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

Received July 30, 2004; accepted November 17, 2004

ABSTRACT:
Among organophosphorothioate (OPT) pesticides, malathion is considered relatively safe for use in mammals. Its rapid degradability 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 activity, 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 (K_mapp1 = 53-67 μM; K_mapp2 = 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, azynphos-methyl, and parathion, characterized by the presence of an aromatic ring in the molecule. Since malathion has linear chains as substituents at the thioether 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 organophosphorothioate (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 invertebrates, 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 dimethylphosphosphate, 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., 1989; Sultatos, 1994), which inhibits acetylcholinesterase (AChE) (Forsyth and Chambers, 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.

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; Vittozzi 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., 2002, 2003). This work has been partially supported by Istituto Superiore di Sanità Projects 3AAS and 3AG/F11. Preliminary results were presented at the 8th European ISSX Meeting in Dijon, France, April 27 to May 1, 2003.

ABBREVIATIONS: OPT, organophosphorothionate; P450, cytochrome P450 isoform; HLM, human liver microsome; CLi, intrinsic clearance; AChE, acetylcholinesterase; iso-OMPA, tetraisopropyl-pyrophosphoramid; Ab, antibody.
The role of CYP2B6 to total hepatic oxon formation seemed to be relevant in a wide range of pesticide concentrations (Tang et al., 2001; Buratti et al., 2003), whereas the contribution of CYP3A4 was significant only at high OPT levels (Mutch et al., 1999; Kappers et al., 2001; Buratti et al., 2002, 2003).

No information is available on human malathion bioactivation, despite the fact that its widespread use makes the potential for human exposure high, both for agriculture workers and for the general population. Exposure may be relevant, especially among children (Fenske et al., 2000, 2002; Adgate et al., 2001) who have excess dietary intake caused by food-to-surface-to-mouth or surface-to-hand-to-food activities (Melnyk et al., 2000). Only limited data have been reported on malathion carboxylesterase activity in human liver (Talcott et al., 1979a).

We have undertaken this study to characterize malathion bioactivation in the human liver and to provide data on the role of human P450s in the formation of toxic metabolites. To study low pesticide concentrations representative of actual human exposure, we took advantage of a method based on AChE inhibition that is able to detect nanomolar levels of oxon (Ma and Chambers, 1994; Buratti et al., 2002).

The OPTs for which human bioactivation has been studied, so far, were characterized by the presence of aromatic rings in the molecule; the similar chemical structure may be the main reason for the involvement of the same P450s in their metabolism. The information on the bioactivation of malathion, which has linear groups as substituents at the thioether sulfur, could indicate whether other OPTs with different chemical structure are bioactivated by the same isof orm(s). This information represents the basis for the selection of metabolic biomarkers to be used to identify individuals potentially characterized by different production of toxic metabolites and consequent different susceptibilities to the toxic effects induced by the entire class of OPT insecticides.

Materials and Methods

Chemicals. 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, (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). Tertraisopropyl-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-reactions 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 experiments. Rats were killed by decapitation, and the whole brain was immediately taken. The European Community Council 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/mg.

HLMs were prepared from a single liver sample (HLMs), 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 (HLMi) or pooled from five donors (HLMi), 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 monoxygenase 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, 1A2, 2A6, 2B6, and 3A4 were measured in HLM2 according to published methods (Chen et al., 1990; Heyn et al., 1996; Krul and Hageman, 1998; Easterbrook et al., 2001).

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 acetylthiocholine hydrolyzed - min·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 1–1.5 μmol of acetylthiocholine hydrolyzed/min) were added, followed by the addition of malathion as 10 μmol of methanol 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 isozyme 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 after 2 min 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 NADP when HLMs were tested. They allow an estimate of AChE total activity in the absence of inhibition (set as 100% cholinergic enzymatic activity). Blanks differed from controls for the absence of acetylcholine.

**Determination of Malathion 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 the 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 procedure for malaoxon formation and detection was followed. In a separate set of experiments, the standard incubation mixture was preincubated 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 controls A, B, and C, respectively. Control A, the presence of Ab was replaced by Tris buffer and was set as 100% metabolic activity. Control B differed from control A for the absence of NADP and was set as 100% cholinergic activity. Blanks differed from controls and samples for the absence of acetylcholine.

**Chemical Inhibition of Malathion Formation.** Isotope-selective cytochrome P450 inhibitors were added to the standard incubation mixture at the following final concentrations: 5 μM orphennadrine, 20 μM furafylline, and 100 μM tolledoxygen, as specific inhibitors of CYP2B6, 1A2 and 3A4, respectively. These inhibitors were chosen on the basis of results obtained in the in vivo experiments, to study the impact of their inhibitory effects on the kinetics of malathion formation. Incubations were carried out with three individual HLMs (HLM1, HLM2, and HLM5) in a range of different malathion concentrations (10–500 μM). Inhibitors were diluted in Tris-HCl buffer except for tolledoxygen, 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 controls A, B, and C, respectively. Control A, the presence of Ab was replaced by Tris buffer and was set as 100% metabolic activity. Control B contained the inhibitor but not NADP and was set as 100% cholinergic activity. Blanks differed from controls and samples for the absence of acetylcholine.

**Data Analysis.** The kinetic parameters Km and Vmax 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 Km and Vmax of HLMs were obtained from the Eadie-Hofstee plot. The intrinsic clearance (CI) was calculated as the Vmax/Km ratio or obtained as the slope of the linear plot 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 Bonferroni post hoc test.

**Results.**

**In Vitro Malathion Bioactivation by cDNA-Expressed Human P450s. Preliminary Studies.** The absence of nonspecific esterases in Supersomes 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 NADP. In the absence of 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 Malathion 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 malathion produced overwhelmed the levels of AChE, reaching 100% inhibi-
hibition. 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 $CL_i$ 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 $CL_i$ 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 $CL_i$ 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 $V_0$ 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 nmol 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, possibly due to unmetabolized malathion or to impurities of the commercial product, was tested by carrying out standard incubations with HLMs in the absence of NADP. Malathion did not affect AChE activity up to 100 μM; at concentrations ≥200 μM, the inhibition was ≤12%. Therefore, ad hoc control incubations in the absence of NADP were run in parallel. The standard experimental conditions used for malaoxon production resulted in the linear range of dependence of the reaction with respect to both incubation time and protein concentrations; indeed, the reaction was linear up to 1 mg of protein/ml and 10-min incubation time (data not shown).

### Characterization of Malaoxon 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 malaoxon 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 malaoxon 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 desulfurization 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 $K_{m,app}$ in the two samples (53–67 μM). However, the efficiency ($CL_i$) 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 $K_{m,app}$ and $CL_i$ 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$_3$ 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$_4$, characterized by quite low levels of each single P450, malathion bioactivation was less efficient. The $CL_i$ 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 malaoxon 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>
<thead>
<tr>
<th>P450</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$CL_i$ (nmol min$^{-1}$ · mg$^{-1}$)</th>
<th>Percentage Contribution$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2C19</td>
<td>5.00 ± 0.01</td>
<td>0.445 ± 0.004</td>
<td>11.2</td>
<td>14.2</td>
</tr>
<tr>
<td>1A2</td>
<td>3.67 ± 0.03</td>
<td>0.78 ± 0.04</td>
<td>4.7</td>
<td>83.5</td>
</tr>
<tr>
<td>2B6</td>
<td>3.6 ± 0.2</td>
<td>3.09 ± 0.07</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>1A1</td>
<td>1.0 ± 0.2</td>
<td>11.8 ± 0.1</td>
<td>0.09</td>
<td>N.D.</td>
</tr>
<tr>
<td>3A4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.025$^b$</td>
<td>1.6</td>
</tr>
<tr>
<td>2C9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.017$^b$</td>
<td>0.3</td>
</tr>
<tr>
<td>2C8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.015$^b$</td>
<td>0.1</td>
</tr>
</tbody>
</table>

$^a$ Values of the relative contributions (percentage) to total malathion desulfuration were calculated from $CL_i$ 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).

$^b$ $CL_i$ values represent the slope of the linear enzymatic activity.
was the only activity correlating with malaoxon formation at 50 μM malathion was no more evident. On the other hand, at high pesticide concentrations, CYP2B6 and 3A4 marker activities showed very high correlation 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).

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 plot of the curves obtained for HLM1 and HLM2 in the presence of the inhibitor fitted a straight line with coefficients (r = 0.902 and r = 0.828, respectively) (Table 4).

When furafylline was used, no malaoxon formation was detectable up to 50 to 100 μM malathion (inhibition ≥90%); at higher malathion concentrations, the activity was much lower than that measured in the absence of the inhibitor (20–50% residual activity). With HLM3, orphenadrine showed a pattern of inhibition similar to the one described in the other two samples (Fig. 3C), whereas furafylline had no effect on HLM1 activity (data not shown), consistently with very low levels of CYP1A2 present in that sample. The effect of troleandomycin was scant and not statistically significant in HLM1 and HLM2 (data not shown), whereas it was quite an efficient inhibitor (about 50% residual activity) in HLM3 (Fig. 3C) at malathion concentrations higher than 200 μM.

The simultaneous presence of the three inhibitors, specific for variability (>200 fold) in oxon formation in the panel of tested samples in the high-affinity phase of the reaction. When 50 μM malathion was used, malaoxon formation ranged from the limit of detection to 60 pmol of oxon · mg protein⁻¹ · min⁻¹ (median ± S.D. = 17 ± 18). At 400 μM malathion there was 18-fold difference in malaoxon levels (58–1035 pmol oxon · mg prot⁻¹ · min⁻¹) (median ± S.D. = 399 ± 254) (Table 4).

When malathion was plotted versus each marker activity for single P450s, only CYP1A2-, 3A4-, and 2B6-related activities showed significant correlation (Table 4). Caffeine N3-demethylation was the only activity correlating with malaoxon formation at 50 μM malathion (r = 0.722). At 400 μM malathion, the same correlation was no more evident. On the other hand, at high pesticide concentrations, CYP2B6 and 3A4 marker activities showed very high correlation coefficients (r = 0.902 and r = 0.828, respectively) (Table 4).
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 desulphuration 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 et al., 2002, 2003), based on chlorpyrifos plasma concentrations (90 nM) measured in human volunteers (Nolan et al., 1984).
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 malaaoxon, 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 malaaoxon were produced. In addition, at 50 \( \mu M \) malathion, which is representative of the high-affinity phase, malaaoxon 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 malaaoxon formation versus malathion concentration in HLM\(_1\) and HLM\(_3\); malaaoxon 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 malaaoxon 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 malaaoxon formation. Results from the kinetic analysis in HLM\(_2\) and HLM\(_3\) showed that when these two isoforms are present at high levels, the C\(_{\text{LH}}\) related to the low-affinity phase is significantly higher (15.5 and 8.0 \( \mu \text{mol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1} \)). In addition, malaaoxon 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 malaaoxon 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 malaaoxon production in HLM\(_1\) and HLM\(_2\), where \( K_{\text{m}} \) 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\(_1\), where the isoform was particularly active.

Although the recombinant CYP2C19 was active, data obtained with

whereas in children, the mean content was about 4.5 nM (range = 3–28 nM) (Adgate et al., 2001). Higher values were reported in tissues, urine, or plasma of workers who applied the insecticide or harvested the crops; their exposure was estimated to be 1 to 3 mg/kg/day and 1 to 270 \( \mu g/kg/day \), respectively (Krieger and Dinoff, 1999), which may give rise to a range of plasma concentrations between 0.03 and 80 \( \mu M \), considering an almost complete absorption. Since in vivo hepatic concentrations are usually higher than circulating ones, malathion concentrations ≤50 \( \mu M \) could be easily achieved in exposed individuals, and therefore, the in vitro concentrations used here may be considered representative of the in vivo situation.

Values of kinetic parameters obtained with recombinant P450s clearly indicated that CYP2C19, 1A2, and 2B6 are very efficient in malaaoxon formation, detectable starting from 0.1 \( \mu M \) OPT. In these conditions, the contribution of CYP3A4, 1A1, 2C8, and 2C9 was negligible; their activity could be more relevant at high substrate concentrations, being linear up to 50 \( \mu M \) malathion. CYP2A6 and 2D6 were not able to catalyze malathion bioactivation. The relative contribution calculated to take into account the average content of each P450 in an ideal human liver suggested a pivotal role in malaaoxon 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 malaaoxon, 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 malaaoxon were produced. In addition, at 50 \( \mu M \) malathion, which is representative of the high-affinity phase, malaaoxon 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 malaaoxon formation versus malathion concentration in HLM\(_1\) and HLM\(_3\); malaaoxon 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 malaaoxon 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 malaaoxon formation. Results from the kinetic analysis in HLM\(_2\) and HLM\(_3\) showed that when these two isoforms are present at high levels, the C\(_{\text{LH}}\) related to the low-affinity phase is significantly higher (15.5 and 8.0 \( \mu \text{mol} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1} \)). In addition, malaaoxon 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 malaaoxon 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 malaaoxon production in HLM\(_1\) and HLM\(_2\), where \( K_{\text{m}} \) 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\(_1\), 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-methenephosphoryl 4'-hydroxylation activity expressed in the panel of HLMs tested, there was no correlation with malaoxon 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; Vittorzzo 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 dimethylthiodiphenylphosphate formation (Fig. 1). Therefore, the bioactivation/detoxification balance may be the crucial step in determining differences in malathion-induced toxicity. However, since carboxylesterase can be strongly inhibited by isomalonial and other impurities (Aldridge et al., 1979; Talcott et al., 1979a,b), differences in malathion production due to different phenotyping can be relevant in interindividual responses to toxic effects consequent to malathion exposure.