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 dichloroacacetate (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 that fasted overnight before dosing. Urine contained DCA and DCA metabolites, including oxalate, glyoxylate, and conjugated glycine (mainly hippurate and phenylacetylglucine). 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.

DCA1 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]C 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 glyoxylic acid, 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[1,2-14C]-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. Carbowax and Permfluor cocktails for use with the tissue oxidizer and Fluoscan II cocktail for use with the radiochemical detector were purchased from Packard Instruments (Chicago, IL). Ectocline scintillation cocktail was purchased from ICN (Costa Mesa, CA). DCA-glycine was prepared as described previously (James and Little, 1983). Phenylacetylglucine was prepared as described previously (James, 1992). The Sigma product had a specific activity of 5.5 mCi/mmol, a reported radiochemical purity of 99.5%, and a measured radiochemical purity of 99%.

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 mm × 250 mm) with C8 guard column (4.6 mm × 50 mm); 0.05-ml sample injection loop; isocratic 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; glyoxylic acid, 3.2 min; glycolate, 3.2 min; acetylglycine, 3.4 min; oxalate, 4.0 min; dichloroacetylglutamic acid, 6.5 min; DCA, 7.1 min; hippuric acid, 10.2 min; phenylacetylglutamic acid, 13.8 min; methyl-DCA, 15.8 min.

GC and GC/MS Analysis. The GC concentrations in plasma from rats that had received unlabeled DCA were measured by the GC method of Chu et al. (1990). Plasma samples from rats that had received [1,2-14C]DCA were analyzed for DCA by GC/MS, using a Hewlett-Packard 5890 Series II Plus GC system, a 972A mass-selective detector, and a Vectra multimedia VL 2/466 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; glyoxylic acid, 11.2 min; oxalate, 11.7 min; acetylglycine, 17.2 min; hippuric acid, 20.2 min; phenylacetylglutamic acid, 21.2 min. For quantitation of DCA in plasma, single ions of molecular mass 59 and 60, corresponding to the [1-13CO3CH3 and [1-13CO2CH3]+ ions, were monitored.
13C fragments, respectively, were monitored for the peak with a retention time matching that of authentic methylated DCA. Standard curves were developed with methylated [13C]DCA standard under identical conditions and were used to quantify 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 13C-labeled methylated metabolite to the mass spectrum of the methylated authentic 14C standard. The presence of 13C in each metabolite peak was confirmed by comparing the 13C/12C ratios in the molecular ion (for methylated glycine conjugates of benzoic, acetic, and phenylacetic acids) or the -COOCH3 fragment (for oxalate and glyoxylate) with the natural abundance of 13C in unlabeled metabolite standards. If the ratio was higher than expected for the natural abundance of 13C, it was assumed that the metabolite arose from 13C]DCA.

NMR Analysis. One sample of urine from a rat that had received a DCA dose containing a mixture of 99% [1,2-13C]DCA and 1% [1-14C]DCA (final specific radioactivity, 0.555 mCi/mmol) was analyzed by 1H and 13C NMR spectroscopy. HPLC analysis, with radiochemical detection, of this urine sample showed that 70.5% of the urinary DCA-derived 14C 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 D2O (0.15 ml), and this solution was examined in a 5-mm tube. The D2O contained a trace of 3-(trimethylsilyl)propionate-2,2,3,3-d4 sodium salt, serving as a chemical shift reference. For both 1H and 13C spectra, each spectrum was each referenced to the 3-(trimethylsilyl)propionate-2,2,3,3-d4 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 13C 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 obtained over 1.4 hr using a standard J-modulated spin-echo sequence, with proton decopling 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 13C NMR spectrum of a saturated D2O solution of authentic hippuric acid was obtained for comparison, using the natural abundance of 13C for signal detection. Spectra were also compared with published 13C 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 = −2ln(likelihood) + 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.

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 [14C]DCA was in expired air as 14CO2, as shown in fig. 1 and table 1. The percentage of the radioactive dose excreted as 14CO2 was quantitated in the young adult rats and varied with access to food and number of DCA doses. It was not possible to quantitate 14CO2 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 CO2 in 24 hr. Less 14CO2 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 [¹³C]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 in urine. There were significant differences between fed and fasted rats in the rate or extent of urinary excretion; therefore, the results are shown based on the number of doses received. Points, mean ± SE. Between 6 and 24 hr, there were significant differences in the rate and extent of urinary excretion in the large rats, compared with the young adult rats (p < 0.05).

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 radiolabeled dose as unchanged DCA (7.4–37.2%) 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 difference 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 [¹³C]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
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. 

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

**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>Hhippuric 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 < 0.05).

† Total recovered in urine is also a glycine conjugate.

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. 

NMR spectroscopy of urine from a fed, repeatedly dosed rat that produced a large amount of U2 (75.0% of urinary 14C) suggested a structure of X-13CH2-13COOH. The proton NMR spectrum (not shown) showed that a significant portion of the proton signal was attributable to a monosubstituted phenyl group, probably a benzamido residue. This observation led to interpretation of the 13C signals from this benzoyl fragment (13C at natural abundance) as probably being connected to the doubly 13C-labeled glycine fragment found in the carbon spectrum (fig. 4). Peaks identified as 1 and 2 in fig. 4 appear as doublets because of 13C coupling, providing further confirmation of the origin of these carbons from [13C]DCA. Thus, the major urinary component was tentatively identified as hippurate. The NMR spectra of this urine sample were consistent with a high concentration of hippuric acid, and peaks in the proton-decoupled 13C spectrum of urine were similar to peaks in the spectrum for authentic hippuric acid obtained under the same conditions and to published spectra for hippuric acid and sodium hippurate. The NMR spectrum of urine also revealed a 13C peak corresponding to oxalate (fig. 4), providing further confirmation of the presence of oxalate in urine.

After the tentative NMR identification of the glycine in hippurate as a DCA metabolite, additional methylated urine extracts were analyzed by GC/MS. Authentic methyl hippurate and a major urinary component had retention times of 20.2 min and molecular ions matching the molecular weight of authentic methyl hippurate (m/z 193). The 20.2-min peak from the urine samples also showed obvious M+2 molecular ions (fig. 5). The M+2/M peak abundance ratios for methyl hippurate from several samples of urine were up to 3-fold higher than for standard methyl hippurate, indicating the presence of two 13C-labeled carbons in the methyl hippurate from urine. Evidence for two other glycine conjugate metabolites of DCA in urine was also gained by GC/MS analysis of methylated urine. Acetylglycine (GC retention
time, 17.3 min) and phenylacetylglucine 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 phenylacetylglucine, in the GC trace and its mass spectrum. The mass spectrum showed a molecular ion for methylphenylacetylglucine 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 methylphenylacetylglucine, 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

FIG. 4. Proton-decoupled NMR spectrum of urine from a rat dosed with a 99:1 mixture of [13C]DCA and [14C]DCA (50 mg/kg).
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 CO$_2$ 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 CO$_2$ and ammonia. Conversion of glycine to CO$_2$ can be accomplished in several ways. During glucogenesis from glycine, the glycine is either converted to serine and then pyruvate or converted to ammonia, $N^5,N^{10}$-methyleneetetrahydrofolate, and CO$_2$ (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 CO$_2$ 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 $^{14}$CO$_2$ 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**

Pharmacokinetic parameters for elimination of DCA from rat plasma after one or two oral doses of DCA (50 mg/kg)

<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 ± 75.4$^a$</td>
<td>1509 ± 248$^b$</td>
</tr>
<tr>
<td>Volume of distribution</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>$\alpha$-Phase half-life (hr)</td>
<td>ND$^c$</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 ± 0.76$^a$</td>
<td>9.72 ± 0.97$^p$</td>
</tr>
<tr>
<td>Time of $C_{max}^{a}$ (hr)</td>
<td>0.27 ± 0.04</td>
<td>1.13 ± 0.46</td>
<td>2.09 ± 0.96</td>
</tr>
<tr>
<td>$C_{max}$ ($\mu$g of DCA/ml)</td>
<td>10.19 ± 1.87</td>
<td>27.22 ± 5.34$^a$</td>
<td>121.2 ± 20.4$^b$</td>
</tr>
</tbody>
</table>

Values shown are mean ± SE ($N = 4$ or 5) for each parameter.  
$^a$ Significantly different from singly dosed rats ($p < 0.02$).  
$^b$ Significantly different from young adult rats ($p < 0.01$).  
$^c$ ND, too rapid to estimate.  
$^{d}C_{max}$, maximal plasma concentration.
holding food is likely to increase glucogenolysis 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 and mitochondria had very little radioactivity from DCA (table 1). 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 monochloroacetate (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 MØ, 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 phenylacetylglycine (HPLC retention time, NMR spectrum of urine, and GC/MS results for methylated urine) and phenylacetylglycine, 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 (Stacpoole 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

| TABLE 4 Subcellular distribution of radioactivity in liver and kidney of rats 1 hr after a single dose of DCA (50 mg/kg) |
|-----------------------------|-----------------------------|-----------------------------|
| Fraction                    | Radioactivity in Each Fraction | % |
| Nuclei and cell debris      | Liver                      | 23.3 ± 3.7                  |
| Mitochondria                | Kidney                     | 18.7 ± 6.2                  |
| Microsomes                  |                            | 7.8 ± 3.9                   |
| Cytosol                     |                            | 7.0 ± 0.7                   |
| Nuclear and cell debris     |                            | 5.8 ± 0.4                   |
| Mitochondria                |                            | 1.2 ± 0.4                   |
| Microsomes                  |                            | 63.0 ± 5.3                  |
| Cytosol                     |                            | 73.1 ± 7.0                  |

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.
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 GR and Page NP (1992) Hepatocarcinogenicity of...


