# Metabolism of Triallate in Sprague-Dawley Rats. 3. In Vitro Metabolic Pathways

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Rat liver enzyme preparations were used to investigate the mechanistic pathways of triallate metabolism. Incubation of [$^{14}$C]triallate with NADPH-fortified microsomes gave rise to metabolites generated via a number of reaction types, including sulfur oxidation/hydrolysis and hydroxylation of the allylic thiol moiety accompanied by rearrangement. Addition of glutathione (GSH) to microsomal incubations resulted in the formation of GSH conjugates arising via conjugate addition of glutathione to metabolic intermediates such as trichloroacrolein. Addition of GSH to microsomal incubations inhibited the binding of triallate-derived radioactivity to microsomal proteins. Metabolite identification was accomplished by mass spectrometry and chemical synthesis and by a novel application of heteronuclear multiple quantum coherence (HMQC) NMR spectroscopy. Elucidation of the in vitro metabolism of triallate provides important mechanistic information regarding triallate metabolism in the laboratory rat.

## INTRODUCTION

The preceding papers (Ridley et al., 1993; Nadeau et al., 1993) describe the metabolism of triallate administered orally to Sprague-Dawley rats. Excretion kinetics varied somewhat according to dose level and sex, but elimination of the dose was rapid and metabolic breakdown of triallate was extensive. Conversion of [$^{14}$C]triallate to expired $^{14}CO_2$ was also observed, consistent with the formation of labile intermediates. Twelve metabolites were identified that arise via oxidation reactions, glutathione conjugation, and breakdown via the mercapturate pathway. This paper describes a study of triallate metabolism using rat liver microsomal and cytosolic enzyme preparations. In addition to employing conventional techniques for metabolite structure elucidation, we have utilized a novel application of the heteronuclear multiple quantum coherence (HMQC) NMR experiment for the determination of metabolite structure in situations where other methods are impractical or unavailable. Our work on the microsomal breakdown of triallate has enabled us to identify reactive metabolites not directly observable in vivo and to establish in considerable detail the mechanistic pathways of triallate metabolism in the laboratory rat.

## MATERIALS AND METHODS

**Chemicals.** [$^{14}$C]Triallate (35.8 mCi/mmol) was synthesized by starting with [1-$^{14}$C]-1,2,3,3-tetrachloro-2-propene (New England Nuclear, Inc., Boston, MA). [1-$^{14}$C]-2,3,3-Trichloro-2-propenesulfonic acid (TCPSA) (37.12 mCi/mmol) was synthesized by starting with [3-$^{14}$C]-1,1,2,3-tetrachloro-1-propene (New England Nuclear). [1-$^{13}$C]Triallate (94.4% isotopic enrichment) was synthesized by starting with [$^{13}$C]methyl iodide (KOR Isotopes, Cambridge, MA). The radiological and chemical purities of these chemicals were determined by HPLC/RAD (high-performance liquid chromatography/radiochemical detection) and GC/FID (gas chromatography/flame ionization detection) analyses and exceeded 98%. The following were purchased from Sigma Chemical Co. (St. Louis, MO): glucose 6-phosphate, glucose-6-phosphate dehydrogenase, glutathione (GSH), and nicotinamide adenine dinucleotide phosphate (NADP). Other chemicals and solvents employed were of the highest purity obtainable from commercial suppliers.

**Liver Homogenate Preparation.** Uninduced male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were anesthetized with $CO_2$ and sacrificed by decapitation (average body weight of 250 g). Livers were removed by lateral abdominal incision, minced with scissors, and mixed with 4 °C potassium phosphate buffer (0.01 M, pH 7.4, containing 1.15% KCl, at a ratio of tissue to buffer of 1:3 (w/v). Further procedures were conducted at 4 °C. Liver suspensions were homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH) and centrifuged at 9000$g$ for 30 min. Supernatants from this centrifugation constituted liver homogenate S-9 fractions, which were centrifuged for 1 h at 100000$g$ to obtain the supernatant cytosolic S-100 fraction. The remaining pellet, or microsomal fraction, was resuspended in buffer and gently dispersed using a glass pestle homogenizer (Kontes, Morton Grove, IL). All enzyme preparations were immediately stored as small aliquots at −70 °C and thawed afterward as needed. Protein concentrations were measured by the Coomassie blue dye-binding assay (Bradford, 1976), using bovine serum albumin as the standard.

**Liver Microsomal Incubations.** Microsomal incubations (1–3 mg of protein) were conducted in open reaction vials containing an NADPH-generating system (Feng and Patanella, 1989), pH 7.4 phosphate buffer (0.2 M), and labeled triallate substrate (0.05 μmol) in a total volume of 1 mL containing 5% methanol. Incubations were stirred at 37 °C in a water bath. Control incubations were conducted with heat-denatured enzymes. Glutathione (0.26–5.0 μmol) was added to incubation reactions conducted with exogenous GSH. Incubations used for generating larger amounts of metabolites for isolation and identification were scaled up 5-fold. All reactions were initiated by addition of substrate and quenched (from 1 min to 24 h) with an equal volume of cold methanol. S-9 incubations were conducted using the procedure described for microsomal incubations.

**Liver Cytosolic Incubations.** Cytosolic incubations (3 mg of protein) were conducted in open reaction vials containing GSH (5.0 μmol), pH 7.4 phosphate buffer (0.2 M), and labeled triallate substrate (0.05 μmol) in a total volume of 1 mL containing 5% methanol. The incubations were stirred in a 37 °C water bath. Control incubations were conducted with heat-denatured enzymes. Reactions were initiated by addition of substrate and were quenched at intervals of 2, 10, 35, 65, and 120 min with an equal volume of cold methanol. Since both triallate and TCPSA were unreactive under these conditions, the activity of the liver cytosolic preparation was confirmed by incubating with alachlor, which reacts rapidly with GSH when incubated with rat liver cytosol (Feng and Patanella, 1989).

**Covalent Protein Binding.** Methanol-precipitated protein pellets from microsomal incubations were washed repeatedly with 80% methanol/water and 50% ethanol/ether until extracted radioactivity was less than 0.1% of the total originally added to the incubation (Dybing et al., 1976). The resulting protein pellet

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was dissolved in 0.25 M NaOH, and a 20-μL sample was withdrawn and dissolved in Atomlight scintillation fluid (New England Nuclear) and counted in a Tracor Analytic Mark III radioactivity counter.

**Metabolite Isolation and Identification.** Metabolites were identified by mass spectrometry, NMR spectroscopy, and comparison of HPLC and GC retention times with those of synthetic standards. Incubations were quenched and centrifuged, and the resulting supernatants were analyzed by reversed-phase HPLC/RAD. Metabolites were separated by reversed-phase HPLC on a Beckman Ultrasphere ODS column (10 mm × 250 mm). GSH conjugates were separated with a 10-min linear gradient of 0–10% acetonitrile in 1% aqueous acetic acid (3 mL/min), followed by a 10-min linear gradient to 100% acetonitrile. Other metabolites were separated with a 15-min linear gradient of 0–100% acetonitrile in 1% aqueous acetic acid (3 mL/min), which was held at 100% acetonitrile for 10 min thereafter. The HPLC effluent was routed directly to a fraction collector for metabolite isolation. The metabolites trichloroacrolein (8) and 2,3,3-trichloro-2-propenethiol (4) were isolated by quenching and extraction of incubations with ethyl acetate or hexane.

**Chromatography and Mass Spectrometry.** HPLC analyses were performed on a system consisting of a Waters 680 gradient controller and two 510 pumps, a Waters 440 UV detector, a Waters 710B Wisp autosampler, a Radiomatic Flo-one radioactivity flow detector equipped with a liquid flow cell (2.5 mL), and an Isco Foxy fraction collector. A Waters 6000A pump was used to deliver Atomflow scintillation fluid (New England Nuclear), which was mixed with the HPLC effluent prior to entering the Flo-one cell.

Gas chromatography chemical ionization (GC/CI) mass spectra were obtained with a Finnigan 4515 quadrupole mass spectrometer and were processed with a Data General Nova 4 computer using INCOS software. The samples were introduced via a splitless injector on a Finnigan 9610 gas chromatograph equipped with a J & W Scientific DB-5 fused silica capillary column (25 m × 0.25 mm). The oven temperature was normally kept at 70 °C for 1 min and was programmed to rise from 70 to 300 °C at a rate of 10 °C/min. The injector temperature was maintained at 220 °C. Helium was employed as the carrier gas at a flow rate of 2 mL/min. Chemical ionization of the gas chromatograph effluent was performed with isobutane (0.5-Torr source pressure). The mass spectrometer was scanned from 120 to 650 amu in 1 s for both positive- and negative-ion analyses.

Liquid chromatography/mass spectrometry (LC/MS) was performed using an HPLC system consisting of a Waters 680 gradient controller and two 510 pumps (Solsten et al., 1990). Samples were introduced into the mass spectrometer via a Waters μBondapak C-18 column (3.9 mm × 300 mm). The mobile phase consisted of a linear gradient of 1% formic or acetic acid in water/methanol (or acetonitrile) increasing from 0–5% to 90–95% methanol (or acetonitrile) over 20 min, at a flow rate of 0.8 mL/min. The liquid chromatograph was interfaced to a Finnigan 4535 quadrupole mass spectrometer by means of a Vestec Model 701 thermospray source operating in the discharge ionization mode. The source block was maintained at 275–280 °C, and the tip heater was maintained at 310–320 °C throughout the analyses. The vaporizer temperature was controlled by a gradient compensator and typically decreased from 160 to 120 °C during the gradient progression. The mass spectrometer was normally scanned from 140 to 700 amu in 1.5 s for both positive- and negative-ion analyses.

Fast-atom bombardment (FAB) mass spectra were obtained with a VG ZAB-HF double-focusing mass spectrometer and were processed with a Digital PDP 11/24 data system using the VG 11/250 software. The samples were deposited by a needle syringe on a thin layer of glycerol on a FAB target and were introduced into the mass spectrometer. Ionization was achieved with an Ion Tech saddle field fast atom gun typically producing 8 kV of xenon atoms at 1-mA emission current. The mass spectrometer was normally adjusted to 1200 amu resolution and was scanned from 300 to 1100 amu at a rate of 10 s/decade mass for both positive- and negative-ion analyses.

**NMR Spectroscopy.** NMR spectra were recorded on Varian VXR-300 and VXR-500 spectrometers without sample spinning and were processed using a Sun data system. The HOD signal at 4.76 ppm was used as an internal reference. $^1$H NMR spectra (299.8 MHz) of synthetic samples were recorded with a 30° radio frequency pulse, using a few milligrams of sample dissolved in 0.7 mL of $D_2O$ (MSD Isotopes, 99.999% D). $^1$H NMR (499.8 MHz) spectra of metabolites and of GSH conjugates were recorded using a 60° radio frequency pulse with four steady-state pulses for suppression of the HOD signal. $^{13}$C NMR spectra (75.4 MHz) of $^{13}$C-enriched metabolites were recorded on a VXR-300 spectrometer using a 40° radio frequency pulse with a broadband proton decoupling pulse and internal referencing to the $^{13}$C methyl signal of acetic acid. Proton-detected heteronuclear multiple quantum coherence (HMQC) NMR spectra of $^{13}$C-enriched metabolites were recorded on a VXR-500 spectrometer equipped with an indirect-detection probe using the previously reported pulse sequence (Bax et al., 1983; Hackett et al., 1990, 1991).

**Synthesis of Metabolite Standards.** Trichloroacrolein (8) (Figure 1) was prepared from 2,3,3-trichloroallyl alcohol (9) (Hatch and McDonald, 1952) using a previously described procedure (Rosen et al., 1980). Triallate sulfoxide 5 and sulfone 6 were prepared from triallate using a previously described procedure (Schuphan and Casida, 1979). The mono and bis glutathione adducts 12–15 were prepared by reaction of GSH with trichloroacrolein (8). GSH (20 mg, 0.065 mmol) was dissolved in $D_2O$ (0.6 mL), and NaOD/$D_2O$ was slowly added to the solution to adjust the pH to 8.5. The solution was transferred to an NMR tube, synthetic 8 (5 mg, 0.032 mmol) was added, and the tube was placed in the NMR probe. Two additional peaks in the aldehyde proton region appeared within 30 min. The same reaction occurred at a slower rate in $D_2O$ at neutral pH. Addition of a trace of $NaBH_4$ to the NMR tube caused the aldehyde proton resonances to disappear. HPLC analysis of the reaction mixture showed two products, which were isolated to yield 14 (5 mg, 0.012 mmol) and 15 (12 mg, 0.017 mmol).

Reaction product 14 was identified as being *N*-[*S*-(1,2-dichloro-3-hydroxy-1-propenyl)-*N*-L-glutamyl-L-cysteinyl]glycine. $^1$H and $^{13}$C NMR spectra were consistent with the proposed structure (see supplementary material). FAB MS of 14: 432 (M + 1, 100%), 434 (M + 3, 76%). The intermediate aldehyde 13, *N*-[*S*-[2-chloro-2-formyl-1-(glycyl-*N*-L-glutamyl-L-cysteinyl)ethenyl]-*N*-L-glutamyl-L-cysteinyl]glycine, which afforded 15 upon treatment with $NaBH_4$, was isolated before reduction by HPLC. The $^1$H NMR spectrum of 13 was consistent with the proposed structure (see supplementary material). Reaction product 15 was identified as being the bis GSH conjugate *N*-[*S*-[2-chloro-1-(glycyl-*N*-L-glutamyl-L-cysteinyl)-3-hydroxy-1-propenyl]-*N*-L-glutamylcysteinyl]glycine. $^1$H NMR spectrum of 15: 4.5–4.6 (m, 2 H), corresponding to C-3 allylic protons, with glutathione proton resonances as previously reported for GSH (Podanyi and Reid, 1988). $^{13}$C NMR spectrum of 15: 138.9 (C-1 position), 127.1 (C-2), 61.3 (C-3), with GSH carbon resonances as expected (Podanyi and Reid, 1988). FAB MS of 15: 703 (M + 1, 100%), 704 (M + 2, 31%), 705 (M + 3, 48%).

## RESULTS

**In Vitro Metabolites in the Absence of Glutathione.** The metabolites produced in microsomal incubations of [$^{14}$C]triallate (Figure 1) varied somewhat in relative amount depending on the particular enzyme preparation used, although a consistent overall pattern of triallate breakdown was observed. Incubations without exogenous GSH afforded hydrolysis and oxidation/reduction products typical of phase 1 metabolism (Hodgson and Dauterman, 1984). Major metabolites are defined here as incubation products consistently observed at levels >5% of the total radioactivity distribution, while minor metabolites were consistently observed at levels <5%.

**2,3,3-Trichloro-2-propenesulfinic Acid (2) and 2,3,3-Trichloro-2-propenesulfonic Acid (TCPSA) (3).** Sulfinic acid 2 was the major microsomal metabolite under oxidative conditions. Negative-ion LC/MS analysis of 2 provided a molecular weight of 208, assigned on the basis of an [M − 1]$^-$ triplet of *m/z* 207, 209, and 211. The latter corresponds to the following isotopic label patterns: $^{12}$C/

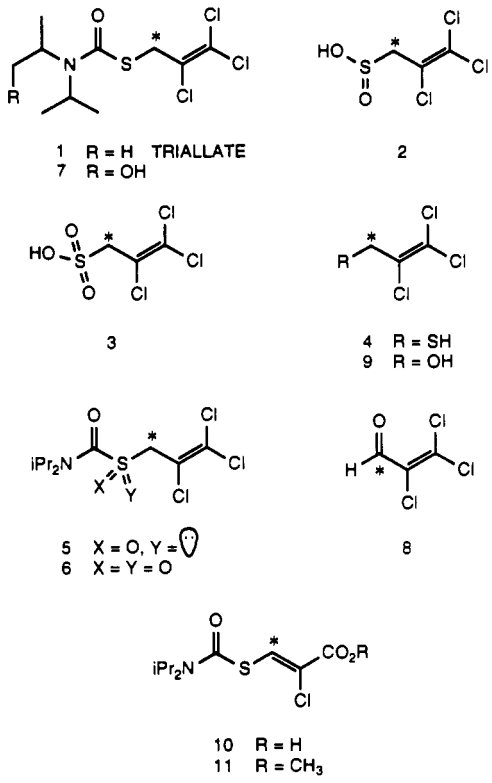



**Figure 1.** Triallate-derived metabolites formed via incubation with rat liver microsomes (fortified with NADPH).

$^{35}Cl$ (207), $^{14}C/^{35}Cl$ and $^{12}C/^{37}Cl$ (209), and $^{14}C/^{37}Cl$ and $^{12}C/^{37}Cl/^{37}Cl$ (211). These peaks were accompanied by other isotopically labeled ion peaks displaying masses and intensities consistent with the presence of 50% $^{14}C$ label and three chlorine atoms. Ions were also observed at $m/z$ 173 and 175, resulting from substitution of a chlorine atom by a hydrogen atom. Metabolite **2** was converted to a methyl sulfone by reaction with methyl iodide in aqueous ethanol. GC/CI/MS analysis of the latter yielded a cluster of three ions $[M + 1]^+$ at $m/z$ 223, 225, and 227, with an isotopic distribution as expected. The sulfonic acid **3** (TCPSA) was observed at levels generally less than 5%, although TCPSA levels increased on prolonged storage of incubation samples, due to air oxidation of **2**. The identity of metabolite **3** was established by HPLC retention time as compared with synthetic TCPSA. TCPSA itself was unreactive in microsomal incubations fortified with NADPH and in cytosolic incubations enriched with exogenous GSH.

**2,3,3-Trichloro-2-propene-2-thiol (4).** Thiol **4** was a minor metabolite produced in S-9 and microsomal incubations. Addition of GSH to incubations increased the formation of metabolite **4**, possibly via reaction of GSH with the transient sulfoxide **5** described below (Mair and Casida, 1991) or via antioxidant activity of GSH. Thiol **4** was formed early in the course of microsomal and S-9 incubations and was then consumed, most likely via oxidation to the sulfur acids **2** and **3**. In vitro metabolite **4** displayed the same HPLC retention time relative to triallate as did an authentic standard (Nadeau et al., 1993). Thiol **4** was isolated from a quenched S-9 incubation mixture by extraction with ethyl acetate and was derivatized to a pentafluorobenzoic acid thioester using pentafluorobenzoyl chloride. GC/MS analysis in the CI mode produced the ion cluster $[M + 1]^+$ expected for a pentafluorobenzoyl ester of **4** (371, 373, 375).

**Triallate Sulfoxide (5) and Sulfone (6).** Sulfoxide **5** and sulfone **6** were transient metabolites under oxidative conditions. Sulfoxide **5** was rapidly consumed after formation, either via hydrolysis to sulfenic acid **21** (see Scheme I) or oxidation to **6**. The identities of **5** and **6** were assigned by comparison of HPLC retention times with those of standards synthesized according to published procedures (Schuphan and Casida, 1979).

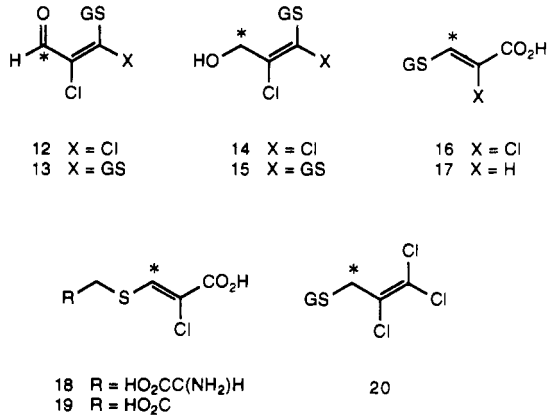
**Hydroxytriallate (7).** Hydroxytriallate (**7**) was a minor product of microsomal incubations and has also been observed as a minor in vivo rat metabolite (Nadeau et al., 1992). LC/MS analysis of **7** displayed the expected ions $[M + 1]^+$ at $m/z$ 320, 322, and 324. The HPLC retention time of in vitro metabolite **7** relative to both triallate and metabolite **11** (see below) corresponded well with that previously observed for the in vivo metabolite **7**.

**Trichloroacrolein (8) and Trichloroallyl Alcohol (9).** Trichloroacrolein (**8**) was present at trace levels in microsomal incubations and was not directly observable by HPLC/RAD. However, **8** was detected by GC/CI/MS analysis of hexane extracts of microsomal incubations. Scanning in the positive-ion mode revealed three intense ions $[M + 1]^+$ at $m/z$ 159, 161, and 163, with intensities reflecting the presence of 50% $^{14}C$ substitution as well as three chlorine atoms. Metabolite **8** coeluted with synthetic trichloroacrolein by GC/MS. Trichloroallyl alcohol (**9**) was a major in vitro metabolite in microsomal incubations, arising via enzymatic reduction of **8**. Negative-ion GC/CI/MS of HPLC-purified **9** displayed ions at $m/z$ 160, 162, and 164, with the expected relative intensities. Metabolite **9** coeluted with an authentic standard by HPLC and GC/MS.

**2-Chloroacrylate Thiocarbamate (10) and Methyl Ester (11).** Metabolite **10** was one of the major products of microsomal incubation of triallate. LC/MS analysis of **10** showed intense doublets at $m/z$ 266 and 268, corresponding to $[M + 1]^+$ for $^{12}C/^{35}Cl$ isotopic substitution and to $[M + 1]^+$ for $^{14}C/^{35}Cl$ and $^{12}C/^{37}Cl$ substitution, respectively. The intensities of these ions were consistent with the presence of one chlorine atom. The proton NMR spectrum of metabolite **10** clearly displayed a vinyl proton at 7.9 ppm as well as isopropyl protons between 1 and 2 ppm. Metabolite **11** was a minor product in microsomal incubations, arising via reaction with cosolvent methanol (5%). HPLC-purified metabolite **10** was converted to **11** by esterification with diazomethane. GC/CI/MS analysis of isolated metabolite **11** provided a molecular weight of 279, on the basis of intense doublets at $m/z$ 280 and 282, which correspond to $[M + 1]^+$ for $^{12}C/^{35}Cl$ isotopic substitution and to $[M + 1]^+$ for $^{14}C/^{35}Cl$ and $^{12}C/^{37}Cl$ substitution, respectively. These two ions and a third ion at 284 ($^{14}C/^{37}Cl$) display masses and intensities consistent with 50% $^{14}C$ label and one chlorine atom. Ions were observed at $m/z$ 128 and 153, resulting from fragmentation of the metabolite into diisopropyl isocyanate ($m/z$ 128) and thiol ($m/z$ 153). Ester metabolite **11** was also identified by LC/MS analysis, which showed expected $[M + 1]^+$ ions at $m/z$ 280, 282, and 284.

**In Vitro Metabolites in the Presence of Glutathione.** Several additional metabolites were observed in microsomal and S-9 reactions conducted with exogenous glutathione (Figure 2), while decreased formation of trichloroallyl alcohol (**9**) and the 2-chloroacrylate thiocarbamate (**10**) was noted. This observation suggested that precursors of the latter were instead reacting with glutathione.

**Bis GSH Allylic Alcohol Conjugate (15).** Model reactions employed synthetic trichloroacrolein and GSH in $D_2O$ at pH 8.5 and produced the successive 1,4-addition products **12** and **13** (Figure 2), as monitored by $^1H$ NMR



12 X = Cl
13 X = GS
14 X = Cl
15 X = GS
16 X = Cl
17 X = H
18 R = $HO_2CC(NH_2)H$
19 R = $HO_2C$
20

**Figure 2.** Triallate-derived metabolites formed via incubation with rat liver S-9 (fortified with NADPH and GSH) and related compounds.

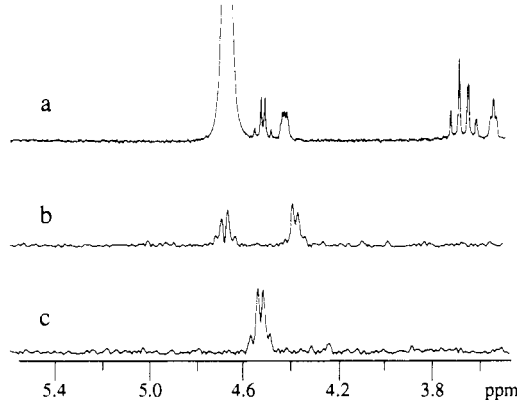



**Figure 3.** 500-MHz $^1H$ NMR spectra (transients in parentheses): (a) synthetic **15** (1024); (b) metabolite **15**, HMQC (8192); (c) metabolite **15** WALTZ $^{13}C$-decoupled HMQC (5120). Spectrum a, displayed in phase-sensitive mode, was acquired via a standard single-pulse sequence, while spectra b and c were acquired as described previously (Bax et al., 1983) and are displayed in absorption mode (acquisition time $t_2$ = 0.25 s; recycle delay = 1.5 s; evolution time = 0 s; delay time $\Delta = 1/(2J_{CH})$; $J_{CH}$ = 140 Hz).

spectroscopy. These aldehydes were reduced to the alcohols **14** and **15** by adding $NaBH_4$ (Giles, 1974). The 500-MHz $^1H$ NMR spectrum of synthetic **15** is illustrated in Figure 3a for the chemical shift region where resonances from both the GSH (Podanyi and Reid, 1988) and allylic alcohol moieties are observed. The structure of synthetic **15** was also confirmed by FAB MS.

Incubations of triallate with S-9, NADPH, and GSH produced two major and several minor GSH conjugates. The HPLC retention times of several incubation products corresponded well with retention times of aldehydes or alcohols produced in model reactions, and one of the major product fractions matched synthetic **15** in retention time. However, FAB MS analysis of HPLC-purified metabolite **15** was unsuccessful due to co-eluting matrix impurities (Hoffmann and Baillie, 1988), and the $^1H$ NMR spectrum of metabolite **15** was complicated by numerous extraneous resonances. [1-$^{13}C$]Triallate was therefore used to prepare in vitro metabolite **15** enriched with $^{13}C$ in the allylic carbon position, which was subjected to the $^1H$-detected HMQC NMR experiment (Bax et al., 1983; Hackett et al., 1990). The one-dimensional HMQC NMR spectra of $^{13}C$-labeled metabolite **15** (45 $\mu$g) are illustrated in Figure 3 and feature resonances for the two methylene protons bonded to the $^{13}C$-enriched carbon, which appear as a doublet of multiplets at 4.53 ppm, with an expected carbon coupling constant of 148.5 Hz. The $^{13}C$ NMR spectrum of metabolite **15** displayed a carbon resonance at 61.3 ppm, the same chemical shift as was observed for the allylic carbon position of synthetic **15**.

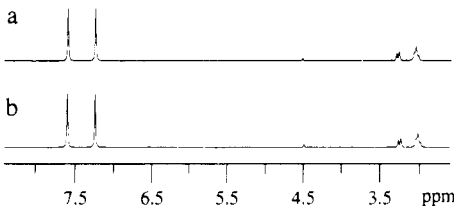



**Figure 4.** 500-MHz $^1H$ NMR spectra (transients): (a) metabolite **16**, HMQC (4096); (b) semisynthetic **16**, HMQC (32798). Spectra were acquired as described previously (Bax et al., 1983) and are displayed in power mode (acquisition time $t_2$ = 0.15 s; recycle delay = 1.5 s; evolution time = 0 s; delay time $\Delta = 1/(2J_{CH})$; $J_{CH}$ = 8.3 Hz).

**2-Chloroacrylate GSH Conjugate (16).** Attempted mass spectral analysis of the other major product observed in incubations with triallate and GSH failed to provide detectable ions. The $^1H$ NMR spectrum of this metabolite fraction (20 $\mu$g) was consistent with the presence of GSH (Podanyi and Reid, 1988) and featured a $^{13}C$-coupled vinyl proton resonance analogous to that observed for the 2-chloroacrylate thiocarbamate (**10**), suggesting a 2-chloroacrylate GSH conjugate structure (Figure 2), by analogy with the in vivo rat metabolites **18** and **19** (Nadeau et al., 1993). The formation of such glutathione-derived conjugates is well precedented (Barnes et al., 1959; Bray et al., 1959).

As before, [1-$^{13}C$]triallate was used to prepare metabolite **16** (20 $\mu$g) enriched with $^{13}C$ in the olefinic carbon position, which was analyzed by one-dimensional HMQC NMR spectroscopy (Figure 4a). To obtain positive confirmation of GSH attachment, the HMQC experiment was optimized for long-range scalar coupling interactions (Bax et al., 1983; Hackett et al., 1991). This permitted visualization of the methylene protons of GSH located three bonds from the $^{13}C$-labeled olefinic carbon nucleus. The $^{13}C$ NMR spectrum of metabolite **16** displayed a carbon resonance at 133.9 ppm, also observed for the C-3 carbon position of semisynthetic **16**, prepared as described below.

**Acrylate GSH Conjugate (17).** It seemed reasonable to infer that GSH conjugate **16** could be formed from thiocarbamate metabolite **10** (Figure 1). However, HPLC-purified metabolite **10** was unreactive toward GSH conjugation in both oxidative and nonoxidative incubation reactions. The synthetic $^{14}C$-labeled GSH thioether **20** (Figure 2) was also unreactive toward microsomal oxidation, ruling out the possibility of GSH conjugation prior to oxidation. Further consideration of the mechanism of formation of metabolite **10** suggested the corresponding acid chloride as a precursor (see Discussion). Treatment of HPLC-purified, $^{13}C$-enriched metabolite **10** with thionyl chloride at room temperature, followed by evaporation and incubation with GSH and S-9 enzymes, gave recovered **10** as well as two major products with slightly differing HPLC retention times. The more slowly eluting product (7 $\mu$g) was identical with the previously isolated metabolite **16** by one-dimensional HMQC experiments (Figure 4b). The faster eluting product (9 $\mu$g) was subjected to HMQC analysis (Figure 5) and displayed GSH proton resonances as well as a signal arising from a doubly coupled proton (6.8 ppm, $J_{CH}$ = 172 Hz, $J_{HH}$ = 10 Hz) attached to a $^{13}C$-labeled acrylate C-3 carbon, by analogy with metabolite **16**. An additional $^1H$-coupled olefinic proton resonance at 5.8 ppm ($J_{HH}$ = 10 Hz) permitted the identification of metabolite **17** as an acrylate GSH conjugate.

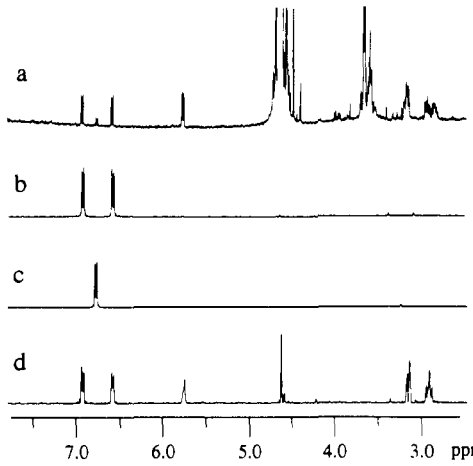



**Figure 5.** 500-MHz $^1$H NMR spectra (transients): (a) metabolite 17 derived from acid chloride **24** (32000); (b) metabolite 17, HMQC (32798); (c) metabolite 17, WALTZ $^{13}$C-decoupled HMQC (32798); (d) metabolite 17, HMQC optimized for long-range coupling (32798). Spectrum a, displayed in absorption mode, was acquired via a standard single-pulse sequence, while spectra b–d were acquired as described previously (Bax et al., 1983) and are displayed in power mode (acquisition time $t_2$ = 0.15 s; recycle delay = 1.5 s; evolution time = 0 s; delay time $\Delta = 1/(2J_{CH})$; $J_{CH}$ = 140 Hz for spectra b and c, 8.3 Hz for spectrum d).

**Table I. Covalent Protein Binding in Microsomal Incubations of Triallate**[a]

| incubation | GSH added, mM | $^{14}$C binding to protein[b] |
|---|---|---|
| microsomes | 0.26 | 0.816 |
| microsomes | none | 1.978 |
| boiled microsomes | 0.26 | 0.032 |
| boiled microsomes | none | 0.031 |

[a] [$^{14}$C]Triallate concentration of 30 μM. [b] Average of four replicates, expressed as nanomoles of triallate equivalents per milligram of protein.

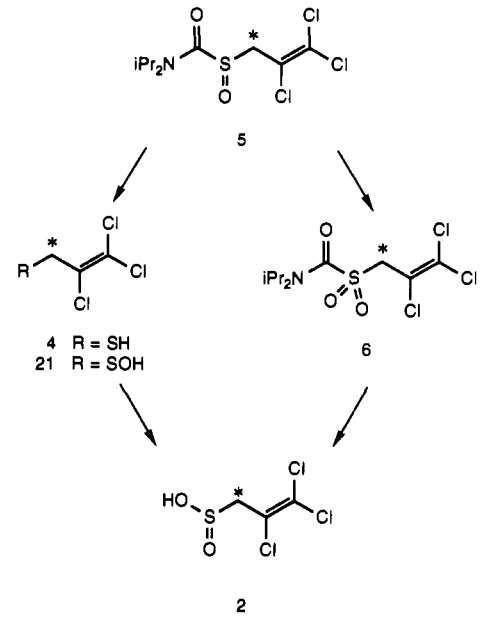
**Binding to Microsomal Protein.** Significant radiolabel incorporation in the microsomal protein fraction was observed during incubation of triallate under oxidative conditions. Protein incorporation was reduced by 50% or more (Table I) in incubations performed in the presence of excess GSH, suggesting that triallate-derived metabolic intermediates react with protein residues in a reaction which can be suppressed by addition of exogenous GSH, which we have shown reacts with such intermediates.

## DISCUSSION

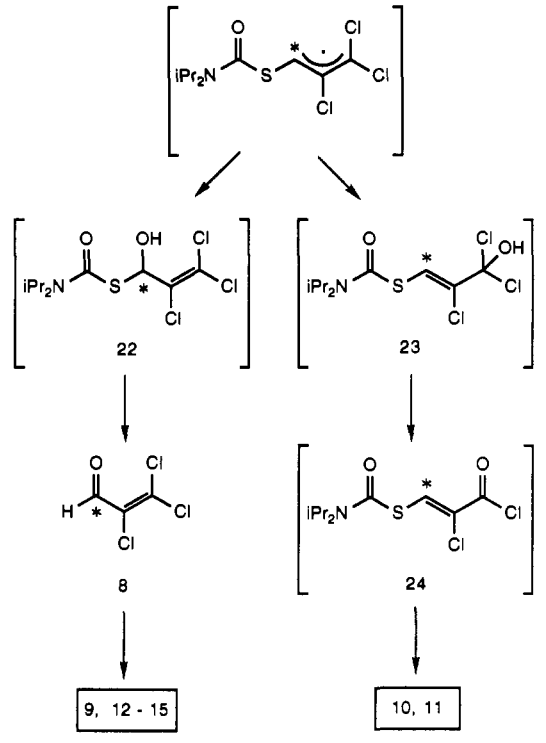
**Comparison of in Vivo and in Vitro Triallate Rat Metabolites.** Similar triallate metabolite distributions are observed in vivo and in vitro (Nadeau et al., 1993). The products of S-oxidation are the most significant, and the sulfuric acid **2** is the major metabolite in both situations. Metabolites **3**, **7**, **9**, and **10** have also been identified in both studies. In vitro metabolite **4** has been demonstrated to play a key intermediary role in vivo (Nadeau et al., 1993) but was not detected in excreta due to its reactivity. Trichloroacrolein (**8**) has likewise not been observed in vivo. Several in vitro metabolites represent precursors of in vivo metabolites formed via the mercapturate pathway. In vitro metabolic pathways include sulfur oxidation/hydrolysis, carbon oxidation, and conjugation with glutathione, as summarized in Schemes I and II.

**Sulfur Oxidation/Hydrolysis: Sulfur Acids 2 and 3 and Thiol 4.** Sulfinic acid **2** is the major product of in vitro triallate breakdown under oxidative conditions. Metabolite **2** likely arises via initial oxidation of triallate

**Scheme I**




**Scheme II**




to sulfoxide **5** (Scheme I), followed by hydrolysis to the rapidly oxidized sulfenic acid **21** (Mair and Casida, 1991). Alternative routes of formation would involve either oxidation of **5** to sulfone **6**, followed by hydrolysis, or direct oxidation of thiol **4**. The sulfonic acid TCPSA **3** is formed via oxidation of **2** to the higher oxidation state and can form by enzymatic conversion and/or air oxidation. In marked contrast to triallate, TCPSA **3** was completely unreactive with rat liver microsomes under oxidative conditions and did not undergo any reaction with GSH. The formation of thiol **4** can be rationalized (Scheme I) by oxidation at sulfur to form sulfoxide **5**, thiocarbamate hydrolysis to sulfenic acid **21**, and disproportionation or reduction to thiol **4** (Allison, 1976). Thiol **4** has been confirmed as a precursor of in vivo methyl sulfone metabolites of triallate (Nadeau et al., 1993). Such metabolites were not produced under the incubation

conditions described in this paper, which do not support S-adenosylmethionine methylation (Weisiger et al., 1980).

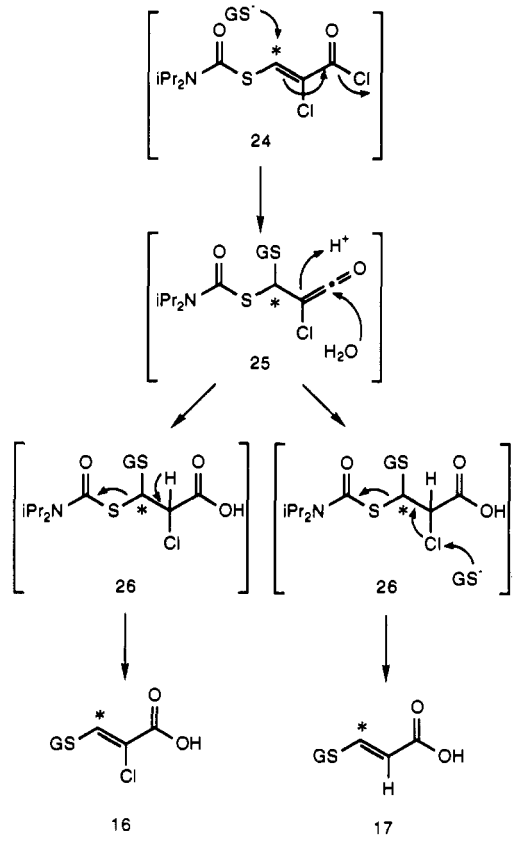
**Formation of Carbon Oxidation Products 8–11 in the Absence of Glutathione.** Metabolites 8 and 9, which retain a trichloropropenyl moiety, presumably form via microsomal hydroxylation of triallate at the C-1 allylic position (Scheme II). The intermediate thiohemiacetal 22 decomposes to diisopropylamine, carbon monoxide, and trichloroacrolein (8). The latter is readily reduced microsomally to the allylic alcohol 9. Trichloroacrolein (8) is thus consumed metabolically as rapidly as it is generated, leading to a negligible steady-state concentration and accounting for the difficulty in detection of 8 both in vitro and in vivo. The 2-chloroacrylate metabolites 10 and 11 form via microsomal hydroxylation of triallate at the C-3 olefinic position (Scheme II) and solvolysis of the resulting acid chloride 24 either to acid 10 or methyl ester 11. The unreactivity of thiocarbamate 10 toward GSH led to the discovery that 24 itself undergoes GSH conjugation, in an unusual example of conjugate addition to an acryloyl chloride (Hackett et al., 1991).

**Formation of Glutathione Conjugates 12–17.** Triallate underwent no reaction with GSH in the absence of microsomes, although microsomal oxidation of triallate generated intermediates capable of undergoing reaction with GSH. Conjugation reactions were observed both with functioning S-9 fractions containing transferase activity and with microsomes enriched with GSH, suggesting that cytosolic transferase activity is not absolutely required for GSH conjugation. However, GSH conjugation was significantly increased by added transferase activity. The aldehydes 12 and 13 (Figure 2) arise via successive 1,4-additions of GSH to trichloroacrolein (Hackett et al., 1990). Subsequent reduction leads to allylic alcohols 14 and 15 (Scheme II). Since these metabolites were not observed in vivo, duplication of these reactions by chemical synthesis was necessary to confirm structure assignments. The 2-chloroacrylate GSH conjugate 16 was formed from thionyl chloride-generated acid chloride 24 (Scheme III), together with the acrylate GSH conjugate 17. Conjugate 16 itself was unreactive under in vitro conditions, eliminating it as a precursor of 17. However, both 16 and 17 could arise via a ketene intermediate resulting from GSH addition to 24 (Scheme III). Addition of water to the ketene, followed by elimination of the thiocarbamate moiety, would produce the 2-chloroacrylate conjugate 16. Alternatively, addition of water and reductive dechlorination via a second equivalent of GSH (Brundin et al., 1982; Seshadri et al., 1981), with simultaneous thiocarbamate elimination, would generate the acrylate 17.

**Binding to Microsomal Protein.** Substantial triallate radiolabel incorporation into the microsomal protein fraction was observed under oxidative conditions, demonstrating the formation of triallate-derived intermediates that can react with protein. Metabolic intermediates formed from triallate should be capable of reacting with protein residues in a reaction that can be suppressed by addition of exogenous GSH, which itself reacts with such intermediates. The GSH-containing metabolites identified in this study can thus serve as models for triallate-derived protein conjugation.

**Conclusions.** The predominant route of triallate degradation in vitro involves oxidation at sulfur to produce sulfur acids 2 and 3, as well as thiol 4. Carbon oxidation of the trichloropropenyl moiety gives rise to metabolites that can undergo conjugation with glutathione. Application of HMQC NMR spectroscopy proved invaluable to the structure elucidation of GSH conjugates formed from

**Scheme III**




reactive intermediates of triallate metabolism. Hydroxylation at the position adjacent to sulfur leads to formation of trichloroacrolein (8), trichloroallyl alcohol (9), and glutathione conjugates of these metabolites. Hydroxylation at the terminal position of the trichloropropenyl moiety leads to the 2-chloroacrylate derivatives 10, 11, 16, and 17. The glutathione adducts 12–17 provide a model for the potential conjugation of triallate-derived metabolites with protein residues. Consistent with this idea is the observed binding of triallate-derived radioactivity in the microsomal protein fraction. Finally, intermediates such as trichloroacrolein (8) and trichloroallyl alcohol (9) could undergo significant breakdown to carbon dioxide, accounting for $^{14}CO_2$ expiration observed in vivo.

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**Supplementary Material Available:** 500-MHz $^1H$ NMR spectra of synthetic standards 13 and 14 and 75-MHz $^{13}C$ NMR spectrum of synthetic standard 14 (4 pages). Ordering information is given on any current masthead page.

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