METABOLISM OF DIPROPYL DISULFIDE BY RAT LIVER PHASE I AND PHASE II ENZYMES AND BY ISOLATED PERFUSED RAT LIVER

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ABSTRACT:

The metabolism of dipropyl disulfide (DPDS), an Allium sulfur compound, was investigated in rat liver cell fractions and in an isolated perfused rat liver. DPDS is oxidized to dipropyl thiosulfinate (DPDSO) by rat microsomes. The contribution of cytochrome P450 enzymes (CYPs) or flavin-containing monooxygenases (FMO) to the formation of DPDSO from its precursor was investigated. In rat microsomes, the reaction followed Michaelis-Menten kinetics with a $K_m = 0.52 \pm 0.1$ mM and a $V_{max} = 5.91 \pm 0.5$ nmol/min/mg of protein, respectively (mean $\pm$ S.E., $n = 4$). Both FMOs and CYPs were involved in DPDS oxidation, although the contribution of CYPs was predominant. Liver microsomes from phenobarbital-treated rats showed a 3.2-fold increase in the rate of formation of DPDSO. Among many CYP isoform-specific inhibitors, only CYP2B1/2 inhibitors decreased the formation of DPDSO and the best correlation between the rate of DPDS oxidation with specific monooxygenase activities was found with a marker of CYP2B1/2 activity. The action of phase II enzymes on DPDS metabolism was studied by incubating DPDS or DPDSO with liver cytosols or microsomes. Two metabolites were formed from DPDS: propylglutathione sulfide conjugate and propylmercaptan, whereas with DPDSO, only the glutathione conjugate was observed. No conjugate compound was detected in the presence of UDP-glucuronosyl transferases. When isolated rat liver was perfused with DPDS, different metabolites were obtained in the output and in the liver tissues: propylmercaptan appeared in the output, whereas methylpropyl sulfide, methylpropyl sulfone, and propylglutathione sulfide were detected in the liver tissue.

Many investigators have reported that plants of the genus Allium such as garlic and onion possess various pharmacological and therapeutic properties, with beneficial effects in the fields of carcinogenesis, cardiovascular diseases, inflammation, allergies, virology, and bacteriology. Specifically, these include: 1) inhibition of the in vitro proliferation of human tumor cells (Sundaram and Milner 1996a); 2) modulation of xenobiotic metabolizing enzymes (Haber et al., 1995; Kim et al., 1996; Siess et al., 1997); 3) activation of cell apoptosis (Sundaram and Milner, 1996b); 4) decrease in the incidence of many cancers (Wargovich et al., 1996; Ernst, 1997); 5) reduction of glycemia and lipemia; 6) antitoxic effects; 7) inhibition of platelet aggregation; and 8) antimicrobial and antiviral effects. For a review, see Dorsch (1996).

The active components present in garlic and onion have been identified to be mainly sulfur-containing compounds. Diallyl disulfide (DADS),1 from garlic and dipropyl disulfide (DPDS), from other Allium species, have often appeared among the most active molecules (Fig. 1).

The chemistry of sulfur compounds from Allium is complex. Monosulfides and polysulfides do not occur in situ in the bulb but are formed when the bulb is disrupted by the action of allinase on-S-alk(en)yl-cysteine sulfoxides, the flavor precursors. The resulting products are very reactive and subsequently undergo nonenzymatic rearrangements to produce a wide range of sulfur compounds that give the characteristic aroma of Allium, with DADS and DPDS being the major polysulfide compounds produced (Lancaster and Boland, 1990).

Although there is considerable knowledge about the biological effects of monosulfides and polysulfides, studies on their bioavailability and metabolism are few. The metabolism of monosulfides such as diallyl sulfide and dipropyl sulfide has been studied in rats, where oxidation of the sulfur atom results in the production of first sulfoxide and then sulfone (Brady et al., 1991; Chen et al., 1994; Nickson and Mitchell, 1994). Metabolism of polysulfides, such as DADS, which contains two atoms of sulfur, depends on the experimental system used to investigate this metabolism. In an isolated perfused rat liver, DADS was reduced to allyl mercaptan (AM) (see Fig. 1) (Egen-Schwind et al., 1992), whereas metabolism of DADS in rat or human liver microsomes occurred by oxidation of one atom of sulfur to give the corresponding thiosulfinate (Fig. 1) (Teyssier et al., 1999; and C. Teyssier, L. Guenot, M. Suschetet, and M.-H. Siess, unpublished results). No thiosulfonate was observed. Although the anticarcinogenic effects of DPDS has been already suggested (Sparnins et al., 1988; Kim et al., 1996; Siess et al., 1997), the metabolism of this compound has not yet been investigated.

In this study we investigated the metabolism of DPDS in rat livers by using either subcellular fractions of liver or an isolated, perfused...
Materials. DPDS (purity of 98%) was obtained from Aldrich Chemical Co. (Strasbourg, France). DPDS was used without further purification. Dipropyl thiosulfinate (DPDPSO) was provided by Pr. J. Auger, Institut de Recherche sur la Biologie de l’Insecte, Université F. Rabelais ( Tours, France). Methylpropyl sulfone (MPSO2) was synthesized as described below, α-Naphthoflavone, cimetidine, 5,5'-dithiobis(2-nitrobenzoate), reduced glutathione (GSH), methimazole, NADPH, orphenadrine, p-nitrophenol, propylmercaptop (PM), quinidine, sulfaphenazole, troleandomycin, and UDPGA were purchased from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France). All other chemicals and reagents used were of the highest commercial quality available.

Animals and Treatments. Weaning male SPF Wistar rats obtained from animal breeding unit of the laboratory were housed individually in wire cages in a room maintained at 22°C with a 12-h light/dark cycle and were fed for 4 weeks with a purified diet. CYP inducers were administrated at the end of this period as described in Table 1.

Tissue Preparation. The animals were sacrificed by cervical dislocation and exsanguination after 16 h of fasting, and 24 h after the last treatment. Hepatic subcellular fractions were prepared as previously described (Haber et al., 1994). Microsomal and cytosolic proteins were quantified using the method of Bradford (Bradford, 1976) using BSA fraction V as a standard. The method was adapted for automatic measurement using a Cobas Fara II analyzer (Roche Instruments, Basel, Switzerland). Cytochrome P450 was assayed according to Omura and Sato (1964).

Metabolism of DPDS by Microsomal Monoxygenases. Rat liver microsomes corresponding to 300 pmol of CYP were incubated with 1.5 mM DPDS, 1 mM NADPH, 50 mM Tris-HCl (pH 7) in a total volume of 500 µl. After 30 min at 37°C, the reaction was terminated by the addition of 320 µl of acetonitrile. After 15 min of protein precipitation, the mixture was centrifuged at 10,500g for 10 min, and 40 µl of the supernatant was analyzed by HPLC as described under HPLC Analysis.

Thermal inactivation of microsomes was performed by a preincubation of the microsomes in Tris-HCl buffer for 10 min at 37°C in the absence of NADPH, whereas control microsomes were preincubated in the presence of NADPH. Then DPDS and NADPH were added to both samples before incubating for 30 min.

Metabolism of DPDS, DPDSO, or PM by cytosolic GST. The reaction mixture contained 1.5 mM of rat cytosolic protein; 1.5 mM DPDS, DPDSO, or PM; and 2 mM [3H]GSH in 50 mM Tris (pH 7) in a final volume of 500 µl. After 30-min incubation, the reaction was stopped by the addition of 1 N HCl, and the supernatant obtained after centrifugation was analyzed by HPLC with a gradient as described under HPLC Analysis.

Metabolism of DPDS or DPDSO by Microsomal UGT. The reaction mixture contained 1.5 mM DPDS or DPDSO, 3 mM [14C]UDPAGA, 1.5 mg/ml microsomal protein, and 5 mM MgCl2 in 50 mM Tris-HCl (pH 7) in a final volume of 500 µl. After 30-min incubation at 37°C, the reaction was stopped as described above and the products of the reaction were analyzed by HPLC with a gradient as described under HPLC Analysis.

Determination of Kinetic Constants. The kinetic constants of phase I and phase II enzymes were determined with the substrates (DPDS, DPDSO, or PM) at concentrations of 0, 0.25, 0.5, 0.75, 1, 2, 4, 6, and 8 mM under the same conditions as described above. Km and Vmax values were estimated by fitting the Michaelis-Menten equation using a nonlinear regression software program (SAS Inc., Cary, NC).

Determination of Phase I or II Enzyme Activities. The activity of the microsomal enzymatic system was determined with 0.75 mg of microsomal protein in the presence of 2.5 mM DPDS, DPDSO, or PM in the usual reaction mixture described under Metabolism by Microsomal Monoxygenases. The activities of the cytosolic enzymatic system were determined with 0.75 mg of cytosol protein in the presence of 2.5 mM DPDS, DPDSO, or PM in the usual reaction mixture described under Metabolism by Cytosolic GST. In case of competition between cytosolic and microsomal enzymatic systems, 0.75 mg of microsomal protein and 0.75 mg of cytosol were added in the presence of 2.5 mM substrate.

Inhibition of DPDS Metabolism. Inhibitors were added to the incubation mixtures before initiation of the reaction. With the mechanism-based inhibitors, aminobenzotriazole and troleandomycin, samples were preincubated for 10 min at 37°C before the addition of DPDS. All inhibitors were dissolved in ethanol, and 0.5 µl of ethanol solution was added to the medium (total volume: 500 µl).

Liver Perfusion. Livers were isolated as described by Sies (1978) under isoflurane anesthesia, and the portal vein was cannulated. The perfusion medium consisted of Krebs-Henseleit bicarbonate buffer, pH 7.4, containing glucose (10 mM final concentration), heated to 37°C and saturated with an oxygen/carbon dioxide mixture (95:5). Biliary outflow was allowed to mix freely with the perfusate. Perfusions were carried out at constant flow (15 ml/min) rates. After a single-pass equilibration period, the experiments were performed with recirculation of the perfusate. A 150-ml reservoir was used for substrate recirculating perfusions. Depending on the experiment, the reservoir contained 20 mg of either DPDS or PM. During control experiments, no sulfur compound was added. The substrate was allowed to mix thoroughly with the buffer. Samples (0.7 ml) were collected at 0, 5, 10, 15, 20, 25, 30, 35, 45, and 65 min following initiation of DPDS or PM perfusion. One hundred microliters of each sample were analyzed by HPLC without any additional treatment. The viability of the liver was assessed by its visual appearance, by checking the
perfusion rate, and by measuring lactate dehydrogenase activity in the perfusate. At the end of the experiment, the liver was dried, weighed, and frozen (−20°C) until analysis.

**Tissue Analysis.** The livers were thawed on ice at 4°C for 2 h, then homogenized in one volume of 50 mM Tris, pH 7. Proteins were eliminated by centrifugation after the addition of trichloroacetic acid. The supernatant was extracted three times with 0.1 volume of dichloromethane. The aqueous phase obtained was analyzed using HPLC with a gradient, whereas the organic layer was analyzed using gas chromatography–mass spectrometry (GC-MS).

**HPLC Analysis.** HPLC analysis was carried out using a Waters system equipped with a pump (model 600), an auto sampler (model 717), a photodiode array UV detector (model 996), a detector (Series A-100 Flo-one, Radiomatic), and a column (Intersil ODS-3, 4.6×150 mm; GL Sciences Inc.). The flow rate was 0.6 mL/min. Analyses of DPDS, DPDSO, and PM were performed with an isocratic phase that consisted of 70:30 (v/v) acetonitrile/water. Propylglycylsulfide (PGS) was analyzed using a gradient system. The mobile phase reservoirs were: A, 1% acetic acid; B, acetonitrile. Mobile phase gradient conditions were as follows: 90% A and 10% B for 3 min, linear gradient to 80% B in 3 min, hold at 80% for 10 min, return to initial conditions in 2 min, and equilibrate for 14 min before the next injection. The spectra were obtained using a photodiode array UV detector from 190 to 300 nm. Metabolite quantities were calculated at 254 nm using standard curves for each metabolite. The HPLC detection of DPDSO was linear from at least 1 ppm to 0.001 ± 0.000 mM, which was determined to be our limit of detection. In the case of PM, the detection was linear from 1.5 ± 0.06 to 0.05 ± 0.01 mM, corresponding to its limit of detection. Data were processed using Waters Millennium software.

**Mass Spectrometry Analysis.** The identification of the DPDS metabolite formed by GST was determined using mass spectrometry. The reaction mixture obtained was analyzed using HPLC with a gradient, whereas the organic layer was analyzed using gas chromatography–mass spectrometry (GC-MS).

**Infrared Analysis.** MPSO2 from CH2Cl2 extract of the livers was identified using infrared analysis. MPSO2 was synthesized using a gradient system. The mobile phase that consisted of 70:30 (v/v) acetonitrile/water. Propylglycylsulfide (PGS) was analyzed using a gradient system. The mobile phase reservoirs were: A, 1% acetic acid; B, acetonitrile. Mobile phase gradient conditions were as follows: 90% A and 10% B for 3 min, linear gradient to 80% B in 3 min, hold at 80% for 10 min, return to initial conditions in 2 min, and equilibrate for 14 min before the next injection. The spectra were obtained using a photodiode array UV detector from 190 to 300 nm. Metabolite quantities were calculated at 254 nm using standard curves for each metabolite. The HPLC detection of DPDSO was linear from at least 1 ppm to 0.001 ± 0.000 mM, which was determined to be our limit of detection. In the case of PM, the detection was linear from 1.5 ± 0.06 to 0.05 ± 0.01 mM, corresponding to its limit of detection. Data were processed using Waters Millennium software.

**Pharmacokinetic Analysis.** The elimination rate constant (k) was determined using linear regression of the natural log of the perfusate drug concentration versus time. The half-life (t1/2) of the compounds in the perfusate was graphically determined. The area under the perfusate drug concentration-time curve (AUC) was estimated using the linear trapezoidal rule. Perfusion clearance (Clp) was calculated by dividing the dose of compound by AUC. The volume of distribution (β phase) was Clp/k.

### Results

**In Vitro, Phase I. Contribution of microsomal monooxygenases to DPDS oxidation.** When DPDS was incubated with rat liver microsomes and NADPH, regardless of the incubation time, only one peak was detected with HPLC. This peak was identified as DPDSO, by comparing its retention time and absorption spectrum with that of synthesized DPDSO in different HPLC systems. When either NADPH or the microsomes were omitted, no DPDSO was detected. The formation of DPDSO was linear over a period of 45 min with respect to the incubation time. An incubation time of 30 min was therefore routinely used. A concentration of 1 mM NADPH was optimal for the reaction. The formation of DPDSO by liver microsomes fitted the Michaelis-Menten equation. The apparent rate constant of the Michaelis-Menten equation was 0.69 ± 0.04 μmol/min/mg of protein (means ± S.E. of four samples).

**Contribution of flavin-containing monooxygenases to DPDS oxidation.** To evaluate the respective roles of FMO and CYP in the oxidation of DPDS, we performed incubations of DPDS in the presence of: 1) 1-aminobenzotriazole, a suicide inhibitor of cytochrome P450 monooxygenases (Ortiz de Montellano and Mathews, 1981), and 2) microsomes containing irreversibly inactivated FMO. The sensitivity of FMO to heat was used to irreversibly inactivate these enzymes (Gut and Conney, 1993; Grothusen et al., 1996). The results indicated that DPDSO formation was inhibited in the presence of 1-aminobenzotriazole (Fig. 2) and that FMO inactivation caused a 21.6% decrease of DPDS oxidation (Table 2). Verification of complete FMO thermal inactivation was done by measuring M0O activity. That thermal treatment did not modify the activity of ethoxycoumarin deethylylase (ECOD) was also checked. This ECOD activity is often used as a standard marker for CYP-mediated reactions because several forms of CYP are involved (Edwards et al., 1984).

**Contribution of different CYP isoenzymes to DPDS oxidation.** To assess further which CYP isoforms are involved in DPDS oxidation, the effects of the following chemical inhibitors were determined: α-naphthoflavone, p-nitrophenol, orphenadrine, quinidine, sultana-

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**TABLE 1**

Outline of liver microsome inductions

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Concentration of the daily dose</th>
<th>Solution</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>1.25 ml/kg</td>
<td>50% acetone/NaCl 0.9%</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>i.p.</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>400 mg/kg</td>
<td>Corn oil</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>24 mg/kg</td>
<td>Corn oil</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>DPDS</td>
<td>150 mg/kg</td>
<td>Corn oil</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>Methylicholanthrene</td>
<td>20 mg/kg</td>
<td>Corn oil</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Sacrifice</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>80 mg/kg</td>
<td>NaCl 0.9%</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Sacrifice</td>
</tr>
</tbody>
</table>

* i.p., intraperitoneal; i.g., intragastric; −, no injection.

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The involvement of CYPs in rats is mainly caused by the isoenzyme CYP2B. The correlation between DPDS oxidation and CYP marker activities was analyzed with a group of 28 individual samples of rat liver microsomes. The thermal inactivation of FMOs was evaluated by measuring MMO activity, whereas the inactivation of CYPs was checked by measuring ECOD activity. Values are the means of four determinations ± S.E.

### TABLE 2

<table>
<thead>
<tr>
<th>Microsome Treatments</th>
<th>DPDS Oxidase</th>
<th>MMO</th>
<th>ECOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal treatment</td>
<td>3082 ± 274</td>
<td>15.76 ± 9.0</td>
<td>0.25 ± 0.0</td>
</tr>
<tr>
<td>Control</td>
<td>3933 ± 264</td>
<td>463.95 ± 62.0</td>
<td>0.25 ± 0.0</td>
</tr>
</tbody>
</table>

The correlation of CYP2B1/2 metabolism by CYP in rats is mainly caused by the isoenzyme CYP2B. The correlation between DPDS oxidation and CYP marker activities was analyzed with a group of 28 individual samples of rat liver microsomes (four samples from each treatment, including the control). The correlation of DPDS formation with marker activities of individual CYPs was calculated. These activities were EROD for CYP1A2, PROD for CYP2B1/2, and PNPH for CYP2E1. The correlations were 0.90 with PROD, 0.20 with PNPH, and 0.06 with EROD. These results confirm the involvement of CYP2B1/2 in the metabolism of DPDS.

### Ex Vivo, Phases I and II. Perfused liver.

In a recirculating system, DPDS disappeared from the perfusion medium and was no longer detectable after 70 min. The disappearance of DPDS from the perfusion medium followed a two-compartment model. Figure 5 shows the semilogarithmic plot of mean concentrations versus time. A second slow distribution phase follows a more rapid first one. The HPLC analysis of the medium showed the formation of a more polar metabolite than DPDS, which was identified as PM. To evaluate the future transformation of PM in the liver, another study was carried out with PM instead of DPDS in the medium reservoir of an isolated perfused liver. PM concentration in the perfusate decreased with time, also following two-phase kinetics (Fig. 5). The calculated pharmacokinetic parameters of both experiments are summarized in Table 5.

The release of lactate dehydrogenase was used to evaluate the viability of the livers. Lactate dehydrogenase activity was constant during the perfusion period (65 U/liter). Neither DPDS nor PM perfusion (data not shown) significantly affected this parameter.

### Contribution of GST to DPDS metabolism.

The involvement of GST was assessed in the presence of [14C]GSH, liver cytosol, with DPDS or DPDSO as substrates. The results are shown in Table 4. In the absence of cytosolic enzymes, no product was observed, although in the presence of the cytosol two new metabolites were observed following HPLC separation. One was identified as propylmercaptan (PM, Fig. 1) by comparison of its absorption spectrum with that of a standard, and the other as PGS (Fig. 1) by mass spectrometry. A likely reaction pathway is that the attachment of one molecule of GSH to DPDS leads to the formation of a conjugate and the release of half of the molecule that corresponds to PM. To determine the possible routes of formation of GSH, we tested DPDS, DPDSO, and PM as substrates with the same reaction conditions as GST. The production rate of PGS was higher (15-fold) when DPDSO was used as the reaction substrate in place of DPDS. Only a limited activity was observed with PM (Table 4). Under conditions of competition between reactions of phase I and II for the transformation of DPDS, DPDSO appeared in a small amount suggesting its transformation to PGS immediately after its formation (Table 4, third column). With consideration of these results, we proposed (Fig. 4) a scheme of transformation of DPDS to PGS with reactions of phases I and II. The formation of PGS from DPDS or DPDSO by liver cytosol fit the Michaelis-Menten equation. The apparent $K_m$ values were 1.26 ± 0.2 or 2.06 ± 0.2 mM, and the apparent $V_{max}$ values were 9.19 ± 0.7 or 175.3 ± 13 nmol/min/mg of protein, respectively, when DPDS or DPDSO was used as the substrate (means ± S.E. of four samples).

In Vitro, Phase II. Contribution of UGT to DPDS metabolism.

The involvement of UGT was analyzed in the presence of [14C]UDP-glucuronic acid and liver microsomes. Both DPDS and DPDSO substrates were tested with this in vitro system. No product formation was detected with any of the incubation conditions or HPLC analyses tested.

Extraction of the liver homogenate gave an aqueous phase containing hydrophilic compounds. The analysis by HPLC of this phase revealed only the presence of PGS. This result is in agreement with those obtained with subcellular fractions.
FMOs and CYPs are the only enzymes present in microsomes that can catalyze NADPH and the oxygen-dependent oxidation of xenobiotics. They often display activities toward many of the same substrates. The inhibition of CYPs by 1-aminobenzotriazole, a suicide inhibitor, as well as the irreversible inactivation of FMOs by heating resulted in a decrease in the rate of DPDS oxidation. These results suggest the contribution of both CYPs and FMOs. However, FMOs appear to be less active than CYPs in this metabolism if the slight decrease in DPDS oxidase activity when FMOs were inactivated in microsomes is taken into consideration. Nevertheless, their abilities to oxidize DPDS is in agreement with their well known specificity for some drugs and other xenobiotics that possess a nucleophile heteroatom such as a sulfur atom. DADS, an equivalent sulfur compound of DPDS, was isolated. The intermediate formed from DPDSO (shown in brackets in the scheme) appears to be highly reactive, immediately producing the scheme. The intermediate formed from DPDSO was isolated. The intermediate formed from DPDSO was oxidized to DPDSO in rat liver microsomes, whereas it is transformed to PGS and PM by liver cytosol and to PM, PGS, MPS, and MPSO₂ in an isolated perfused rat liver. The microsomes contain both phase I (CYPs and FMOs) and phase II (UGTs) enzymes. Their respective efficiencies are dependent on the cofactor added. The cytosol contains phase II enzymes such as quinone reductases and GSTs. The intact liver contains phases I and II enzymes as well as all of the required cofactors.

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Three approaches were used to identify the CYP isoenzymes involved in DPDS oxidation by liver in microsomes. These were based on: 1) selective chemical inhibitors, 2) CYP induction, or 3) CYP marker activity correlation. Of all inhibitors tested, inhibition was observed only with orphenadrine, a specific inhibitor of CYP2B1/2. The maximum inhibition observed was about 55%. This result is consistent with the fact that FMOs are also present in microsomes and that they contributed to the oxidation DPDS. In fact, FMOs decreased the apparent inhibition rate of each inhibitor, because these enzymes are insensitive to the CYP inhibitors. The incubations of DPDS performed with microsomes from rats treated with several CYP inducers suggested the involvement of CYP2B in the formation of DPDSO. DPDS is known to induce the isoform CYP2B (Siess et al., 1997), and so it is likely that the xenobiotic DPDS induces the isoenzyme responsible for its metabolism. Phenobarbital is described to induce CYP2B1/2 and CYP3A (Wrighton et al., 1985; Waxman and Azaroff, 1992). Analysis of the rate of formation of DPDSO by dexamethasone-induced microsomes shows that CYP3A does not seem to be involved. In addition, the inhibition of CYP3A1 by troleandomycin has had no effect on the rate of DPDSO formation. The last approach used to identify the isoenzymes involved in DPDS oxidation was the study of the correlation between DPDSO formation rate and the level of various CYP isoenzymes. These levels were determined by CYP marker activities. The highest correlation rate obtained was between DPDS oxidase activity and PROD, the marker activity of CYP2B1/2. The three approaches used to determine CYP involvement strongly suggest the involvement of CYP2B1/2 in DPDS oxidation by rat liver microsomes.

Phase II enzymes play a central role in detoxification reactions in the liver. They neutralize, by conjugation, toxic compounds if they possess specific chemical functions. Thus the substrates of phase II enzymes are not only phase I metabolites. Studies were undertaken with not only DPDSO but also DPDS as potential substrates of GST. No conjugation was observed in the presence of UGT. To our knowledge, no glucuronide conjugate has ever been observed with sulfur compounds from Allium. GST led to the formation of PGS and PM when DPDS was the substrate, and only PGS when DPDSO was the substrate. This indicates that PM is not the intermediate in the formation of a conjugate from DPDSO. On the basis of these observations we have proposed a scheme for the metabolism of DPDS to GSH conjugates (Fig. 5). DPDS and DPDSO could be conjugated by GST to PGS by different ways of transformation. The intermediate PM, formed only during the reaction between DPDS and GST, was isolated. The intermediate formed from DPDSO (shown in brackets in the scheme) appears to be highly reactive, immediately producing another molecule of PGS (Jin and Baillie, 1997). de Rooij et al. (1996) have already observed the conjugation of thiol-containing compounds from Allium with AM from garlic. With regard to the activity of each reaction and to kinetic parameters, DPDS would be preferentially oxidized to DPDSO and then conjugated to PGS, which is the class-

### Discussion

These studies have shown that DPDS is metabolized in rat liver. DPDS is oxidized to DPDSO in rat liver microsomes, whereas it is transformed to PGS and PM by liver cytosol and to PM, PGS, MPS, and MPSO₂ in an isolated perfused rat liver. The microsomes contain both phase I (CYPs and FMOs) and phase II (UGTs) enzymes. Their respective efficiencies are dependent on the cofactor added. The cytosol contains phase II enzymes such as quinone reductases and GSTs. The intact liver contains phases I and II enzymes as well as all of the required cofactors.

FMOs and CYPs are the only enzymes present in microsomes that can catalyze NADPH and the oxygen-dependent oxidation of xenobiotics. They often display activities toward many of the same substrates. The inhibition of CYPs by 1-aminobenzotriazole, a suicide inhibitor, as well as the irreversible inactivation of FMOs by heating resulted in a decrease in the rate of DPDS oxidation. These results suggest the contribution of both CYPs and FMOs. However, FMOs appear to be less active than CYPs in this metabolism if the slight decrease in DPDS oxidase activity when FMOs were inactivated in microsomes is taken into consideration. Nevertheless, their abilities to oxidize DPDS is in agreement with their well known specificity for some drugs and other xenobiotics that possess a nucleophile heteroatom such as a sulfur atom. DADS, an equivalent sulfur compound of DPDS found in garlic, has also been shown to be oxidized by FMOs and CYPs (Teyssier et al., 1999).

Three approaches were used to identify the CYP isoenzymes involved in DPDS oxidation by liver in microsomes. These were based on: 1) selective chemical inhibitors, 2) CYP induction, or 3) CYP marker activity correlation. Of all inhibitors tested, inhibition was observed only with orphenadrine, a specific inhibitor of CYP2B1/2. The maximum inhibition observed was about 55%. This result is consistent with the fact that FMOs are also present in microsomes and that they contributed to the oxidation DPDS. In fact, FMOs decreased the apparent inhibition rate of each inhibitor, because these enzymes are insensitive to the CYP inhibitors. The incubations of DPDS performed with microsomes from rats treated with several CYP inducers suggested the involvement of CYP2B in the formation of DPDSO. DPDS is known to induce the isoform CYP2B (Siess et al., 1997), and so it is likely that the xenobiotic DPDS induces the isoenzyme responsible for its metabolism. Phenobarbital is described to induce CYP2B1/2 and CYP3A (Wrighton et al., 1985; Waxman and Azaroff, 1992). Analysis of the rate of formation of DPDSO by dexamethasone-induced microsomes shows that CYP3A does not seem to be involved. In addition, the inhibition of CYP3A1 by troleandomycin has had no effect on the rate of DPDSO formation. The last approach used to identify the isoenzymes involved in DPDS oxidation was the study of the correlation between DPDSO formation rate and the level of various CYP isoenzymes. These levels were determined by CYP marker activities. The highest correlation rate obtained was between DPDS oxidase activity and PROD, the marker activity of CYP2B1/2. The three approaches used to determine CYP involvement strongly suggest the involvement of CYP2B1/2 in DPDS oxidation by rat liver microsomes.

Phase II enzymes play a central role in detoxification reactions in the liver. They neutralize, by conjugation, toxic compounds if they possess specific chemical functions. Thus the substrates of phase II enzymes are not only phase I metabolites. Studies were undertaken with not only DPDSO but also DPDS as potential substrates of GST. No conjugation was observed in the presence of UGT. To our knowledge, no glucuronide conjugate has ever been observed with sulfur compounds from Allium. GST led to the formation of PGS and PM when DPDS was the substrate, and only PGS when DPDSO was the substrate. This indicates that PM is not the intermediate in the formation of a conjugate from DPDSO. On the basis of these observations we have proposed a scheme for the metabolism of DPDS to GSH conjugates (Fig. 5). DPDS and DPDSO could be conjugated by GST to PGS by different ways of transformation. The intermediate PM, formed only during the reaction between DPDS and GST, was isolated. The intermediate formed from DPDSO (shown in brackets in the scheme) appears to be highly reactive, immediately producing another molecule of PGS (Jin and Baillie, 1997). de Rooij et al. (1996) have already observed the conjugation of thiol-containing compounds from Allium with AM from garlic. With regard to the activity of each reaction and to kinetic parameters, DPDS would be preferentially oxidized to DPDSO and then conjugated to PGS, which is the class-

### Table 3

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Specificity</th>
<th>Percentage of Control Activity</th>
<th>References*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenol (200 μM)</td>
<td>CYP2E1</td>
<td>99.9 ± 8.1</td>
<td>Gardner et al. (1997)</td>
</tr>
<tr>
<td>Orphenadrine (200 μM)</td>
<td>CYP2B1/2</td>
<td>47.4 ± 7.7</td>
<td>Shu and Hollenberg (1997)</td>
</tr>
<tr>
<td>α-Naphthoflavone (10 μM)</td>
<td>CYP1A</td>
<td>103.7 ± 8.9</td>
<td>Newton et al. (1995)</td>
</tr>
<tr>
<td>Cimetidine (100 μM)</td>
<td>CYP2C11</td>
<td>88.5 ± 4.7</td>
<td>Umehara et al. (1997)</td>
</tr>
<tr>
<td>Sulfaphenazole (100 μM)</td>
<td>CYP2C6</td>
<td>76.5 ± 6.6</td>
<td>Umehara et al. (1997)</td>
</tr>
<tr>
<td>Quinidine (10 μM)</td>
<td>CYP2D1</td>
<td>96.1 ± 11.6</td>
<td>Kobayashi et al. (1989)</td>
</tr>
<tr>
<td>Troleandomycin (200 μM)</td>
<td>CYP3A1/2</td>
<td>80.2 ± 13.5</td>
<td>Gardner et al. (1997)</td>
</tr>
</tbody>
</table>

* These references describe the specificity of the used inhibitors.
sical route in biotransformation of xenobiotics. A similar scheme has already been proposed for DAS metabolism (Jin and Baillie, 1997).

In the isolated perfused rat liver, DPDS is also reduced to PM. The perfused liver preserves the integrity of the organ. All the enzymes remain active, and can compete for the transformation of DPDS. Because PM was the sulfur compound detected in the perfusate after the passage of DPDS in the liver, at least a small amount of DPDS would be transformed to PM. In addition, DPDS would also be oxidized to DPDSO by the action of monooxygenases and would be immediately conjugated with GSH to form PGS. In this case, DPDSO would not be detected after liver perfusion. It was not possible for us to perfuse a liver with DPDSO to study its ex vivo degradation, because we could not obtain enough of this compound. Egen-Schwind et al. (1992) observed AM when the perfusate contained DADS. At the end of the experiment, the perfused livers were homogenized and extracted, and PGS was detected in the aqueous phase. Two other metabolites, MPS and MPSO₂, were observed in the organic phase. They were detected following perfusion with DPDS and PM, although in higher amounts in the latter case. This suggests that PM is an intermediate in the formation of these compounds, in agreement with the presence of only one sulfur atom in PM as well as MPS and

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Microsomes</th>
<th>Cytosol</th>
<th>Microsomes + Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product</td>
<td>Activity</td>
<td>Product</td>
<td>Activity</td>
</tr>
<tr>
<td>DPDS</td>
<td>DPDSO 4.51 ± 0.3</td>
<td>PM 5.22 ± 1.0</td>
<td>PGS 76.79 ± 2.9</td>
</tr>
<tr>
<td>PM</td>
<td>uld*</td>
<td>PM 5.41 ± 0.1</td>
<td>PGS 1.65 ± 0.3</td>
</tr>
<tr>
<td>PGS</td>
<td>uld*</td>
<td>PGS 76.79 ± 2.9</td>
<td>PGS 1.65 ± 0.3</td>
</tr>
</tbody>
</table>

* uld, under limit of detection (<0.001 ± 0.000 mM for DPDSO; 0.05 ± 0.01 mM for PM).

![Fig. 4. Proposed scheme for the metabolism of DPDS to GSH conjugate.](image)

The compound shown in the brackets has not been seen but represents the proposed intermediate. Dotted arrows represent hypothetical routes. The width of each arrow symbolizes the importance of the route.

![Fig. 5. Perfusate drug concentration following addition of 20 mg of DPDS or PM to the reservoir of an isolated perfused rat liver.](image)

Each data point represents the mean of four experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>20</td>
<td>0.01</td>
</tr>
<tr>
<td>30</td>
<td>0.001</td>
</tr>
</tbody>
</table>

![TABLE 5: Kinetics of DPDS and PM in an isolated perfused rat liver](table)

Twenty milligrams of DPDS or PM was added to the liver perfusion medium. Results are means of four replicate experiments ± S.E.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DPDS</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-life (min)</td>
<td>4.2 ± 0.5</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Clearance (ml/min)</td>
<td>388 ± 52</td>
<td>50.6 ± 14.2</td>
</tr>
<tr>
<td>AUC (min·μg/ml)</td>
<td>54 ± 6</td>
<td>481 ± 110</td>
</tr>
<tr>
<td>Volume of distribution (ml)</td>
<td>2282 ± 113</td>
<td>350 ± 56.8</td>
</tr>
</tbody>
</table>

MPSO₂. We hypothesize that methylation of PM gives MPS. Transmethylation from S-adenosyl methionine to thiols is a well known route in drug metabolism and clearly represents a detoxification step (Weisiger and Jakoby, 1980).

The perfused liver preparation also gives pharmacokinetic information. We compared the pharmacokinetics of DPDS and PM during 65 min of rat liver perfusion. Both substances rapidly disappeared from the perfusion medium (within a few minutes). This demonstrates the large capacity of the liver to retain these compounds following presentation via the hepatic portal vein. This suggests that the liver extensively metabolizes these compounds. The calculated volume of distribution confirms the large tissue distribution reflected by the biphasic profile.

In conclusion, DPDS is extensively metabolized in rat liver. De-
pending on the experimental system used, several metabolites are formed. DPDSO, PM, and PGS are observed in the presence of liver subcellular fractions. PM, PGS, MPS, and MPO$_{2}$ are formed in the perfused liver. The results observed in the perfused liver are assumed to mimic what occurs in vivo. However, in the in vitro studies have enabled the identification of the intermediate DPDSO and the characterization of enzymes involved.

Acknowledgments. We thank Pr. Jacques Auger for providing DPDSO and Christian Ginies, Sophie Bouzigues, Sabine Louet, Marie-France Vernevaut, and Joëlle Chevalier for their technical assistance.

References


### TABLE 6

<table>
<thead>
<tr>
<th>Compound</th>
<th>Perfusion, Relative Abundance</th>
<th>EI*</th>
<th>Mol. wt.</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPDS</td>
<td>PM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPS</td>
<td>2.664</td>
<td>24.625</td>
<td>41, 48, 61, 90</td>
<td>90</td>
</tr>
<tr>
<td>MPO$_{2}$</td>
<td>3.777</td>
<td>28.587</td>
<td>41, 43, 65, 81, 94, 122</td>
<td>122</td>
</tr>
</tbody>
</table>

* Main fragments were classified by order of appearance.