the same line, starting from urinary alkylyphosphate levels, a body burden of 208 μg/kg azinphos-methyl (corresponding to 6.5 μM in plasma) was estimated in orchard workers at the steady state (Carrier and Brunet, 1999). Similar conclusions may be drawn for malathion: it has been reported that in urine of the general U.S. adult population, malathion monoacid is detectable up to 3 μM (Kutz et al., 1992),

**Table 3**

<table>
<thead>
<tr>
<th>Activity</th>
<th>50 μM Mean</th>
<th>400 μM Mean</th>
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<tbody>
<tr>
<td>Caffeine N-demethylation (CYP1A2)</td>
<td>400 μM</td>
<td>58–1035</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation (CYP2D6)</td>
<td>18–492</td>
<td>244</td>
</tr>
<tr>
<td>Dextromethorphan N-demethylation (CYP3A4)</td>
<td>24–205</td>
<td>86</td>
</tr>
<tr>
<td>Tolbutamide methyl-hydroxylation (CYP2C9)</td>
<td>131–736</td>
<td>307</td>
</tr>
<tr>
<td>S-Mephenytoin N-demethylation (CYP2B6)</td>
<td>23–306</td>
<td>115</td>
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<tr>
<td>S-Mephenytoin 4-hydroxylation (CYP2C19)</td>
<td>10–464</td>
<td>112</td>
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<tr>
<td>Chlorzoxazone 6-hydroxylation (CYP2E1)</td>
<td>625–2977</td>
<td>1879</td>
</tr>
<tr>
<td>Lauric acid 12-hydroxylation (CYP4A11)</td>
<td>582–2318</td>
<td>1310</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation (CYP1A1)</td>
<td>144–3407</td>
<td>1316</td>
</tr>
</tbody>
</table>

**Table 4**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malathion</td>
<td>0.722**</td>
</tr>
<tr>
<td>50 μM Malathion</td>
<td>0.846</td>
</tr>
<tr>
<td>400 μM Malathion</td>
<td>0.042</td>
</tr>
</tbody>
</table>

**Table 5**

<table>
<thead>
<tr>
<th>Inhibition (%)</th>
<th>Malathion</th>
<th>Anti-CYP3A4</th>
<th>Anti-CYP1A2</th>
<th>Anti-CYP2B6</th>
<th>Anti-CYP2A6</th>
<th>Anti-CYP2E1</th>
<th>Anti-CYP2D6</th>
<th>Anti-CYP1A1</th>
<th>Multiple Abs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM1</td>
<td>50 μM</td>
<td>11 ± 2</td>
<td>72 ± 5***</td>
<td>0 ± 7</td>
<td>5 ± 2</td>
<td>0 ± 3</td>
<td>4 ± 2</td>
<td>1 ± 3</td>
<td>78 ± 3***</td>
</tr>
<tr>
<td>400 μM</td>
<td>58 ± 8***</td>
<td>11 ± 9</td>
<td>50 ± 8***</td>
<td>3 ± 8</td>
<td>0 ± 7</td>
<td>6 ± 8</td>
<td>4 ± 7</td>
<td>88 ± 6***</td>
<td></td>
</tr>
<tr>
<td>HLM2</td>
<td>50 μM</td>
<td>0 ± 12</td>
<td>71 ± 5***</td>
<td>71 ± 1***</td>
<td>1 ± 10</td>
<td>4 ± 9</td>
<td>0 ± 5</td>
<td>2 ± 5</td>
<td>83 ± 7***</td>
</tr>
<tr>
<td>400 μM</td>
<td>70 ± 5***</td>
<td>15 ± 9</td>
<td>57 ± 1***</td>
<td>1 ± 6</td>
<td>0 ± 10</td>
<td>2 ± 4</td>
<td>0 ± 4</td>
<td>93 ± 5***</td>
<td></td>
</tr>
<tr>
<td>HLM3</td>
<td>50 μM</td>
<td>6 ± 3</td>
<td>69 ± 7***</td>
<td>9 ± 2</td>
<td>4 ± 7</td>
<td>3 ± 5</td>
<td>3 ± 7</td>
<td>0 ± 8</td>
<td>73 ± 8***</td>
</tr>
<tr>
<td>400 μM</td>
<td>59 ± 6***</td>
<td>4 ± 6</td>
<td>48 ± 5**</td>
<td>1 ± 3</td>
<td>0 ± 4</td>
<td>0 ± 3</td>
<td>2 ± 9</td>
<td>92 ± 7***</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** All correlations are significant at p < 0.001.

**References:**

1. Buratti ET AL.
2. Caffeine N-demethylation (CYP1A2)
3. Dextromethorphan O-demethylation (CYP2D6)
4. Dextromethorphan N-demethylation (CYP3A4)
5. Tolbutamide methyl-hydroxylation (CYP2C9)
6. S-Mephenytoin N-demethylation (CYP2B6)
7. S-Mephenytoin 4-hydroxylation (CYP2C19)
8. Chlorzoxazone 6-hydroxylation (CYP2E1)
9. Lauric acid 12-hydroxylation (CYP4A11)
10. Coumarin 7-hydroxylation (CYP1A1)
11. Correlation Coefficient (r)
12. Immunoinhibition of malaoxon formation
13. **p < 0.01; ***p < 0.001.
oxon formation for CYP1A2 at low pesticide concentrations and a substantial contribution of CYP3A4 at higher malathion levels. Much of these qualitative data were confirmed by using HLMs, where the different isoforms are concurrently present and compete with one another for the substrate. Within the tested panel, malathion was efficiently bioactivated, and HLMs were able to produce substantial amounts of malaoxon, although with a high level of interindividual variability (>200-fold). Kinetic characterization of malathion desulfuration in four different HLMs showed that one or two components with different affinity for the pesticide were present, depending on the relative specific P450 content and indicating the involvement of multiple P450s. The presence of two phases in oxon formation has been previously shown with other OPTs, both with human (Buratti et al., 2003) and rat (Ma and Chambers, 1994) liver microsomes, when a low-to-high range of pesticide concentrations was tested. The similarity of the $K_{\text{m,app}}$ ($K_{\text{m,app}} = 53–67 \mu M$), calculated for the high-affinity phase, suggested the involvement of a single isoform, identified as CYP1A2. Indeed, the high-affinity component was present only in HLM$_1$ and HLM$_2$ endowed with high levels of CYP1A2-marker activity, independently of the relative content of other P450s. In HLM$_3$, which showed the highest levels of 2B6, 3A4, and 2C19, and low 1A2 activity (ratios = 28, 26, and 17, respectively), the reaction consisted of only one phase that was linearly dependent on malathion concentration, up to 400 $\mu M$, although high amounts of malaoxon were produced. In addition, at 50 $\mu M$ malathion, which is representative of the high-affinity phase, malaoxon formation correlated only with CYP1A2-related activity and was dramatically inhibited by anti-CYP1A2 Abs in three HLMs. In the presence of furafylline, the high-affinity phase was no longer evident in the kinetics of malathion formation versus malathion concentration in HLM$_1$ and HLM$_2$; malathion was undetectable at malathion concentrations ≤50 to 100 $\mu M$. The low-affinity phase seemed to be not significantly affected by furafylline, indicating a limited contribution of CYP1A2 at high pesticide concentrations, as was also suggested by lack of correlation between malathion formation and CYP1A2 and lack of inhibition by anti-CYP1A2 Abs at 400 $\mu M$ malathion. The immunoinhibition measured in HLM$_2$ with anti-CYP2B6 Ab, and the inhibitory effect of orphenadrine in HLM$_3$ at low malathion concentrations, seems to suggest some involvement of this P450 to the high-affinity phase, likely relevant only when CYP2B6 is highly expressed or CYP1A2 content is scant, as in the case of the tested samples.

The low-affinity component of malathion bioactivation was not saturated up to a 500 $\mu M$ concentration of pesticide in the tested samples; results from the different approaches indicated CYP2B6 and 3A4 as the major isoforms responsible for malathion formation. Results from the kinetic analysis in HLM$_1$ and HLM$_2$ showed that when these two isoforms are present at high levels, the CL$_i$ related to the low-affinity phase is significantly higher (15.5 and 8.0 $\mu l \cdot min^{-1} \cdot g^{-1}$). In addition, malathion formation positively correlated with CYP2B6- and 3A4-marker activities. Since the expression of these two cytochrome P450 isoforms is under coordinate control, their catalytic activities correlate with each other ($r = 0.944$); thus, it could be misleading to form a conclusion on their role in malathion formation. However, the bioactivation reaction was inhibited by both anti-CYP2B6 and -3A4 Abs, clearly confirming the involvement of both isoforms at 400 $\mu M$ malathion to be representative of high pesticide concentrations. The presence of orphenadrine, inhibiting CYP2B6, completely prevents the low-affinity phase of malathion production in HLM$_1$ and HLM$_2$, where $K_m$ and $V_{\text{max}}$ were almost identical to the values obtained for the high-affinity phase without the inhibitor. Troleandomycin better evidenced the role of CYP3A4 in the low-affinity phase in HLM$_3$, where the isoform was particularly active.

Although the recombinant CYP2C19 was active, data obtained with
HLMs did not establish a clear role for this isoform in malathion bioactivation. The absence of the high-affinity phase in HLM3, with a CYP2C19/CYP1A2 ratio = 17, suggested a negligible contribution at low pesticide concentrations. Moreover, despite the broad range of S-mephenytoin 4'-hydroxylation activity expressed in the panel of HLMs tested, there was no correlation with malathion formation. Results obtained with other OPTs were controversial: some authors considered the role of CYP2C19 relevant in OPT dearylation rather than in oxon formation (Tang et al., 2001; Vitozzi et al., 2001; Buratti et al., 2003); others suggested some involvement only when hepatic content of the CYP2C family is extremely high, with a simultaneous low CYP3A4 level (Mutch et al., 1999), whereas it has been considered as the major catalyst for diazoxon production at high diazinon concentrations (Kappers et al., 2001). However, the lack of a selective chemical inhibitor of either monoclonal anti-CYP2C19 Ab makes it difficult to conclude a role for this isoform, although the whole body of results indicates a scant contribution to malathion bioactivation.

Differently from the limited action of human hepatic paraoxonase at a low level of oxon, due to its $K_{m}$ value (0.29 mM) (Gonzalvo et al., 1998), human carboxylesterase seems to be quite efficient in detoxifying malathion (USEPA, 2000). Moreover, cytochrome P450 itself may contribute to malathion detoxication, catalyzing dimethyldithio- phosphate formation (Fig. 1). Therefore, the bioactivation/detoxication balance may be the crucial step in determining differences in malathion-induced toxicity. However, since carboxylesterase can be strongly inhibited by isomalafoxan and other impurities (Aldridge et al., 1979; Talcott et al., 1979a,b), differences in malathion production due to different phenotypes can be relevant in interindividual responses to toxic effects consequent to malathion exposure.

The present results showed the involvement of CYP1A2 in malaoxon formation at low malathion concentrations, representative of conditions of actual human exposure, whereas the CYP2B6 and 3A4 contribution was relevant at higher pesticide levels, very likely reached during poisoning episodes. These data are in line with those obtained with other OPTs, namely, azinphos-methyl, chlorpyrifos, diazinon, and parathion (Buratti et al., 2002, 2003), all presenting an aromatic ring in the molecule. Since malathion structure is different, being characterized by linear substituents at the thioether sulfur, it can be hypothesized that at actual exposure levels, although differing in their chemical structure, OPTs are mainly bioactivated by CYP1A2 and only to a lesser extent by CYP2B6. These isomers can be regarded as good candidates as metabolic biomarkers of susceptibility. Indeed, individuals with high levels of CYP1A2, readily inducible by smoking, caffeine, and aromatic amines, or bearing variant CYP2B6 alleles, would be expected to produce higher oxon levels and consequently to be more susceptible to adverse effects induced by this class of pesticides.

References
Aldridge WN, Miles JW, Mount DL, and Verschoyle RD (1979) The toxicological properties of malathion and its inactivation by isomalafoxan and other impurities (Aldridge et al., 1979; Talcott et al., 1979a,b), differences in malathion formation at high diazinon concentrations (Kappers et al., 2001). However, the lack of a selective chemical inhibitor of either monoclonal anti-CYP2C19 Ab makes it difficult to conclude a role for this isoform, although the whole body of results indicates a scant contribution to malathion bioactivation.

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