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Running title

In vitro metabolism of DADS

Abstract.

1. The metabolism of diallyl disulphide (DADS) has been investigated *in vitro* with rat and human liver cell subfractions and *ex vivo* with an isolated perfused rat liver.

2. DADS was oxidized to diallylthiosulphinate by rat liver microsomes with an apparent K_m = 0.86 ± 0.1 mM and an apparent V_{max} = 0.47 ± 0.12 nmol min^{-1} mg^{-1} protein (mean ± S.E.). Both cytochrome P-450 (CYP) and flavin-containing monooxygenases were involved, with CYP2B1/2 and CYP2E1 being the most active CYP enzymes.

3. In rat and human, microsomal oxidation of allylmethyl sulphide (AMS) to allylmethyl sulphoxide (AMSO) and allylmethyl sulphone ($AMSO_2$) also occured, although at a low rate. DADS was also metabolised to allylglutathione sulphide (AGS) and allyl mercaptan (AM). In addition, diallylthiosulphinate reacted non-enzymatically with glutathione to form AGS.

4. When an isolated rat liver was perfused with DADS, the metabolites AM, AMS, AMSO, $AMSO_2$ and AGS were detected primarily within the liver tissue, with only small amounts of metabolites found in bile and perfusion medium. The pharmacokinetic parameters for DADS were $t_{1/2}$ = 6.09 min; $AUC_{0-\infty}$ = 4.77 min mmol l^{-1}; clearance = 34.22 ml min^{-1}.

5. A scheme for the metabolism of DADS in rat and human is proposed.

Introduction.

Garlic has been used for a long time in traditional medicine, being recommended for a broad range of disorders such as headache, fever, inflammation, insomnia and cough (Rivlin 2001). More recently, investigations have been undertaken to provide a scientific basis for this medicinal use. Several studies have shown that organosulphur compounds contained in garlic were linked with its biological activities, including prevention of cardiovascular diseases and cancer, antibacterial, antifungal, antithrombotic, hypotensive, and hypolipidemic properties (Reuter 1995, Milner 2001, Amagase *et al*. 2001). These compounds act through mechanisms such as modulation of drug metabolizing enzymes, inhibition of cell proliferation, inhibition of genotoxicity, immune system enhancement (reviewed by Le Bon and Siess 2000) and antioxidant activities (Phelps and Harris 1993, Prasad *et al*. 1996). Information on the bioavailability and the metabolism of the organosulphur compounds from garlic is essential to explain its health benefits. Most of the garlic organosulphur compounds are derived from alliin (S-allyl-cysteine-sulphoxide) which is converted to DADSO (diallyl thiosulphinate or allicin, figure 1) by the enzyme allinase when a garlic clove is crushed or cut. DADSO is an unstable compound, which breaks down into numerous organosulphur products such as ajoene and various sulphides (Block 1992). Among these sulphides, DADS (diallyl disulphide, figure 1) is one of the major volatile degradative garlic compounds which accounts for 40-60 % of garlic oil. However, metabolic studies of DADS are scarce, although a few have been reported. Studies in mice using radioactive DADS indicated that the uptake of radioactivity peaked in the liver 90 min after i.p. injection and rapidly disappeared (Pushpendran *et al*. 1980). With similar kinetics, we recently reported that after administration of DADS to rats, the metabolites AM (allyl mercaptan, figure 1), AMS (allylmethyl sulphide, figure 1), AMSO (allylmethyl sulphoxide, figure 1) and $AMSO_2$ (allylmethyl sulphone, figure 1) were detected in tissues such as stomach, plasma, liver and

urine (Germain *et al*. 2002). The pharmacokinetic parameters were determined for all these compounds except DADS which disappeared too rapidly in the body. In this latter *in vivo* experiment the observed metabolites were in good agreement with previous observations in rat hepatocytes, in which DADS was reported to be converted to AM and AMS (Sheen *et al*. 1999). In a study using perfused rat liver, DADS was metabolised to DADSO and AM (Egen-Schwind *et al*. 1992). In an other system using human microsomes, DADS was efficiently oxidized to DADSO (Teyssier *et al*. 1999) which could rapidly and non-enzymatically react with glutathione (Miron *et al*. 2000). In human, AM and DADS have been detected in breath after garlic consumption (Laakso *et al*. 1989, Minami *et al*. 1989, Cai *et al*. 1995, Taucher *et al*. 1996). AMSO and $AMSO_2$ have been detected in an *in vivo* rat study. In regard to these results, the hepatic metabolism seems to be predominant for the sulphur compounds DADS and DADSO (Teyssier *et al*. 1999, Egen-Schwing *et al*. 1992). AM and AMS are synthesized in the liver. They could be also transformed by the liver. For AMSO and $AMSO_2$, there is less evidence that the liver plays a role in their metabolism. They were detected in different tissues during an *in vivo* study (Germain *et al*. 2002). However during the perfusion of a liver with DADS these compounds were not detected (Egen-Schwing *et al*. 1992). Then the role of the liver in the metabolism of AMSO, $AMSO_2$ and AM, AMS is not clearly established.

Our goal was to determine DADS metabolic pathways in the liver by three approaches 1) an *in vitro* study of phase I and phase II enzymes from rat liver tissues; 2) an *in vitro* study of phase II human liver enzymes (Teyssier *et al*. 1999); 3) an isolated rat liver perfused with DADS to determine pharmacokinetic parameters of DADS, and to identify the metabolites formed in the liver. Based on these results, a possible metabolic pathway in the liver is proposed in rat and human.

[Insert figure 1 about here]

Methods.

Materials

DADS (purity 80%), AMS and AM were obtained from Aldrich Chemical Co. (Strasbourg, France). DADS was further purified by distillation to 92.2% before use and the impurities were identified by HPLC as diallyl sulphide and diallyl trisulphide. Pure DADSO was provided by Professor Auger (Institut de Recherche sur la Biologie de l'Insecte, Université F. Rabelais, Tours, France). AMSO and $AMSO_2$ were synthesized by oxidation of AMS with sodium metaperiodate in a methanolic solution thawed on ice overnight (Furniss *et al*. 1989, Germain *et al*. 2002). The reaction gave a mixture of AMSO (85%) and $AMSO_2$ (15%) as estimated by GC-MS. [glucuronyl-^{14}C]-UDPGA (272.5 mCi mmol^{-1} specific activity) and [glycine-2-^{3}H]-GSH (44.8 Ci mmol^{-1} specific activity) were purchased from NEN (Boston, United States). [^{3}H-CH_3]-S-adenosyl-methionine (85 Ci mmol^{-1} specific activity) was purchased from Isotopchim (Ganagobie-Peyruis, France). All other chemicals and reagents were of the highest commercial quality available.

Animals and treatments

Weaning male SPF Wistar rats (4-week-old) were purchased from Janvier (Le genest Saint Isle, France). They were maintained for four weeks in a controlled animal area (22°C with a 12-h light/dark period), and they were fed with a purified diet during the last week (ref 20118S from Harlan, Gannat, France). For cytochrome P-450 monooxygenase (CYP) *in vivo* induction, inducers were administered to some rats at the end of this period as previously described (Teyssier and Siess 2000).

Human liver samples

Human liver samples were provided by Professor Favre (Département de Chirurgie Digestive Thoracique et Cancérologique, General Hospital of Dijon, France) according to a protocol approved by the local ethic committee. They provided from women and men of a mean of age of 58.5 ± 7.4 (± S.E.), operated because of hepatic carcinoma or metastases. They did not follow a chemotherapy or radiotherapy. The samples were frozen in liquid nitrogen and stored at -80°C until further use for subcellular fraction preparation.

Subcellular fraction preparation

The animals (average body weight 201 g) were sacrificed by cervical dislocation, followed by exsanguination 24 h after the last treatment (16 h of fasting). Human and rat hepatic subcellular fractions were prepared as previously described (Haber *et al*. 1994). Microsomal and cytosolic protein contents were quantified by the method of Bradford (1976) using bovine serum albumin fraction V as a standard. The method was adapted for automatic measurements performed with a Cobas Fara II analyser (Roche Instruments, Basel, Switzerland). CYP was assayed according to Omura and Sato (1964).

Phase I experiments

For microsomal monooxygenases reactions, liver microsomes corresponding to 300 pmol of CYP were incubated with 1.5 mM substrate, 1 mM NADPH, 50 mM Tris-HCl pH 7.0 in a 500-µl final volume. The reaction rate was linear over a period of 60 minutes with respect to the protein concentration and the incubation time. After 30 min (60 min for AMS) at 37°C the reaction was stopped by the addition of acetonitrile (or dichloromethane for AMS). After protein precipitation, the mixture was centrifuged and the supernatant analyzed by HPLC (see below for HPLC analysis). The AMS incubation medium was analyzed by GC-MS after dichloromethane extraction.

Heat inactivation of microsomes (Kitchell *et al*. 1978) was performed by pre-incubation in Tris-HCl buffer for 10 min at 37°C in the absence of NADPH whereas control microsomes (non-inactivated) were pre-incubated in the presence of NADPH. DADS and NADPH were then added to both samples before incubation for 30 min. A positive control of the complete FMO thermal inactivation was done by measuring methimazole oxidase activity. The ethoxycoumarin deethylase activity was used to check the absence of thermal effect on CYP (Edwards *et al*. 1984). These two activities were measure as described in (Teyssier and Siess 2000).

Phase II experiments

For determination of cytosolic glutathione-S-transferase (GST) activity, the incubation mixture contained 0.75 mg of cytosolic protein, 1.5 mM substrate (DADS, DADSO or AM), and 5 mM [^{3}H]-GSH or cold GSH in 50 mM Tris pH 7.0 in a 500-µl final volume. After 30 min incubation at 37°C, the reaction was stopped by the addition of 1 M HCl and the supernatant obtained after centrifugation was analyzed by HPLC.

For determination of microsomal UDP-glucuronyl transferase activities, the incubation mixtures with the substrates DADS, DADSO or AM were conducted as previously described (Teyssier and Siess, 2000). The products of the reaction were analyzed by HPLC.

For cytosolic methyl transferase reactions, the incubation mixture consisted of 50 mM Tris-HCl pH 7.4, 1.2 mM $MgCl_2$, 200-1000 µM of S-adenosyl-methionine and 0.05 µCi of [^{3}H-CH_3]-S-adenosyl-methionine, 1 mg/ml cytosolic or microsomal protein, 1 mM dithiothreitol and 1.5 mM substrate (DADS, DADSO or AM) in a final volume of 500 µl. The incubation was carried out at 37°C for 30 min to 2 hours and was stopped by addition of acetonitrile. After elimination of precipitated proteins by centrifugation, the supernatant was analyzed by HPLC. The same assays were also conducted in the absence of $MgCl_2$ or dithiothreitol. The

positive control used phenylthiol and luteolin as substrates. The reaction was validated by HPLC detection of the methylated compounds.

Determination of kinetic constants

The kinetic constants of phase I or phase II enzymes were determined with DADS at concentrations of 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 8 mM under the conditions specified above. Apparent K_m and V_{max} values were estimated by fitting the Michaelis-Menten equation using a non-linear regression program of the SAS software (Cary, NC, USA).

Chemical inhibition study

The substrate concentration was identical in separate inhibition experiments and in "Phase I experiments" section (1.5 mM). The used concentrations for each inhibitor are indicated with their specificities. They were chosen as low as possible to get an inhibition in the range of the specificity of the inhibitor. The inhibitors used were α-naphthoflavone (CYP1A1/2; 0 to 20 µM; Teyssier *et al.* 2000), chlorzoxazone (CYP2E1; 0 to 2.0 mM; Takana 2001), diethyldithiocarbamate (CYP2E1; 0 to 100 µM; Shu and Hollenberg 1997), nifedipine (CYP3A1/2; 0 to 500 µM; Bourrié *et al*. (1996)), orphenadrine (CYP2B1/2; 0 to 500 µM; Teyssier *et al.*, 2000), quinidine (CYP2D1; 0 to 20 µM; Teyssier *et al.* 2000), tolbutamide (CYP2C6; 0 to 500 µM; Gardner *et al.* 1997), tranylcypromine (CYP2B1/2 and CYP2E1; 0 to 500 µM; Belanger *et al*. 1982) and troleandomycin (CYP3A1/2; 0 to 500 µM; ; Teyssier *et al.* 2000). Inhibitors were added to the incubation mixtures prior to initiation of the reaction. With the mechanism-based inhibitors 1-aminobenzotriazole and troleandomycin, samples were preincubated for 10 min at 37°C before the addition of DADS. All inhibitors were dissolved in an ethanolic solution and controls received the equivalent concentration of pure ethanol.

Liver perfusion

Liver was isolated from rats of around 300 g body weight as previously reported (Teyssier and Siess 2000). Surgery was performed to collect the bile (with a PE10 cannula) during liver perfusion with a Krebs-Henseleit bicarbonate buffer (pH 7.4). After a single-pass equilibration period, the experiment was performed with recirculation of a new solution of perfusate (volume of 150 ml) containing 1 mM DADS. Perfusate samples (0.5 ml) were collected at 0, 5, 10, 15, 20, 30, 40, 50, 60, 75 and 90 min following initiation of DADS perfusion, and subsequently analyzed by HPLC by direct injection as described below. The viability of the liver was assessed by its visual appearance, by checking the perfusate flow and by measuring lactate dehydrogenase activity in the perfusate. At the end of the experiment, the liver, the bile and the perfusate were store at -80°C until further analyses. They were then thawed on ice at 4°C for 1 hour, and the liver was homogenized in two-fold volumes of water. 100 µl of the HPLC standard *p*-cymene (200 ng ml^{-1}) were added to each sample. Proteins and cell debris were eliminated by addition of trichloroacetic acid followed by centrifugation. The collected supernatant was extracted three times with distilled dichloromethane. The aqueous phase obtained was analyzed by HPLC whereas the organic layer, enriched with a second standard (nonane at 200 ng ml^{-1}), was analyzed by GC-MS.

The DADS pharmacokinetic parameters were calculated with the Kinetica® software (InnaPhase, Champs sur Marne, France) using a non-compartmental method. The elimination rate constant (k_{el}) was determined by linear regression of the log perfusate concentrations in the elimination phase β. Area under the concentration versus time curve (AUC) was calculated by the trapezoidal rule and extrapolated from the last point to infinity with k_{el}. The half-life ($t_{1/2}$) was determined from the phase β in the log-linear regression plot. Clearance

was deduced from the dose/AUC ratio and the distribution volume from the clearance/k_{el} ratio.

HPLC analysis

HPLC analysis was carried out using a Waters (Saint Quentin-en-Yveline, France) system equipped with a pump (model 600), an auto sampler (model 717), a photodiode array UV detector (model 996) or an UV detector (model 2487) associated to a Serie A-100 Flo-one detector from Radiomatic and an Uptisphere 5 µm ODB column (4.6 x 150 mm, GL Sciences Inc., Tokyo, Japan) thermostated at 25°C. The flow rate was 0.7 ml min^{-1}. Data were processed by Waters Millennium software. Analysis of the metabolites after microsomal oxidation of DADS and assays of methylation of AM were performed in an isocratic phase which consisted of 70:30 (v/v, acetonitrile/water). Allylglutathione sulphide (AGS, figure 1) was analyzed using an isocratic phase consisting in 30:70 (v/v, acetonitrile/1% acetic acid in water). The spectra were obtained with a photodiode array UV detector from 190 to 300 nm, or with a flo-one detector associated with an UV detector (at 254 nm) for radiolabeled compounds. DADSO and AM were quantified at 254 nm using linear concentration standard curves for each metabolite. For DADSO, the HPLC detection was linear from 12 ± 0.465 to 1.5 ± 0.014 nmoles, the latter being the limit of detection. For AM, the detection was linear from 7.0 ± 1.46 nmole to 0.5 ± 0.14 nmole (detection limit). The quantification of AGS was carried out with the standard curve of chemically synthesized AGS from radiolabeled GSH. The detection was linear from 10 ± 0.25 nmole to 0.1 ± 0.05 nmole (detection limit).

Mass spectrometry analysis

Mass spectrometry analysis of AGS was carried out in the positive-ion mode on an Esquire~LC mass spectrometer equipped with an electrospray interface (Bruker, Strasbourg,

France). To detect AGS, the phase II incubation medium, after protein precipitation, was directly analyzed by direct infusion at 200 µl h^{-1}. Analyses were performed at a capillary voltage of 4 kV, and helium (constant velocity fixed at 35 cm/s) was used at an operating pressure of 30 psi. In-source collisions were used to induce some fragmentation of selected precursor ions.

GC-MS analyses of AM, AMS, AMSO and $AMSO_2$ were carried out using an Agilent 6973 quadripole mass spectrograph equipped with an Agilent 6890 gas chromatograph. Analyses were performed in a positive-ion mode with an electronic impact of 70 eV. Helium was employed as carrier gas. A DB-1701 (J&W Scientific, Folsom, US) capillary GC column (30 m x 0.32 mm, 1 µm film thickness) was used. The samples were injected onto the column at 35°C. After 2 min, the oven temperature was raised to 180°C at the rate of 5°C/min, and then at 10°C/min to 220°C. The samples were detected in scan (from 29 to 220 *m/z*) and in SIM (Selected Ion Monitoring) modes. Detection of AM, AMS, AMSO, $AMSO_2$ and DADS was performed with the ions at *m/z* 74, 88, 104, 120 and 146 respectively. Purity was continuously checked by the control of the ratio between this identifying ion and another one specific for each compound. As already presented, the concentration of each compound in the biological samples were calculated by the ratio between the area of the organosulphur compound and the product of the *p*-cymene (internal standard for extraction) and the nonane area (internal standard for final volume) (Germain et al., 2002).

Results

[Insert figure 2 about here]

In vitro oxidation of DADS and its metabolites in rat

To gain an insight into DADS biotransformation, the reaction products avising from phase I and II systems were investigated separately. In the presence of NADPH, liver microsomes catalyzed DADSO formation from DADS, with kinetics fitting the Michaelis-Menten equation. The apparent K_m was 0.86 ± 0.1 mM and the apparent V_{max} was 0.47 ± 0.12 nmol min^{-1} mg^{-1} protein (means ± S.E. of four samples). To determine which monooxygenase is responsible of the DADSO formation, we examined the effects of FMO inactivation by heat or CYP inhibition using 1-aminobenzotriazole, ubiquitous CYP suicide inhibitor. Heat inactivation led to a 48% inhibition of activity (figure 2) and aminobenzotriazole produced up to 65% inhibition suggesting the contribution of both types of monooxygenases in DADS oxidation to DADSO.

To identify precisely which CYP enzymes were involved in this reaction, the effects of specific chemical inhibitors (figure 3) and inducers (figure 4) were evaluated. Extensive inhibition was observed with orphenadrine (specific inhibitor of CYP2B1/2), chlorzoxazone and diethyldithiocarbamate (inhibitors of CYP2E1) and tranylcypromine (inhibitor of both CYP2B1/2 and CYP2E1). The rate of DADSO production by microsomes from animals treated with inducing agents was compared to that observed with microsomes isolated from untreated animals. DADSO formation was increased significantly ($p<0.05$) in microsomes prepared from livers of rats pre-treated with phenobarbital (CYP2B inducer and lightly CYP2C7, CYP3A1/2, 3.37-fold greater). The other treatments inducing CYP1A (methylcholanthrene), CYP2E1 (acetone), CYP3A (dexamethasone) or CYP4A (clofibrate) did not produce any significant changes in DADSO formation (figure 4). These results of

inhibition and induction of CYP enzymes suggest that DADS metabolism by CYP in rats is mainly due to CYP2B and to some extent, CYP2E1.

[Insert figures 3 and 4 about here]

We also investigated if AM and DADSO could be oxidized by microsomal enzymes. These potential oxidations were not observed whatever the conditions of incubation or detection (data not shown).

To explain the formation of AMSO and $AMSO_2$ detected *in vivo* (Germain *et al*. 2002), AMS was incubated in presence of microsomes and NADPH. Even after 60 min of incubation, the amounts of AMSO and $AMSO_2$ detected were very low (data not shown) and hence, kinetics parameters could not be accurately determined.

Conjugation of sulphur compounds in rat

The involvement of the UDP-glucuronyl transferases was analyzed in the presence of [^{14}C]-UDP-glucuronic acid, liver microsomes and DADS, DADSO or AM as substrates. No glucuronide conjugates were detected whatever the incubation conditions or HPLC analysis systems (data not shown).

To determine how AMS was formed we first hypothesised a methylation of AM to AMS. However incubation in the presence of [^{3}H-CH_3]-S-adenosyl-methionine, produced no methylation of AM with liver cytosol. Positive controls were performed with phenylthiol and luteolin. Some methyltransferases are known to be microsomal enzymes, and hence the methylation of AM was also tested in the presence of microsomes. No formation of AMS was observed. The stability of AMS was checked in the various incubation media or during the steps preceding the HPLC injection. In the last assay, DADS or DADSO have been also

tested with any success as substrates in the previous reaction media with microsomal or cytosolic enzymes.

The involvement of GST was first assessed in the presence of [^{3}H]-GSH, liver cytosol, with DADS, DADSO or AM as substrates. When DADS was used as the substrate, two new metabolites were obtained only in presence of cytosol. They corresponded to AM and another metabolite, which subsequent to mass spectrometry analysis, was in agreement with the structure of AGS. In the ms/ms experiment, this ion with *m/z* of 380.0 was fragmented and gave a major peak with *m/z* of 251.0, corresponding to AGS without the glutamic residue. The formation of AGS fitted the Michaelis-Menten equation with an apparent K_m of 9.03 ± 2.6 mM and an apparent V_{max} of 0.25 ± 0.04 nmol min^{-1} mg^{-1} protein (means ± S.E. of 4 samples). When AM was assayed in the GST reaction, no product was detected. By contrast, when DADSO was incubated with GSH, a spontaneous AGS formation occurred in absence of cytosolic proteins, suggesting a non enzymatic reaction between DADSO and GSH.

Contribution of phase I and II enzymes to sulphur compound metabolism in human

[Insert figure 5 about here]

Experiments similar to those made in rats were performed with human enzymes. In the presence of incubation medium for glutathione conjugation, DADS was metabolised to AM and AGS. Figure 5 shows the HPLC radio-chromatography obtained after such an incubation. The formation of AGS from DADS by liver cytosol fitted the Michaelis-Menten equation. The apparent K_m was 1.48 ± 0.08 mM and the apparent V_{max} was 0.08 ± 0.00 nmol min^{-1} mg^{-1} protein (means ± S.E. of four samples). No glucuronide conjugates of DADS, DADSO or AM were detected whatever the incubation conditions or HPLC analysis systems (data not shown).

No metabolites were determined when AM, DADS or DADSO was incubated with cytosolic and microsomal enzymes in presence of S-adenosyl-methionine. On the contrary, monooxygenases oxidized AMS in AMSO and $AMSO_2$ (data not shown).

Ex vivo *metabolism of DADS in the isolated perfused rat liver*

[Insert figure 6 and table 1 about here]

During perfusion in a recirculating system, DADS was monitored by HPLC and its concentration was determined over a 75-min period, after which DADS was no longer detectable. Figure 6 shows the semilogarithmic plots of DADS disappearance versus time and the deduced pharmacokinetic parameters are presented in table 1. DADS disappearance exhibits a biphasic pattern suggesting its extensive tissue distribution, consistent with the calculated distribution volume (312 ml). The elimination half-life of DADS was about 6 min. In order to assess the metabolites appearing during the experiment, the perfusate was analyzed by HPLC using conditions to detect either organosulphur compounds or the conjugates. As far as organosulphur compounds were concerned, a metabolite, identified as AM by comparison with the spectrum and retention time of the standard, was detected within the first minutes of perfusion. It slowly disappeared until 60 minutes suggesting its transformation to another product. DADSO was not detected at any time during the experiment. In the conjugate elution conditions, we observed the continuous increasing production of AGS.

[Insert figures 7 and 8 about here]

After perfusion, livers, perfusates and collected bile contents were analyzed by either HPLC (conjugates) or GC-MS (sulphurcompounds). The metabolites were identified by both methods through direct comparisons to synthesized standards. AGS was the only conjugate detected by HPLC and was found in both the liver and the perfusate. On the contrary many organosulphur compounds were detected as shown in figure 7. Their relative partition

between the three compartments is shown in figure 8. Only DADS and AM were detected in the bile. In the liver, AM and DADS were identified in trace amounts, and AMS, AMSO and $AMSO_2$ in larger quantities. In the perfusate, all organosulfur compounds were also recovered with extremely low concentrations compared to liver levels.

Discussion

After the *in vivo* administration of DADS to rats AM, AMS, AMSO and $AMSO_2$ have been previously detected in various tissues: (Germain *et al*. 2002). To further investigate the enzymes producing these metabolites, *in vitro* studies were first undertaken in rat and human subcellular fractions. In the presence of rat microsomes, DADS was a good substrate for phase I reactions, leading to DADSO formation. This latter compound did not seem to be subject to further oxidation and both CYPs and FMOs appeared to be involved in this reaction. Two complementary approaches using selective chemical inhibitors or CYP induction implied that CYP2B1/2 was responsible for DADS oxidation with a contribution by CYP2E1. The phase I metabolism of DADS is similar between rats and human with regard to the formed metabolites whereas the kinetic parameters and the enzymes involved are different. The intrinsic clearance (V_{max}/K_m) was 0.54 µl min^{-1} mg $protrein^{-1}$ for rat whereas it reached a maximum value of 30.32 µl min^{-1} mg $protrein^{-1}$ for human enzymes (Teyssier *et al*. 1999). In humans the major enzyme involved is CYP2E1, which represents 7% of total hepatic CYP (Shimada *et al*. 1994). In rats, CYP2B1/2 is the main enzyme involved and represents 37.7% of total hepatic CYP (Waxmam *et al*. 1985). Taking into account the percentage each CYP and the observed intrinsic clearance between the two animal species, human CYP2E1 is apparently much more efficient in catalysing the oxidation of DADS to DADSO. In the same way, FMOs seemed to be more active in rats DADS oxidation than in humans. In adult rat liver, the two major forms are FMO1 and FMO3 whereas FMO1 is almost absent in adult human liver (Yeung *et al*. 2000, Lattard *et al*. 2002). This suggests that FMO1 is more efficient than FMO3 in this oxidation. Microsomal monooxygenases catalyzed also the transformation of AMS into AMSO and $AMSO_2$, although at very low rates in both species. By analogy, Nnane and Damani (1999, 2002) have reported that the rate of oxidation of ethylmethyl sulphide and diphenyl sulphide to sulphones was less than 0.1%.

Phase II reactions were carried out in presence of many potential substrates such as DADS, DADSO and AM. Only enzymatic glutathione conjugation of DADS was observed, producing AGS and AM. A likely reaction pathway to explain the formation of AM is glutathione conjugation of DADS, and the subsequent release of half of the molecule corresponding to AM. AGS was also produced through a non-enzymatic reaction between DADSO and GSH, a phenomenon previously described (Miron *et al*. 2000). This indicates that AM is not the intermediate in the formation of a conjugate from DADSO.

[Insert figure 9 about here]

On the basis of these observations we propose a scheme for the metabolism of DADS in rats and humans (figure 9). This scheme is in agreement with the general metabolism of xenobiotics containing sulphur compounds (Damani 1987). Generally *S*-oxidation is predominant, explained by the readily accessible lone pair of electrons on the divalent sulphur of most sulphur compounds. Damani proposed that S-methylation of thiol groups is frequent and is followed by S-oxygenation to water-soluble sulphoxides and, in some case, sulphones. This probably represents the most important detoxification pathway for foreign thiols as well as for thiols generated *in vivo* from cystein conjugates. However, we were unable to show *in vitro* formation of AMS by methylation in the presence of either cytosolic and microsomal protein extracts. Nevertheless, we propose this reaction to be the link between AM and AMS which is further oxidized to AMSO and $AMSO_2$. Sulphoxides and sulphones are hydrophilic and generally chemically stable and are therefore the ultimate metabolites detected in the urine of animals treated with sulphide compounds such as DADS (Germain *et al*. 2002) and DPDS (results not published). In this proposed scheme, DADS has two possible routes of biotransformation: oxidation to DADSO or conjugation producing AM plus AGS.

The isolated perfused rat liver is a system which largely preserves the integrity of the organ. The enzymes are present in the same proportions as the whole organism and can compete for

the transformation of DADS. In such a system, the sulphur compounds formed from DADS were AM, AMS, AMSO and $AMSO_2$, in addition to AGS from conjugation. DADSO which appeared to be an important metabolite of DADS *in vitro*, was not found at any time in the perfused liver and among the *in vivo* metabolites of DADS after *in vivo* administration (Germain *et al*. 2002). Two possibilities could explain its absence *in vivo* and *ex vivo*: 1/ this compound could only be formed *in vitro* in the total absence of glutathione. 2/ the rate of formation of DADSO is lower than its rate of transformation into AGS thus interfering with its detection. In agreement with this last hypothesis, DADSO has been described to rapidly disappear from the circulation after intravenous injection (Lawson and Wang 1993). The metabolites identified in the isolated perfused liver herein are in accordance with previous reports: AM was observed in the isolated perfused liver (Egen-Schwind *et al*. 1992). Surprisingly the latter authors did not identify other metabolites. AM and AMS have previously been identified as metabolites of DADS in rat hepatocytes (Sheen *et al*. 1999). Excepted for AGS, the panel of metabolites presented in this study is strictly identical to those recovered in liver of rat receiving an oral DADS administration (Germain *et al*. 2002). It is important to draw attention that this is the first time AGS is described as metabolite of DADS. Unfortunately, this was not confirmed in our *in vivo* study as our analytical conditions were then unsuitable to detect conjugates.

Pharmacokinetic parameters are available for the sulphur metabolites of DADS but the rapid disappearance of DADS from the circulation prevented an accurate determination of its kinetics (Germain *et al*. 2002). DADS pharmacokinetic parameters were determined using the isolated rat liver and indicated a rapid biotransformation of DADS during liver perfusion, mainly into AM and AGS as shown by the direct analysis of the perfusate. The large DADS distribution volume suggested substantial tissue retention, confirmed by the organosulphur compound composition of liver tissue, bile and perfusate. The route of elimination of AM,

AMS, AMSO $AMSO_2$ can be predicted from the analysis of the fluids (perfusate and bile) leaving the perfused liver. DADS and AM were eliminated by the bile and the perfusate whereas AMSO and $AMSO_2$ were eliminated only by the perfusate. AMS was detected only as trace in the perfusate and totally absent in the bile. The comparison of the proportion of AMS stored in the liver or eliminated through the perfusate highlighted the low elimination of this compound through the fluids indicating that metabolism was the only clearance mechanism for this compound in this system. Nevertheless, AMS metabolism was low as it accumulates in the liver. In addition, a metabolic elimination should also occur for AM, which was not eliminated by the fluids. The conjugate AGS was detected in the perfusate. Surprisingly, AGS was never found in the biliary samples during our studies, although its polar structure and molecular weight would favour it.

In conclusion, DADS is extensively metabolized in the rat and human liver. The same metabolites were formed in both species. DADSO is formed by CYPs and FMOs. Conjugation with glutathione takes place with DADS and DADSO to produce AGS. In the case of DADS, this conjugation produces also a thiol, AM, which could be methylated to AMS and then is oxidized by monooxygenases to AMSO and $AMSO_2$. These metabolites, except DADSO, are observed in the rat liver perfused with DADS. Garlic is intensively used in folk medicine and DADS and DADSO are often described as important active compounds of garlic (Le Bon and Siess 2000, Milner 2001). With regard to their rapid metabolism by the liver, we suggested that the beneficial effects of garlic are not only mediated by these sulphur compounds. Among the potential active compounds AGS has been reported to exhibit high antioxidant properties (Rabinkov *et al.* 2000), whereas nothing has been reported on the health properties of some metabolites such as AMSO and $AMSO_2$.

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TABLE 1

Pharmacokinetical parameters of DADS in the isolated perfused rat liver.

DADS was perfused in the liver at the concentration of 1 mM.

k_{el} (min^{-1})	0.13 ± 0.03
$AUC_{0-\infty}$ (min $mmol.l^{-1}$)	4.77 ± 0.80
Half-life, β (min)	6.09 ± 1.26
Clearance (ml min^{-1})	34.22 ± 5.6
Volume of distribution (ml)	311.9 ± 0.08

Results are means of four replicate experiments ± S.E.

DADS was perfused in the liver at a concentration of 1 mM, as described in Materials and Methods.

Legends for figures.

Figure 1: *Molecular structures of sulphur compounds investigated.*

Figure 2: *Effects of inactivation of FMOs or CYPs on DADS oxidase activity*.

A : thermal inactivation of FMOs ; B : chemical inhibition. Results are the percentage of control (without inactivation) expressed as means ± S.E. (n = 4).

Figure 3: *Inhibition of DADS oxidation by chemical CYP inhibitors in rat liver microsomes.*

Values are the percentage of control (without inhibitor) expressed as means ± S.E. (n = 4). The inhibited CYP isoforms are specified for each inhibitor.

Figure 4: *Effect of CYP inducers on DADSO formation by rat liver microsomes.*

Value marked with an asterisk differs significantly (Dunnett's test) from the control corresponding value ($p \leq 0.05$). The induced CYP isoform is indicated under each inducer. Results are means ± S.E. (n = 4). AC : acetone; CL : clofibrate; DX : dexamethasone; MC : 3-methylcholanthrene; PB : phenobarbital.

Figure 5: *HPLC profile of an incubation of DADS with human cytosol and radio-labelled GSH, obtained with a flo-one detector associated with UV detector at 254 nm.*

Figure 6: *DADS concentration in the perfusate following addition of 1 mM DADS to the reservoir of an isolated rat perfused liver.*

Each data point represents the mean of four experiments.

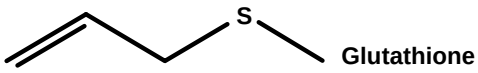
Figure 7: *Total GC-MS ionic current chromatogram of an extract of rat liver.*.

The liver had been previously perfused by 1mM DADS in a recirculating system for 90 min.

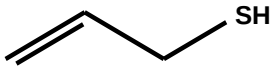
Figure 8: *Relative proportions (in percent) of sulphur compounds in different compartments of an isolated rat perfused liver.*

The sulphur compounds in the liver, the bile and the perfusate were extracted at the end of the perfusion experiment, and analyzed by GC-MS. 100 % represents the sum of normalized areas of all detected products in the three compartments. Results are means ± S.E. (n = 4).

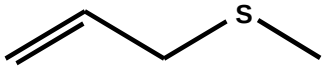
Figure 9: *Proposed biotransformation pathways of DADS in rat and human*. Dotted arrow represents a hypothetical route. MMO : microsomal monooxygenase; MT: methyl transferase.



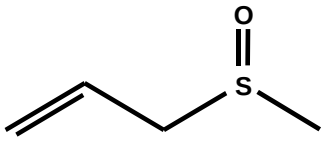
AGS, allylglutathione sulphide



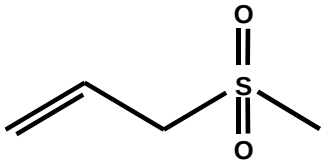
AM, allyl mercaptan



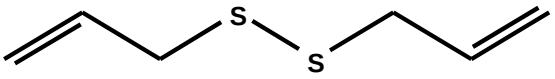
AMS, allylmethyl sulphide



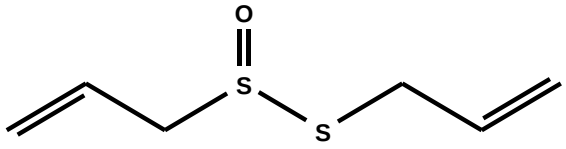
AMSO, allylmethyl sulphoxide



$AMSO_2$, allylmethyl sulphone



DADS, diallyl disulphide



DADSO, allicin or diallyl thiosulphinate

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