PHARMACOKINETICS AND METABOLISM OF [14C]DICHLOROACETATE IN MALE SPRAGUE-DAWLEY RATS

Identification of Glycine Conjugates, Including Hippurate, as Urinary Metabolites of Dichloroacetate

MARGARET O. JAMES, ZIMENG YAN,¹ RACHEL CORNETT, V. MURALI K. M. JAYANTI,² GEORGE N. HENDERSON, NATALIA DAVYDOVA, MICHAEL J. KATOVICH, BRAD POLLOCK, AND PETER W. STACPOOLE

Departments of Medicinal Chemistry (MOJ, RC, VMKMJ) and Pharmacodynamics (MJK), College of Pharmacy, and Department of Medicine, Division of Endocrinology and Metabolism (ZY, GNH, ND, PWS), Department of Health Policy and Epidemiology (BP), and Department of Biochemistry and Molecular Biology (PWS), College of Medicine, University of Florida

(Received February 13, 1998; accepted July 9, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

Pathways of metabolism of dichloroacetate (DCA), an investigational drug for the treatment of lactic acidosis in humans and a rodent hepatocarcinogen, are poorly understood. In this study, rats were given, by gavage, one or two 50 mg/kg doses of NaDCA. DCA labeled with 14C (carboxy carbon) or 13C (both carbons) was used in studies of disposition and pharmacokinetics, respectively. The effect of fasting for 14 hr before dosing was studied. Expired air, urine, feces, and tissues were collected from [14C]DCA-dosed rats. Urine was analyzed by HPLC, GC/MS, and NMR spectroscopy. Plasma samples were analyzed by GC/MS. DCA plasma elimination half-lives were 0.1 ± 0.02 and 5.4 ± 0.8 hr in young adult rats (180–265 g, 3–4 months of age) given one or two doses of DCA, respectively, and 9.7 ± 1 hr in large, 16-month-old rats given two DCA doses. The percentage of the DCA dose excreted as CO2 varied from 17 to 46% and was lower (p < 0.001) in fed rats, compared with rats fasted overnight before dosing. Urine contained DCA and DCA metabolites, including oxalate, glyoxylate, and conjugated glycine (mainly hippurate and phenylacetylglycine). More unchanged DCA was excreted by large rats pretreated with DCA (mean, 20.2% of the dose) than by young adult rats given one dose of DCA (mean, 0.5%). This study confirmed that CO2, glycine, and oxalate are major products of DCA metabolism, it demonstrated that one dose of DCA altered the elimination of a subsequent dose, and it showed that age or body size, as well as access to food, significantly affected DCA metabolism in rats.

DCA has been shown to have numerous biological effects, ranging from desirable therapeutic effects in humans (lowering blood levels of lactic acid and glucose) (Stacpoole, 1989) to undesirable toxic effects in rats (e.g., testicular abnormalities, birth defects, and liver cancer) (Smith et al., 1992; Toth et al., 1992; DeAngelo et al., 1991; Stacpoole et al., 1998). DCA is being used in clinical trials to lower blood lactic acid levels in children with congenital lactic acidosis (Stacpoole et al., 1997) or with lactic acidosis resulting from severe malaria infection (Krishna et al., 1995). It has been used experimentally in humans to lower lactic acid levels during liver transplantation (Shangraw et al., 1994). The effective human therapeutic dose range is 12.5–37.5 mg/kg, administered twice-daily (Stacpoole et al., 1997). DCA is also an environmental contaminant, formed by chlorination of surface water and oxidation of chlorinated solvents. It has been found in chlorinated municipal drinking water at concentrations up to 0.160 mg/liter (Uden and Miller, 1983) and is a minor metabolite of the industrial solvents trichloroethylene and perchloroethylene (Jolley, 1985; Abbas and Fisher, 1997). It has been suggested that environmental exposure of humans to DCA may be hazardous, because DCA is a mouse carcinogen when administered in the drinking water for 60–104 weeks, at concentrations of 500-5000 mg/liter (Herren-Freund et al., 1987; Daniel et al., 1992). At present, however, the relevance of the rodent toxicological characteristics of DCA to its human toxicological profile is unknown.

Although the biological effects of DCA have been widely studied, there have been few studies of the human or rodent pharmacokinetics and metabolism of DCA. Studies of DCA pharmacokinetics in humans showed that DCA was rapidly and completely absorbed after oral administration, that the elimination half-life of DCA from blood increased after the first dose was given, and that this effect persisted for several weeks (Curry et al., 1985; Henderson et al., 1997). In Fischer 344 rats (mean body weight, 344 g) given single oral doses of 5, 20, or 100 mg of [1,2-14C]DCA/kg, 23–29% of the dose was recovered as CO2 and 19–24% was excreted in urine (Larson and...
Bull, 1992). The urine contained 1–2% of the dose as DCA, and the remaining urinary radioactivity was reported as nonchlorinated organic acids. In another study, there was evidence for dose-dependent effects on the fate of DCA. Fischer 344 rats (180–240 g) given single oral doses of 28.2 or 282 mg of [14C]DCA/kg excreted 25–35% of the dose as CO₂ and 12–35% in urine; 20–36% of the dose was recovered in rat tissues (Lin et al., 1993). The rats that received 282 mg/kg excreted less of the 14C as CO₂ and more in urine, and the percentage of unmetabolized DCA in urine ranged from 0.6% of the dose for the 28.2 mg/kg group to 20% of the dose for the 282 mg/kg group (Lin et al., 1993). Other urinary metabolites were reported to be glyoxylate, glycolate, and oxalate, although these metabolites were not unequivocally identified (Lin et al., 1993). These previous studies suggested that dose and possibly size influence the fate of DCA in rats. The present study further investigated the disposition, metabolism, and elimination of DCA in rats. The objectives were to determine whether repeat doses of DCA altered the fate of DCA in rats, as in humans, to identify the urinary metabolites, and to examine the influence of body size and overnight fasting on the pharmacokinetics and metabolism of DCA.

**Materials and Methods**

**Chemicals.** The two sources of [1-14C]DCA used involved the custom synthesis of NaDCA by Sigma Radiochemicals (St. Louis, MO) and the purchase of free acid from American Radiolabeled Chemicals (St. Louis, MO). The Sigma product had a specific activity of 5.5 mCi/mmol, a reported radiochemical purity of 99%, and a measured radiochemical purity of 97%. The American Radiolabeled Chemicals product had a specific activity of 55.5 mCi/mmol, and the reported and measured radiochemical purities were >99.9%. The free DCA was converted to the sodium salt by equimolar addition of NaOH. Na[1,2-13C]DCA was custom-synthesized by Cambridge Isotope Laboratories (Cambridge, MA) at 100% isotope-enriched and was shown by GC/MS to be >99.5% chemically and isotopically pure. Unlabeled NaDCA (>99.8% chemically pure) purchased from TCI America (Portland, OR) was used to dilute the [14C]DCA to conserve radiolabeled compound. Carbosorb and Permaflour columns for use with the tissue oxidizer and Floscint II cocktail for use with the radiochemical detector were purchased from Packard Instruments (Chicago, IL). Eclomune scintillation cocktail purchased from ICN (Costa Mesa, CA). DCA-glycine was synthesized by stirring 0.75 g of dichloroacetyl chloride with 1 g of glycine-t-buty1 ester and 2 g of sodium carbonate, in 25 ml of acetonitrile, at 60°C for 2 hr. After cooling, the mixture was filtered and the filtrate was evaporated to dryness. The precipitate was suspended in ether and filtered, and the filtrate was allowed to evaporate in a hood. The product, DCA-glycine-t-buty1 ester, was hydrolyzed in trifluoroacetic acid/water (9:1) to yield DCA-glycine. The structure of the DCA-glycine was confirmed by 1H NMR (Jayanti, 1995). Phenylacetylglutamine was prepared as described previously (James et al., 1972). All other chemicals used were of the purest grade available from Millipore (Woburn, MA). Fisher Scientific (Orlando, FL), Sigma Chemical Co. (St. Louis, MO), or Aldrich Chemical Co. (St. Louis, MO).

**Animals.** Male Sprague-Dawley rats were used in all studies. Most of the rats weighed 180–265 g and were 3–4 months of age, but one group of four larger rats (body weight, 580–690 g; age, 16 months) was also used. Rats were normally fed Purina laboratory chow ad libitum and maintained on a 12-hr light/dark cycle. In some studies of distribution, metabolism, and excretion, the chow was removed at 4 p.m. the day before dosing (fasted rats). All rats were allowed free access to water. DCA was administered by oral gavage of a solution of NaDCA in water. Doses were given between 10 a.m. and noon.

In studies of distribution, metabolism, and excretion, the rats were given 50 mg/kg DCA with 280–400 μCi/kg 14C. The rats were maintained for 1 or 24 hr in all-glass metabolism cages (Stanford Glass, Palo Alto, CA) equipped to collect CO₂, urine, and feces. Excretion of 14C in urine and CO₂ was monitored at 1–3-hr intervals by counting samples of the urine and the Carbosorb used to trap the CO₂. In most studies, rats were sacrificed at 24 hr, blood was sampled, and organs were dissected, weighed, and analyzed for 14C content by complete oxidation of duplicate samples of each tissue, in a Packard tissue oxidizer (Packard Instruments, Chicago, IL). In one study, singly dosed rats were sacrificed 1 hr after the dose and subcellular fractions were prepared from samples of liver and kidney, as described below. To determine the effects of repeat doses on the distribution, metabolism, and excretion of DCA, rats received a dose of unlabeled DCA on a given day and a dose of [14C]DCA (as described above) at the same time on the next day and were sacrificed 24 hr after the radiolabeled dose.

The pharmacokinetics of appearance and disappearance of DCA in plasma were monitored in rats fitted with indwelling jugular vein cannulae (Harms and Ojeda, 1974). Rats were anesthetized with a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) (im 0.7 ml/kg). A silastic catheter (i.d., 0.025 inches; o.d., 0.047 inches) was inserted into the jugular vein for drug infusion and blood sampling. The cannula was exteriorized between the shoulder blades, filled with a heparin solution (10 units/ml), and sealed with a stylet. The cannulae were inserted 2 days before administration of DCA, and patency was ensured by twice-daily flushing with a solution of heparin in saline (100 units/ml). Rats used in pharmacokinetic studies only were dosed with unlabeled NaDCA or [1,2-13C]DCA (50 mg/kg). Samples of blood (0.3–0.4 ml) were obtained at time 0 and at regular intervals after dosing. After sampling, a volume of heparinized saline equal to the volume of the sample taken was returned through the catheter, to prevent undue fluid loss. A group of large rats (body weight, 580–690 g) and some smaller rats received a mixture of Na[1-14C]DCA and Na[1,2-13C]DCA (1:99) and were studied for pharmacokinetics, distribution, metabolism, and excretion.

**Preparation of Subcellular Fractions.** Samples of liver (5 g) and kidney (1.5 g) were rinsed three times in 4 volumes of ice-cold 0.15 M KCl/0.05 M potassium phosphate, pH 7.4, and were then homogenized in 4 volumes of the ice-cold buffer. Subcellular fractions were prepared by differential centrifugation, as described previously (James and Little, 1983).

**Analytical Methods.**

**HPLC Analysis.** Urine samples for each time point were filtered through a 0.45-μm nylon filter (Centrisart; Rainin Instruments) and analyzed by ion-pair reverse-phase HPLC. The following conditions were used: C18 column (4.6 × 250 mm) with C8 guard column (4.6 × 50 mm); 0.05-mM sample injection loop; isotropic mobile phase, 70% 0.005 M tetrabutylammonium sulfate (PICA, low UV); Waters)/30% methanol; flow rate, 1 ml/min; on-line detection, UV absorbance at 220 nm (Dynamax; Rainin Instruments) and radiochemical detection (Flo-one Beta; Packard Instruments). It was necessary to equilibrate the reverse-phase column with the ion-pair-containing mobile phase for at least 40 min before injection of samples, for reproducible analysis of DCA and metabolites. Standard solutions of DCA, as well as known and suspected DCA metabolites, were prepared in the mobile phase at concentrations of 0.2–2.5 mg/ml, and retention times were monitored by UV detection at 220 nm. The retention times of the standard compounds were as follows: glycine, 3.0 min; glyoxylate, 3.2 min; glycotate, 3.2 min; acetylglutamine, 3.4 min; oxalate, 4.0 min; dichloroacetylglycine, 6.5 min; DCA, 7.1 min; hippuric acid, 10.2 min; phenylacetylglutamine, 13.8 min; methyl-DCA, 15.8 min.

**GC and GC/MS Analysis.** The DCA concentrations in plasma from rats that had received unlabeled DCA were measured by the GC method of Chu et al. (1992). Plasma samples from rats that had received [1,2-13C]DCA were analyzed for DCA by GC/MS, using a Hewlett-Packard 5890 Series II Plus GC system, a 5972A mass-selective detector, and a Vectra multimedia VL2 4/66 computer with ChemStation software (Yan et al., 1997). GC/MS was also used to verify the identity of metabolites present in urine. Urine or plasma samples were spiked with 4-chlorobutyric acid as an internal standard and were derivatized by heating with an equal volume of a 14% solution of boron trifluoride in methanol at 115°C for 15 min. The methylated derivatives were extracted into methylene chloride. A portion of the methylene chloride extract was injected onto a Carbowax column (HP-Wax, 30 m × 0.25 mm with 0.15-μm film thickness; phase ratio, 420), with helium carrier gas (flow rate, 1.21 ml/min) and an inlet pressure of 9 psi. The GC system temperature was maintained at 35°C for 4 min, followed by a linear gradient to 100°C at 3°C/min and then to 240°C at 50°C/min. The temperature was maintained at 240°C for 5 min. Under these conditions, the retention times of the methyl esters of DCA and metabolites were as follows: DCA, 10.8 min; glyoxylate, 11.2 min; oxalate, 11.7 min; acetylglutamine, 17.2 min; hippuric acid, 20.2 min; phenylacetylglutamine, 21.2 min. For quantitation of DCA in plasma, single ions of molecular mass 59 and 60, corresponding to the [13C4]COOCH3 and [13C8]COOH.
$^{13}$COOCH$_3$ fragments, respectively, were monitored for the peak with a retention time matching that of authentic methylated DCA. Standard curves were developed with methylated [1$^{13}$C]DCA standard under identical conditions and were used to quantitate the plasma peaks, as described previously for human studies (Yan et al., 1997). Each of the urinary metabolites was identified by determining the GC retention time and by matching the complete mass spectrum of the $^{13}$C-labeled methylated metabolite to the mass spectrum of the methylated authentic $^{13}$C standard. The presence of $^{13}$C in each metabolite peak was confirmed by comparing the $^{13}$C/$^{12}$C ratios in the molecular ion (for methylated glycine conjugates of benzoic, acetic, and phenylacetic acids) or the $\text{-COOCH}_3$ fragment (for oxalate and glyoxylate) with the natural abundance of $^{13}$C in unlabeled metabolite standards. If the ratio was higher than expected for the natural abundance of $^{13}$C, it was assumed that the metabolite arose from $^{13}$C[DCA].

**NMR Analysis**. One sample of urine from a rat that had received a DCA dose containing a mixture of 99% [1,2-$^{13}$C]DCA and 1% [1-$^{14}$C]DCA (final specific radioactivity, 0.555 mCi/nmol) was analyzed by $^1$H and $^{13}$C NMR spectroscopy, HPLC analysis, with radiochemical detection, of this urine sample showed that 70.5% of the urinary DCA-derived $^{14}$C was in the form of an unidentified metabolite with a retention time of 10.5 min. This urine sample also contained 0.5% parent DCA, 18.6% oxalate, and 9.6% other polar metabolites and had 300 µg of DCA-molar equivalents/0.6 ml of urine. To adjust magnetic field homogeneity and to maintain a stable field-frequency lock, the urine sample (0.6 ml) was mixed with D$_2$O (0.15 ml), and this solution was examined in a 5-mm tube. The D$_2$O contained a trace of 3-(trimethylsilyl)propionate-2,3,3-$\delta_3$ sodium salt, serving as a chemical shift reference. For both $^1$H and $^{13}$C spectra, each carbon reference for the 3-(trimethylsilyl)propionate-2,3,3-$\delta_3$ sodium salt resonance at 0.0 ppm.

For proton NMR spectroscopy, signals were acquired with a standard presaturation pulse sequence to reduce the proton resonance of water in the sample. The free induction decay was then processed with an exponential line-broadening of 0.25 Hz before Fourier transformation (Fourier number, 32,768 points).

For carbon NMR spectroscopy, four $^{13}$C spectra were acquired (a proton-coupled spectrum and three proton-decoupled spectra). One proton-decoupled spectrum was acquired with continuous proton decoupling (Wideband, Alternating-phase, Low-power Technique for Zero-residual splitting, power, 40 dB), using a standard one-pulse sequence and the following parameters: observed pulse, 6 µsec; tip angle, 60°; acquisition time, 2 sec; postacquisition delay, 8 sec; spectral width, 265 ppm (centered at approximately 108 ppm). Ten thousand transients were accumulated over a 27.75-hr period. The free induction decay was processed with an exponential line broadening of 1 Hz before Fourier transformation. A second spectrum was acquired over 1.4 hr using a standard J-modulated spin-echo sequence, with proton decoupling gated off during the modulation delay and on during acquisition and the postacquisition delay. A third, semiquantitative, spectrum was acquired over 8.5 hr with proton irradiation gated off during the postacquisition delay, to suppress the nuclear Overhauser effect, and on during acquisition, to allow proton decoupling. The proton-coupled spectrum was obtained over 11.4 hr using the pulse sequence and parameters described above, except that proton irradiation was gated off during acquisition, to allow proton coupling, and on during the postacquisition delay, to maintain the nuclear Overhauser effect.

The $^{13}$C NMR spectrum of a saturated D$_2$O solution of authentic hippuric acid was obtained for comparison, using the natural abundance of $^{13}$C for signal detection. Spectra were also compared with published $^{13}$C NMR spectra for hippuric acid and sodium hippurate (Aldrich).

**Pharmacokinetic Modeling**. Pharmacokinetic modeling was conducted with the PCNONLIN program (Statistical Consultants, Lexington, KY). The initial data analysis was performed using the RSTRIP program (MicroMath, Salt Lake City, UT). After the initial analysis with the RSTRIP program, DCA plasma levels were fitted to one-, two-, and three-compartment models, using the nonlinear regression program PCNONLIN (version 3.0). AIC, where AIC = $N \ln (\text{SSE}) + 2p$ (where $N$ is the number of observations, $p$ is the number of model parameters, and SSE is the residual sum of squares), was used to compare the three models (Akaike, 1978; Akcay and Rose, 1980; Torres-Molina et al., 1992). The model with the lowest AIC would be the most efficient. It was found that some of the data best fit a one-compartment pharmacokinetic model and other sets best fit a two-compartment model. The pharmacokinetic parameters were obtained using the appropriate model for each rat.

**Statistical Analysis**. Statistical analyses of the experimental data were performed with the general linear models procedure of the Statistical Analysis System (SAS Institute, 1989). Feeding status and number of doses were modeled as separate main effects. A cross-products term was included to assess potential interactions. In no instance did the interaction term have a significant impact on the comparison of main effects; therefore, the reported analyses are of the main effects only. The outcome variables were the percentage of the dose excreted as $^{14}$CO$_2$, the total percentage of the dose excreted in urine, and the percentage of the dose excreted as each of the separated metabolites or metabolite groups.

**Results**

**Excretion in Expired Air**. The extent of metabolism of DCA and the routes of excretion of DCA metabolites varied with rat size, preexposure to DCA, and feeding status. A major route of elimination of radioactivity from [1$^{14}$C]DCA was in expired air as $^{14}$CO$_2$, as shown in fig. 1 and table 1. The percentage of the radioactive dose excreted as $^{14}$CO$_2$ was quantitated in the young adult rats and varied with access to food and number of DCA doses. It was not possible to quantitate $^{14}$CO$_2$ excretion by the large fed rats given two doses of DCA, because they were too large for the metabolism cages. For rats that were allowed free access to food, 17–26% of the radiolabeled dose was excreted as CO$_2$ in 24 hr. Less $^{14}$CO$_2$ was expired in 24 hr by fed,
repeatedly dosed rats, compared with fed, singly dosed rats, and the initial rate of CO₂ excretion was lower (fig. 1). In the first 6 hr after receiving a single dose of [¹⁴C]DCA, fed rats excreted 2.93 ± 0.43% of the dose/hr as CO₂, whereas repeatedly dosed rats excreted 1.67 ± 0.32%/hr (significantly different, p < 0.05).

The rats that were fasted for 18–20 hr before dosing excreted much more of the [¹⁴C]DCA as CO₂ in 24 hr, compared with fed rats (p < 0.0001). In the fasted groups, the initial rates of excretion of ¹⁴CO₂ were similar for rats given one or two doses of DCA (fig. 1) and were 4.84 ± 0.83%/hr and 5.76 ± 0.87% for singly and repeatedly dosed rats, respectively.

**Urinary Excretion and Identification of Urinary Metabolites.**

There were major differences between the young adult rats and the large rats with respect to the percentage of the dose excreted in urine over 24 hr and the percentage of the dose excreted as parent DCA (fig. 2, table 2). Access to food did not affect the percentage of the dose excreted in urine for the young adult rats; therefore, the data for total urinary excretion of ¹⁴C were pooled for singly dosed rats and for repeatedly dosed rats (fig. 2). All rats excreted approximately 7% of the dose in urine in the first 6 hr. The rate of urinary excretion decreased after 6 hr for the young adult rats, whereas the large rats continued to excrete DCA and metabolites in urine, in an almost linear manner, up to 24 hr (fig. 2).

The possible contaminants of DCA, namely monochloroacetate and trichloroacetate, as well as the known metabolites of DCA, i.e. oxalate, glyoxylate and glycine, were well separated from DCA by reverse-phase HPLC using an ion-pair reagent. Monochloroacetate was not, however, separated from oxalate, and glyoxylate was not separated from glycolate or glycine in this system. Typical chromatograms for urine samples from rats given single or repeat doses of DCA are shown in fig. 3.

The amount of unchanged DCA excreted in urine varied with dosing schedule and rat size. Large rats allowed free access to food excreted more of the radioactive dose as unchanged DCA (7.4–37.2% of the dose) and more of the dose in urine (18.5–43.8%) than did any other group. There were differences in total urinary excretion and excretion of parent DCA between the groups of differently treated young adult rats, but these were not as marked as the differences between large rats and all young adult rats. The young adult, singly dosed rats excreted the smallest amounts of parent DCA in urine (table 2).

The previously identified DCA urinary metabolite oxalate was found in all urine samples (fig. 3). Urinary oxalate accounted for 1.1–7.6% of the dose, and there were no significant differences among the groups of rats in the percentage of the dose excreted in urine as oxalate. The presence of oxalate derived from DCA in urine was verified by GC/MS analysis of selected urine samples from rats given [¹⁴C]DCA. All urine samples also contained broad peaks from 2.8 to 3.4 min, corresponding to the retention times of glycine, acetylglycine, glycolate, and glyoxylate. GC/MS showed that <0.5% of the dose was present in urine as monochloroacetate and glyoxylate.

Three unknown radiolabeled acidic metabolites (U1–U3) were consistently found in urine samples (fig. 3). The amount of U1 (retention time, 8 min) excreted in urine was greatest for young adult rats that were fasted before the dose. U2 (retention time, 10.2 min) accounted for >1% of the administered dose of DCA in all but two urine
DCA metabolites found in 24-hr urine samples from male Sprague-Dawley rats treated with one or two doses of NaDCA (50 mg/kg)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Metabolite Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasted</td>
</tr>
<tr>
<td></td>
<td>% of dose</td>
</tr>
<tr>
<td>DCA</td>
<td>0.66 ± 0.51</td>
</tr>
<tr>
<td>Oxalate</td>
<td>2.48 ± 0.49</td>
</tr>
<tr>
<td>Glyoxylate and other polar metabolites</td>
<td>3.84 ± 0.82</td>
</tr>
<tr>
<td>U1</td>
<td>0.88 ± 0.73</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>1.31 ± 0.74</td>
</tr>
<tr>
<td>Phenylacetylglycine</td>
<td>0.45 ± 0.34</td>
</tr>
<tr>
<td>Total recovered in urine</td>
<td>9.8 ± 0.67</td>
</tr>
</tbody>
</table>

The groups of rats were as described for table 1. Fed rats were allowed free access to food throughout the study. Fasted rats had food withheld overnight before dosing by oral gavage in the morning. Values shown are mean ± SD.

* Repeatedly dosed rats were significantly different from singly dosed rats (p < 0.005), and fed rats were significantly different from fasted rats (p < 0.01).

Feeding status affected the amounts of metabolites excreted by fed rats was greater than that excreted by fasted rats (p < 0.01).

Large fed rats were significantly different from all other rats (p < 0.01).

Repeatedly dosed rats were significantly different from singly dosed rats (p < 0.05).

The amounts of labeled hippurate excreted were very variable, and the mean amount excreted by fed rats was greater than that excreted by fasted rats (p = 0.05).

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.

Reverse-phase, ion-pair HPLC was performed as described in Materials and Methods. The traces shown are from the radiochemical detector, set to detect 14C.
time, 17.3 min) and phenylacetylglutamic acid were positively identified as metabolites containing [13C]glycine in two urine samples from rats given [13C]DCA. Fig. 5 shows the peak at 21.2 min, resulting from phenylacetylglutamic acid, in the GC trace and its mass spectrum. The mass spectrum showed a molecular ion for methylphenylacetylglutamic acid at m/z 207 and an M+2 ion at m/z 209. In the urine sample shown in fig. 5, the M+2/M ion abundance ratio was 2.4-fold greater than was found for the synthetic standard for methylphenylacetylglutamic acid, indicating two 13C-labeled carbons.

Elimination of DCA from Plasma. There was no effect of feeding on the plasma uptake or elimination of DCA, so data for rats allowed free access to food were pooled with data for rats restricted from food overnight. GC and GC/MS methods gave similar results for DCA concentrations. DCA was very rapidly absorbed after oral administration (table 3, fig. 6). The peak DCA plasma concentrations varied among groups of rats and were considerably higher for the large, repeatedly dosed rats than for the young adult rats. The rate of elimination of DCA from plasma varied markedly between the rat groups receiving single and repeat doses and between young adult and large rats receiving repeat doses (fig. 6). The elimination half-life for singly dosed rats was 0.11 ± 0.05 hr, that for repeatedly dosed, young adult rats was 5.4 ± 1.8 hr, and that for repeatedly dosed, large rats was 9.7 ± 1.9 hr. Other pharmacokinetic parameters are shown in table 3.

Tissue Distribution of Radioactivity. Between 25 and 42% of the administered [14C]DCA dose remained in the body after 24 hr (table 1). Liver, muscle, skin, and the gastrointestinal tract contained most of the remaining radioactivity. Although there was interindividual variability in the amounts left in tissues, there were no significant between-group differences in these values. The percentage of radioactivity in the livers of the repeatedly dosed, large rats was very variable, ranging from 6 to 15.6% of the dose.

To further investigate the initial distribution of the radioactivity in the expected sites of biotransformation and excretion of DCA, namely liver and kidney, subcellular fractions were prepared from livers and kidneys of rats sacrificed 1 hr after a single dose of DCA. The distribution of radioactivity in each fraction was determined. In both organs, most of the radioactivity was in the cytosolic fraction (table 4). In liver, roughly equal amounts of 14C were in microsomes and mitochondria; in kidney, very little radioactivity (<2%) was associated with microsomes. Radioactivity was also located in the fraction pelleted at 600g (nuclei and cell debris). The nature of the radioactivity associated with each subcellular fraction was not investigated.

Discussion

Excretion of DCA as CO2. Previous studies with Fischer 344 rats showed that expired CO2 was a major route of excretion of DCA (Larsen and Bull, 1992; Lin et al., 1993). Preliminary investigations in this laboratory with Sprague-Dawley rats also showed that a large percentage of the DCA dose was expired as CO2 (Jayanti et al., 1994). In a recent report, Gonzalez-Leon et al. (1997) showed that, in Fischer 344 rats, the rate and extent of conversion of [14C]DCA to 14CO2 were dependent on dose and pretreatment with DCA. Rats exposed to 2 g/liter DCA in the drinking water before being given a radiolabeled dose of 5, 20, or 100 mg/kg DCA excreted CO2 more slowly than did rats that were not pretreated. Over a 24-hr period, less total DCA was excreted as CO2 by pretreated rats given 20 or 100 mg/kg DCA, but for rats given the lowest dose (5 mg/kg DCA) the total amount of CO2
excreted in 24 hr was similar to that for control rats. The access to food of the Fischer 344 rats was not stated.

DCA can be converted to CO₂ through its major metabolite, glyoxylate, by several routes. The major routes are shown in fig. 7. Glyoxylate can be directly decarboxylated or may be converted to glycine by transamination (Bais et al., 1991; Danpure and Purdue, 1995). The glycine may then be utilized as the free amino acid or degraded to CO₂ and ammonia. Conversion of glycine to CO₂ can be accomplished in several ways. During glucogenesis from glycine, the glycine is either converted to serine and then pyruvate or converted to ammonia, N⁵,N¹⁰-methylenetetrahydrofolate, and CO₂ (Voet and Voet, 1995). Previous studies in this laboratory showed that a major primary pathway of DCA metabolism is dechlorination to glyoxylate and that, in rats allowed free access to food, pretreatment with DCA reduced the cytosolic conversion of DCA to glyoxylate (James et al., 1997). Our finding in the present study that the rate of excretion of CO₂ was slower in the fed, DCA-pretreated rats than fed controls is consistent with reduced glyoxylate formation in these rats.

Surprisingly, the extent of metabolism of a 50 mg/kg dose of DCA to ¹⁴CO₂ was much greater in rats that were fasted overnight before dosing than in rats that were allowed free access to food. This was true for rats given one or two doses of DCA. This raises the possibility that DCA pretreatment does not reduce the activity of the dechlorinating enzyme in fasted rats. Arguing against this possibility is the finding that DCA pretreatment resulted in increased urinary excretion of parent DCA (table 2) and prolonged plasma elimination of DCA in fed and fasted rats (fig. 6). An alternative explanation is that with-

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>One Dose (N = 4)</th>
<th>Two Doses (N = 5)</th>
<th>Two Doses, Large Rats (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>232 ± 12</td>
<td>224 ± 13</td>
<td>617 ± 45</td>
</tr>
<tr>
<td>AUC (mg · hr/liter)</td>
<td>11.72 ± 1.68</td>
<td>240.8 ± 75.4⁶</td>
<td>1509 ± 248⁶</td>
</tr>
<tr>
<td>Volume of distribution (liters/kg)</td>
<td>0.68 ± 0.07</td>
<td>0.39 ± 0.14</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Absorption half-life (hr)</td>
<td>0.37 ± 0.03</td>
<td>1.45 ± 0.64</td>
<td>1.30 ± 0.69</td>
</tr>
<tr>
<td>α-Phase half-life (hr)</td>
<td>ND⁷</td>
<td>0.16 ± 0.04</td>
<td>0.22 ± 0.05</td>
</tr>
<tr>
<td>Elimination half-life (hr)</td>
<td>0.11 ± 0.02</td>
<td>5.38 ± 0.76⁶</td>
<td>9.72 ± 0.97⁷</td>
</tr>
<tr>
<td>Time of Cₘ₅₉ (hr)</td>
<td>0.27 ± 0.04</td>
<td>1.13 ± 0.46</td>
<td>2.09 ± 0.96</td>
</tr>
<tr>
<td>Cₘ₅₉ (µg of DCA/ml)</td>
<td>10.19 ± 1.87</td>
<td>27.22 ± 5.3⁴</td>
<td>121.2 ± 20.4⁷</td>
</tr>
</tbody>
</table>

Values shown are mean ± SE (N = 4 or 5) for each parameter.

⁶ Significantly different from singly dosed rats (p < 0.02).

⁷ Significantly different from young adult rats (p < 0.01).

⁸ ND, too rapid to estimate.

⁹ Cₘ₅₉, maximal plasma concentration.
TABLE 4
Subcellular distribution of radioactivity in liver and kidney of rats 1 hr after a single dose of DCA (50 mg/kg)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity in Each Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver (%)</td>
</tr>
<tr>
<td>Nuclei and cell debris</td>
<td>23.3 ± 3.7</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>7.8 ± 3.9</td>
</tr>
<tr>
<td>Microsomes</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>Cytosol</td>
<td>63.0 ± 5.3</td>
</tr>
</tbody>
</table>

Data shown are mean ± SD (N = 4 individual rats). Rats were treated with [14C]DCA (50 mg/kg) 1 hr before sacrifice and preparation of subcellular fractions.

holding food is likely to increase glucogenogenesis from glycine (and other free amino acids), resulting in greater excretion of CO₂ in the food-deprived rats. The extent of excretion of CO₂, as a metabolite of DCA, depends on several factors, including dose, prior exposure to DCA, and nutritional status.

Tissue Distribution of DCA and Metabolites. Radioactivity from DCA was widely distributed in tissues of rats sacrificed as early as 1 hr after the dose (table 1). Liver and muscle were major initial distribution sites, and liver retained radioactivity from DCA for at least 24 hr after the dose. Kidneys also exhibited high concentrations of radioactivity, but because of their size the percentage of the dose was small. At the 1-hr time point, both liver and kidney cytosolic fractions had the highest concentrations of radioactivity, consistent with the localization of an enzyme for conversion of DCA to glyoxylate (Lipscomb et al., 1995; James et al., 1997). Liver microsomes and mitochondria had similar percentages of radioactivity, whereas in the kidney microsomes had very little radioactivity from DCA (table 4).

To date no microsomal pathways of metabolism of DCA have been identified. It is likely that DCA metabolites formed in the cytosol, such as glycine, are incorporated into newly synthesized microsomal proteins. At this point, there is no evidence that the radioactivity in hepatic proteins is derived from adducts with intact DCA or chlorinated metabolites, rather than from incorporation of the DCA carbons into the carbon pool.

Another possible pathway for DCA metabolism was conversion to nonpolar metabolites such as esters with cholesterol or other lipids, as has been demonstrated for monocloroacetate (Bhat and Ansari, 1989). Our preliminary studies showed that <1% of the radioactivity in liver was bound to isolated lipid fractions (Jayanti, 1995; Lou Z and James MO, unpublished observations).

Identification of Glycine Conjugates as Urinary Metabolites of DCA. This study is the first to report that glycine conjugates are urinary metabolites of DCA. Based on the positive identification of hippurate (HPLC retention time, NMR spectrum of urine, and GC/MS results for methylated urine) and phenylacetlyglycine (HPLC retention time and GC/MS results) as urinary metabolites of DCA, table 2 reports the percentage of the dose excreted in urine as hippuric acid and phenylacetlyglycine, with the glycine coming from DCA. Unidentified urinary metabolite U1 may also be a glycine conjugate, possibly a-hydroxyhippurate, but it was not present in large enough amounts for identification in the subset of rats given doubly labeled [13C]DCA and [14C]DCA. Unlike other xenobiotic carboxylic acids, which are often excreted as glycine or glucuronide conjugates, we found no evidence that DCA itself directly forms either glycine or glucuronide conjugates. Glycine arising from the metabolism of DCA to glyoxylate (fig. 8) appears to be available to glycine N-acetyltransferase, which is located in the mitochondrial matrix (James and Bend, 1978; Kelley and Vessey, 1993). Some of the glyoxylate formed in the hepatic cytosol (James et al., 1997) may be transported to the mito-

chondria and transaminated in this organelle. Alternatively, glycine formed in another cellular compartment may be taken up by mitochondria, where some is decarboxylated and some is used in glycine conjugation. Studies with liver and kidney homogenates from mice have shown that glyoxylate, as a glycine precursor, is as effective as glycine in supporting hippurate synthesis from benzoic acid in vitro (Qureshi et al., 1989). Other studies have shown that the availability of glycine limits the formation of hippurate from benzoic acid (Beliveau and Brusilow, 1987), perhaps because the hepatic concentration of glycine is close to the KM for glycine (3 mM) of benzoyl-CoA/glycine N-acetyltransferase (Gregus et al., 1992; Nandi et al., 1979). The apparent KM for glycine of rat renal phenylacetly-CoA/glycine N-acetyltransferase is 20 mM (James and Bend, 1978), suggesting that glycine availability is critical for formation of phenylacetlyglycine from phenylacetic acid.

Urinary Excretion of DCA and Metabolites. The importance of urine as a route of elimination of DCA varied with rat size, feeding status, and prior exposure to DCA. With young adult rats given one dose of DCA, <1% of the dose was excreted unchanged in urine; with large rats given two doses of DCA, an average of 20% of the second DCA dose was excreted unchanged in urine (table 2). With young adult rats given two doses of DCA, the excretion of unchanged DCA in urine varied with feeding status but was <5% of the second dose. The finding of extensive urinary excretion of unchanged DCA, together with reduced plasma elimination of DCA, suggests that DCA metabolism is very slow in the large rats.

The major urinary metabolites from the singly and repeatedly dosed young adult rats that were fasted before DCA dosing were oxalate, glyoxylate, and other very polar metabolites. Three metabolites that formed strong ion-pairs with tetrabutylammonium and were originally designated U1, U2, and U3 (U2 and U3 are now known to be hippurate and phenylacetlyglycine, respectively) were found in all of these urine samples. Feeding shifted the ratios of the urinary metabolite peaks, such that hippuric acid, with the glycine arising from DCA, became the major urinary metabolite, accounting for 1.1–12.3% of the 14C dose excreted by fed rats. The source of the increased hippurate found in the fed rats was not clear. Although sodium benzoate is widely used as a food preservative, the rat chow used in these studies did not list benzoate as an ingredient. Benzoic and phenylacetic acids are produced endogenously during the catabolism of phenylalanine (Jones et al., 1978; Dwivedy and Shah, 1982). Our results suggest that more benzoate was produced in rats that were fed ad libitum, with more phenylacetic acid in rats fasted overnight.

Pharmacokinetics of DCA in Rats. As has been found in humans (Staapoole et al., 1998), the present study showed that a single dose of 50 mg/kg DCA dramatically impaired the elimination of a subsequent DCA dose in young adult male Sprague-Dawley rats. Although no DCA was detectable in singly dosed rats by 12 hr after the dose, rats given a second dose the next day had detectable levels of DCA in plasma up to 24 hr after the dose (fig. 6). Another striking finding from the present study was that the pharmacokinetics of DCA in large rats given two doses of DCA were very different from those in similarly treated young adult rats. The peak plasma concentrations were 5-fold higher in the large rats, and the elimination half-life was slowed from 5.4 to 9.7 hr. The urinary excretion of unchanged DCA was also different in the large rats (see above). It is not clear whether the observed differences in DCA metabolism in the two repeatedly dosed groups were the result of size or age, because the large rats used were both older and larger.

A previous study with three male Sprague-Dawley rats (160–170 g) given a single iv bolus dose of 100 mg/kg DCA reported elimination half-lives of 2.4–4.4 hr, although examination of the data suggested...
somewhat shorter half lives of 1.5–4 hr (Lukas et al., 1980). Rats given single oral doses in the present study showed much shorter elimination half-lives (0.11 ± 0.02 hr), raising the possibility that dose may influence the rate of DCA elimination.

Other reported studies of DCA pharmacokinetics in animals were conducted in Fischer 344 rats. Recent work by Gonzalez-Leon et al. (1997) showed that the half-life of elimination of an iv bolus dose of 100 mg/kg DCA from male Fischer 344 rats (body weight, 287 ± 18 g) was slowed from 2.4 ± 0.2 hr to 10.8 ± 2.0 hr by prior administration of 2 g/liter DCA in the drinking water for 2 weeks. The present study shows that the impairment of DCA elimination, presumably resulting from inhibition of DCA metabolism, occurs after only one DCA dose. The main site of DCA metabolism in humans is the liver (Shangraw et al., 1994), which is a major site of uptake of DCA in rats, as demonstrated in this study. In vitro studies in this laboratory confirmed that administration of DCA (50 mg/kg) to fed rats for 2 days before the preparation of hepatic cytosol resulted in slower dechlorination of DCA to glyoxylate (James et al., 1997). It is not known whether the impairment of DCA metabolism is the result of reversible or irreversible inhibition or destruction of the dechlorinating enzyme.

In summary, these studies have confirmed that a single dose of DCA markedly affects the plasma elimination of a subsequent dose of DCA in rats, and they have shown that feeding status, size, and age influence DCA metabolism. Rats that were fasted overnight before the radiolabeled dose excreted much more of the dose as CO2 than did rats allowed free access to food. The glycine formed from transamination of glyoxylate, the major initial dechlorinated metabolite of DCA, was available for conjugation with carboxylic acids such as benzoic, phenylacetic, and acetic acids, and the glycine conjugates formed were major metabolites in urine.

Acknowledgments. The authors are grateful to Jim Rocca, Center for Structural Biology, University of Florida, for assistance with the NMR studies and to Bin Xu and Dr. Meide Pan for technical assistance.

References
Daniel FB, DeAngelo AB, Stober JA, Olsen GB and Page NP (1995) Hepatocarcinogenicity of
Dichloroacetate metabolism in rats


