Metabolism of 2,4-Dichlorophenoxyacetic Acid in Laying Hens and Lactating Goats

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2,4-Dichlorophenoxyacetic acid (2,4-D) labeled with ^{14}C was found to be rapidly eliminated by laying hens and lactating goats dosed orally for 7 consecutive days at 18 mg/kg of food intake and for 3 consecutive days at 483 mg/kg of food intake, respectively. Excreta of hens and goats contained >90% of the total dose within 24 h after the final dose. Tissue residues were low and accounted for <0.1% of the dose in these animals. For hens, the residues in muscle, liver, and eggs (0.006−0.030 ppm) were lower than those found in fat and kidney (0.028−0.714 ppm), 2,4-D equivalents. The tissue with highest residue in goat was the kidney at 1.44 ppm, 2,4-D equivalents. Milk, liver, composite fat, and composite muscle had significantly lower residue levels of 0.202, 0.224, 0.088, and 0.037 ppm, respectively. The most abundant tissue residue was 2,4-D and acid/base releasable residues of 2,4-D. A minor metabolite was identified as 2,4-dichlorophenol.

Keywords: *2,4-Dichlorophenoxyacetic acid; 2,4-D; laying hens; lactating goats; metabolism; herbicide*

INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is a systemic foliar herbicide commonly used to control annual and perennial weeds in small grains and grassland. 2,4-D is often formulated as either an aqueous salt [e.g., dimethylamine (DMA) or sodium (Na)] or an ester [e.g., 2-ethylhexyl (2-EHE)]. Forms of 2,4-D are typically registered for use on grassland; agricultural areas growing commodity crops such as wheat, rice, oats, corn, and sugarcane; aquatic areas; and noncrop areas.

Early literature reports stated that phenoxyacetic acids were rapidly excreted unchanged by animals (*1*). Within 24 h, 96% of orally administered dose of [^{14}C]-2,4-D was recovered from the urine of a sheep (*2*). Clark et al. (*3*) evaluated tissue residues in sheep and cattle following orally administered doses of either 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), or silvex [2-(2,4,5-trichlorophenoxy)propionic acid]. They reported the presence of chlorophenoxy acids and the related phenol metabolites in muscle, fat, liver, and kidney tissues. The highest residues were found in the kidney, with the muscle and fat containing several orders of magnitude less residue. Because 2,4-D is widely used on cereal crops and grasslands, other livestock may be exposed to 2,4-D residues through their diet. Therefore, this paper describes the distribution and identity of 2,4-D residues in the eggs, tissues, and excreta of laying hens and in the milk, tissues, urine, and feces of lactating goats.

MATERIALS AND METHODS

Chemicals. The test material for both the hen and goat studies was a mixture of uniformly ^{14}C-ring-labeled 2,4-D and non-radiolabeled analytical standard of 2,4-D prepared by Dow AgroSciences, LLC, Indianapolis, IN. Appropriate amounts of the ^{14}C-labeled and nonlabeled 2,4-D were combined to produce test substances with specific activities of 3.15 and 1.02 mCi/mmol and radiochemical purities of >99% and >97% for the hen and goat studies, respectively. Authentic standards for metabolites *o*-chlorophenoxyacetic acid (*o*-CPA), *p*-chlorophenoxyacetic acid (*p*-CPA), 2,4-D, 2,4-dichlorophenol (2,4-DCP), and 2,4-dichloroanisole (2,4-DCA) were furnished by Dow AgroSciences or obtained from Aldrich Chemical Co. and Sigma Chemical Co. All other reagents, solvents, and liquid scintillation cocktails (Ready Gel and Ultima Gold) were obtained from commercial suppliers.

Preparation of Dosing Capsules. For the hen study [^{14}C]-2,4-D was dissolved in ethanol/water (95:5), and portions were transferred to gelatin capsules containing cellulose powder. The capsules were sealed and placed in frozen storage. The dose capsules for the goat study were prepared by weighing an appropriate amount of the test material into empty gelatin capsules. The capsules were then filled with cellulose powder, sealed, and stored frozen. In the hen study, before and after the dosing period, a single capsule was analyzed, and 2,4-D was shown to be stable in the capsule matrix for 8 days while stored frozen (average recovery was 95 ± 0.8%).

Animal Handling and Dosing. White Leghorn laying hens were obtained from Schnuk's Agri-Foods, Inc. (Hawk Point, MO). The hens were 29 weeks old and in their first laying cycle. After a two-week acclimation period, hens that met average egg production and weight requirements were randomly selected and assigned to groups. The average hen weight over the course of the study for each group ranged from 1.5 to 1.6 kg. Four groups of five hens each were used, one control and three treated. Egg production was consistent for all groups, averaging 4.5 eggs per group per day. [^{14}C]-2,4-D was administered orally once a day for seven consecutive days. Hens in the treated groups received on average 1.4 mg of 2,4-D/kg of body weight/hen/day (18 ppm, based on average food intake).

Lactating Alpine goats were obtained from a dairy goat operator in Elmer, MO. After a 12-day acclimation period, the goats that met the selection requirements of being a minimum of 1 year old, producing at least 950 g of milk per day and weighing between 40 and 60 kg each, were randomly selected as either a control or treated animal. The prepared capsules

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were administered orally via a stainless steel balling gun once a day for three consecutive days. The treated goat received a dose on average of 23 mg of 2,4-D/kg of body weight/day (483 ppm, based on average food intake). The control hens and goat were treated with placebo capsules and served as a source of control tissue for each study.

Sample Collection, Preparation, and Analysis. *Poultry.* Eggs were collected twice a day, morning and evening. Evening collections were stored refrigerated overnight and combined with the next day's morning eggs as a single sample. Egg whites and yolks were pooled by groups into tared plastic jars and weighed. The eggs were then homogenized and stored frozen. Excreta were collected once daily from aluminum foil cage liners, pooled by treatment group, and homogenized with water for radiochemical analysis by liquid scintillation counting (LSC). Within 22−24 h after the final dose, the hens were sacrificed by cervical dislocation. Tissue samples were collected from each animal upon necropsy, with each tissue type pooled by treatment group. Tissues collected were breast and thigh muscle, fat, heart, liver, kidney, and gizzard. Tissues and eggs were homogenized in the presence of dry ice using either a Hobart meat grinder or an Osterizer blender. Aliquots were solubilized or combusted for radiochemical analysis.

Goats. Milk was collected twice daily, morning and evening, and refrigerated. On day 3, a composite milk sample was prepared by combining a 200-mL aliquot from the evening and morning milkings. Urine and feces were collected as separate samples daily, weighed, and stored frozen. Frozen feces were homogenized in a grist mill with dry ice. Prior to sacrifice, two 10-mL aliquots of blood (from the jugular vein) were collected into heparinized tubes. These blood samples were centrifuged into plasma and red blood cells and stored frozen. Goats were sacrificed within 24 h of the last dose by electrocution and samples were collected upon necropsy. Tissues collected were omental and perirenal fat, liver, kidney, semimembranous muscle, longissimus doris muscle, triceps muscle, gall bladder contents, intestines and contents, rumen, and reticulum and contents. Tissues were homogenized with dry ice using either a Hobart meat grinder or an Osterizer blender. Equal amounts of each muscle (semimembranous, longissimus doris, and triceps) and equal amounts of each fat (omental and perirenal) were ground together to form composite muscle and fat samples, respectively. Aliquots of the urine, feces, and tissues or composite tissues were solubilized or combusted for radiochemical analysis.

Quantification of Radioactivity. Replicate combustions (three, 0.1−0.5 g) of all egg, feces, and nonfat tissues were performed using either a Harvey OX-500 biological material oxidizer or a Packard 307 Tricarb oxidizer. Performance of the oxidizers was monitored daily by combustion of a ^{14}C standard or fortification of control tissue with a [^{14}C]-2,4-D standard. The average recovery for the fortification of control tissues with a [^{14}C]-2,4-D standard was >98%. Fat samples were prepared for radioanalysis by the solubilization of ~0.5 g of tissue in 8 mL of Carbosorb (Packard) at 100 °C for ~48 h, followed by the addition of 12 mL of Permafluor (Packard) and LSC. The average recovery for control fat fortified with [^{14}C]-2,4-D was >99%. Milk samples were prepared for direct radioanalysis by placing 0.5 g of milk into 9 mL of deionized water and adding 10 mL of scintillation cocktail. Radioactivity was measured by using a TM Analytical Delta 300-6891 LSC system with a Toshiba T100 portable PC. Counting efficiency was determined using the external standard ratio (ESR) technique. Sample counting was terminated either after 10 min or after a total accumulation of 200000 counts ($2\sigma = 0.5\%$), whichever occurred first. Sample counting for HPLC fractions was terminated after 5 min, or a total accumulation of 200000 counts. Scintillation fluids used for LSC were either Ready Gel or Ultima Gold.

Extraction of ^{14}C Residues. *Poultry.* In general, kidney, eggs, and liver tissue subsamples were acidified with either trifluoroacetic acid or HCl prior to extraction with diethyl ether (hereafter referred to as ether). Additional processing of the initial ether extracts and/or postextracted tissues included (1) acid or base hydrolysis, (2) acetonitrile (ACN)/hexane partitioning, and/or (3) base extraction followed by ether/acid back extraction. The ^{14}C residues in egg tissue were solubilized without acidic or basic hydrolysis.

Specifically for kidney, the acidic, aqueous phase (pH 2) resulting from the initial extraction of kidney tissue was heated (100 °C, 16 h). The resulting hydrolysate was partitioned against ether, the ether phase was concentrated, the concentrate was purified by TLC, and the TLC-isolated ^{14}C fraction was reconstituted in aqueous ACN. The aqueous phase from acid hydrolysis was adjusted to pH 10 with NaOH and heated at 100 °C for 18 h. Following this step, the mild base hydrolysate was acidified and partitioned against ether. The ether phase was concentrated, purified by TLC, and reconstituted in aqueous ACN for HPLC analysis. The aqueous phase from the mild base hydrolysate was separated into an ACN isolate and a residual tissue isolate. The latter was treated with strong base (SB) (1 M KOH, 100 °C, 22 h) to give a further SB-ether isolate, which was combined with the ACN isolate for HPLC analysis.

Homogenized fat tissue was acidified and refluxed in ACN. The remaining aqueous/fat mixture was adjusted to pH 12 with NaOH and held at 100 °C overnight. After basic hydrolysis, the aqueous solution was acidified to pH 2 with HCl and partitioned against ether. The resulting ether fraction was concentrated and hydrolyzed a second time using KOH/methanol. This mixture was partitioned against ACN and hexane.

Following these extraction/solubilization steps, extracted tissues were analyzed for total radioactive residue (TRR) by combustion analysis. The extracts were analyzed by LSC and/or HPLC/TLC for quantification and characterization/identification purposes. Breast and thigh muscle tissues were not extracted because the TRR values were low (<0.008 ppm of 2,4-D equivalents).

Goats. The ^{14}C residue in kidney was isolated as outlined in Figure 1. Muscle tissue was first extracted with ACN/H_2O (4:1). Partitioning of the ^{14}C residue between aqueous acid and chloroform and then between ACN and hexane was used to prepare the soluble ^{14}C residue for HPLC analysis.

Milk samples were acidified with H_2SO_4 and extracted with ether. The ether-soluble ^{14}C residues were partitioned between ACN and hexane followed by acid hydrolysis of these residues (2 N HCl, 100 °C, 4 h). The acid hydrolysate was worked up via ether extraction with HPLC analysis of the released residue.

Homogenized fat tissue was macerated with ether, and the ether extract was concentrated. The concentrated ether was partitioned against ACN and hexane, followed by basic hydrolysis of the hexane phase (0.5 M NaOH at 90 °C for 12 h). The ^{14}C residues in the hydrolysate were purified by ether extraction and partitioning against hexane/ACN with subsequent analysis by HPLC. The pellet remaining after initial ether extraction was extracted with ACN. The ACN was partitioned against hexane, followed by basic hydrolysis of the hexane phase (NaOH, 100 °C, 4 h) with purification as above, and subsequent analysis by HPLC. Post-ACN-extracted fat pellet was base hydrolyzed (0.5 M NaOH, 95 °C, 26 h), acidified, and partitioned against ether prior to HPLC analysis.

Liver samples were first incubated with pancreatin enzyme (pancreatin, 0.02 M Tris buffer, pH 7.5, 38 °C, 18 h), and then the enzyme/liver mixture was acidified to pH 1 and extracted with ACN. The ACN-soluble ^{14}C residues were subsequently partitioned against $CHCl_3$ and between ACN and hexane. The ACN-extracted solid was hydrolyzed with acid (1 N HCl, 100 °C, 16 h) and partitioned between ACN and hexane.

Urine was directly analyzed by HPLC.

Thin-Layer Chromatography (TLC). All TLC separations were carried out using normal phase Merck silica gel 60 F-254 [glass (20 × 20 cm) with a 250-μm particle size and a 0.25- or 1.0-mm gel thickness]. The solvent system used for analysis was hexane/2-propanol (1:1, v/v) with 5% acetic acid. Radioactive zones were located using an Ambis Radioanalytical Imaging system or a Radiomatic model RS radio TLC scanner.

High-Performance Liquid Chromatography (HPLC). Three HPLC systems were utilized in each study: (1) a

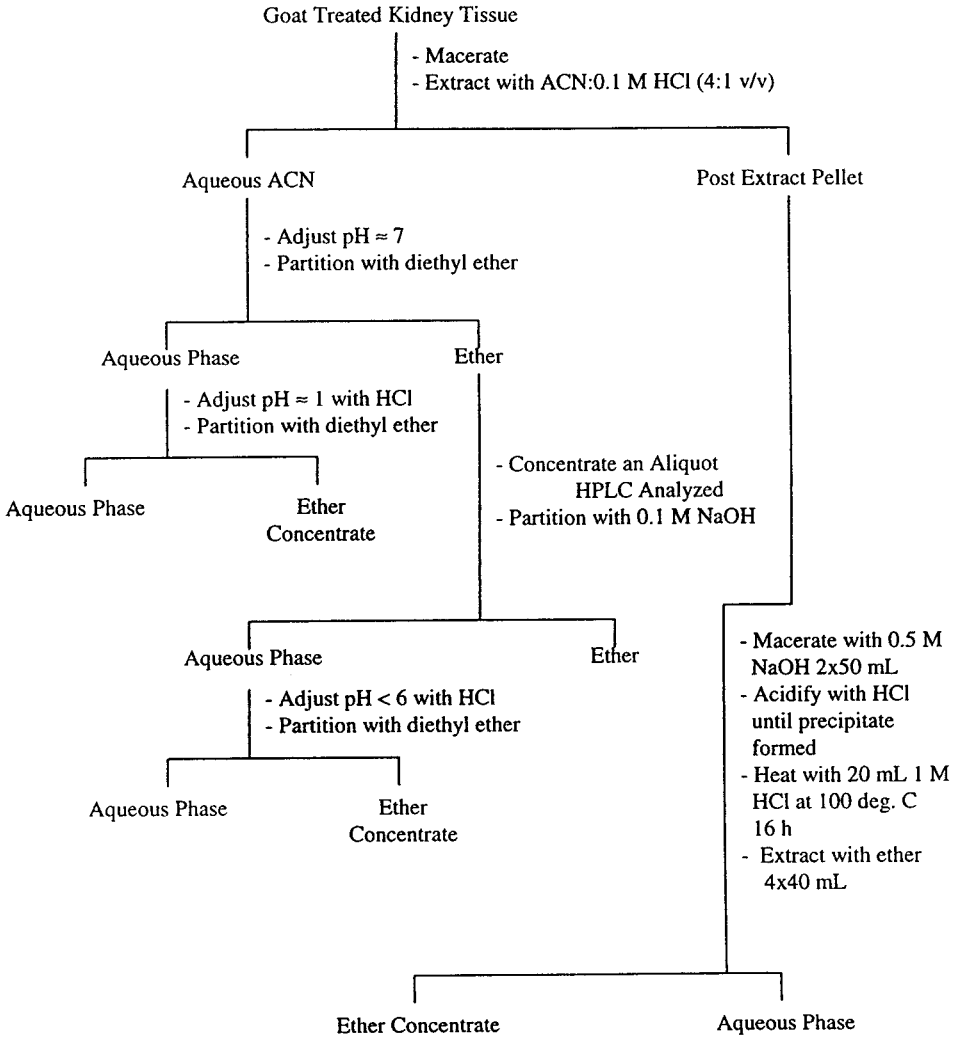

Figure 1. Extraction scheme for the isolation of goat kidney metabolites.

Shimadzu HPLC with an SCL-6B or 6A system controller, an SPD-6A UV detector, and two LC-6A pumps; (2) a Varian HPLC Star 9020 workstation with a 9010 gradient pump and a 9050 UV detector; and (3) a Waters Baseline system with two model 510 pumps, a model 486 variable wavelength UV detector, a SIMS data acquisition module, an NEC 386sx computer with Waters Baseline software, and a Rheodyne model 7125 injector. All of the systems were equipped with a Gilson model 202C fraction collector to collect the column eluant. Analysis was conducted using a Waters Nova-pak C-18 or C-8 column, 8 mm × 10 cm, with UV absorbance at 280 nm, flow rate of 1.5 mL/min, solvent system A = water/ACN (80:20, v/v) containing 2.5% glacial acetic acid, and solvent system B = ACN containing 2.5% glacial acetic acid; the gradient started at 20% B and ran to 55% B over 30 min and then from 55% B to 100% B over 5 min, and was held at 100% B for an additional 15 min. Detection of radioactivity was accomplished using LSC of the collected fractions. Typical retention times for reference standards on the Waters C-18 column were 20.0, 21.4, 26.6, 28.4, and 38.3 min, respectively, for o-CPA, *p*-CPA, 2,4-D, 2,4-DCP, and 2,4-DCA. Typical retention times for reference standards on the Waters C-8 column were 16−18 and 18−20 min, respectively, for 2,4-D and 2,4-DCP.

Mass Spectrometry Analysis. Gas chromatography−mass spectrometry (GC-MS) was performed using a Finnigan-Mat 5100 quadrupole mass spectrometer interfaced with a Finnigan model 9611 gas chromatograph. The mass spectrometer was operated in the electron impact (EI) mode with ionizing voltage set at 70 eV. The separations were performed on a J&W DB-5, 30-m fused silica capillary column.

RESULTS

Distribution of Total Radioactivity. *Poultry.* The TRR recovered in the hen matrices and the percent of dose recovered are summarized in Table 1. TRR is reported in parts per million (ppm) based on 2,4-D acid equivalents. Approximately 92% of the dose was eliminated in the excreta, with <0.1% of the dose found in each of the other matrices.

Goat. The TRR levels found in the goat matrices and the percent of dose recovered in these matrices are shown in Table 2. Consistent with the hen results, the lowest radioactive residue was in the composite muscle and the highest in the kidney. Approximately 90% of the dose was excreted in the urine (82%) and feces (8%).

Isolation, Characterization, and Identification of ^{14}C Residues in Poultry and Goats Matrices. *Poultry Matrices.* The ^{14}C residues in the edible tissues with TRR >0.01 ppm and in day 7 eggs were further characterized by extraction analysis and chromatography.

Egg. The mean ppm residue levels for the various groups increased steadily from 0.003 ppm at day 2 to 0.018 ppm on day 7 (composited from various groups). The majority (0.015 ppm, 85%) of the ^{14}C residues were recovered in the initial ether extract with 16% (0.003 ppm) of the residues remaining unextractable. Matrix interferences made the direct HPLC analysis of the

Table 1. [^{14}C]-2,4-D Equivalents and Percent Total Dose Recoveries in Body Tissues and Other Matrices of Laying Hens

sample	av ppm[a]	SD	% total dose recovered
egg 1[b]	<MQL		<0.1
egg 2	0.003	0.001	<0.1
egg 3	0.006	0.0	<0.1
egg 4	0.010	0.0	<0.1
egg 5	0.012	0.003	<0.1
egg 6	0.017	0.001	<0.1
egg 7[c]	0.018	0.001	<0.1
fat	0.028	0.005	<0.1
kidney	0.71	0.07	<0.1
liver	0.03	0.01	<0.1
breast muscle	<MQL		<0.1
thigh muscle	0.006	0.002	<0.1
heart	0.02	0.01	<0.1
gizzard	0.1	0.05	<0.1
excreta[d]	17.8	0.7	91.7
total dose recovered			91.8

[a] Numbers listed are the means of the three groups of dosed hens. Groups 1−3 were replicates of five hens each. The tissues from each group were analyzed in triplicate. The dose levels were the same for each group of hens. MQL, minimum quantifiable limit. [b] First day of dose. [c] Last day of dose. [d] Range of ppm values for the day 1−7 samples.

Table 2. [^{14}C]-2,4-D Equivalents and Percent Total Dose Recoveries in Body Tissues and Other Matrices of a Lactating Goat

sample	ppm[a]	% total dose recovered
milk	0.202	0.024
liver	0.224	0.008
kidney	1.44	0.008
composite fat	0.088	0.016
composite muscle	0.037	0.021
urine	274−320[b]	82.1
feces	23.9−54.5[b]	7.73
total		90.0

[a] Numbers listed are the means of triplicate determinations of pooled samples from a single goat. [b] Range of ppm values for the day 1−3 samples.

ether-extractable residues from day 7 eggs impossible. HPLC analysis after cleanup extraction exhibited three radioactive components (Figure 2). 2,4-D (23%, 0.004 ppm) and 2,4-DCP (7.3%, 0.001 ppm) were identified by comparison to standards and by co-injection of a mixture of these standards. The third component (0.0005 ppm, 3%), eluting at 18.0−19.0 min, was not identified.

Fat. The TRR found in composited fat was 0.027 ppm 2,4-D equivalents. The initial ACN extraction recovered 22% (0.006 ppm) of TRR. Base hydrolysis of the ACN-extracted tissue released the remaining ^{14}C residue (82% TRR or 0.022 ppm); however, after cleanup, only 31% of TRR was analyzable by HPLC with 25% of TRR (0.007 ppm) identified by cochromatography as 2,4-D residues.

Liver. The TRR found in the composite liver sample was 0.030 ppm. A majority (54%, 0.016 ppm) of the liver ^{14}C residues were recovered in the initial ether extract. Analysis of the TLC-purified ^{14}C residues contained in the ether extract by HPLC resulted in the identification of 2,4-D (18%, 0.005 ppm) and 2,4-DCP (4.4%, 0.001 ppm) by cochromatography with the radiolabeled standards.

Kidney. Hen kidney contained higher residue levels than any other tissue or egg. The initial ether extract solubilized 12% of TRR (0.08 ppm). TLC cleanup indicated the presence of only 2,4-D. HPLC analysis recovered 1.4% of the TRR (0.01 ppm) as the parent compound 2,4-D. Another three minor components (total <0.003 ppm) were also detected by HPLC but not characterized further. Mild acid hydrolysis of the residual ether-extracted tissue gave 58% of TRR. TLC cleanup indicated only 2,4-D was present. HPLC analysis identified 57% of the TRR (0.41 ppm) as 2,4-D from the mild hydrolytic step. This ^{14}C component was derivatized using diazomethane, and the derivative was analyzed by GC-MS. The resulting mass spectral data confirmed the identity of the derivatized component as 2,4-D methyl ester. HPLC analysis of the mild base ^{14}C fraction tentatively identified the residue as 2,4-D (17% of TRR, 0.12 ppm). Although 5% of the TRR was solubilized by the ACN/strong base hydrolytic action, only 1.5% TRR was identified as 2,4-D by elution time comparison. Overall, extraction of the kidney tissue recovered 92% of the TRR (0.67 ppm), of which 77% (0.55 ppm) was identified as 2,4-D.

Goat Matrices. The ^{14}C residues in urine, milk, and all edible tissues were characterized by extraction and subsequent analysis by TLC and HPLC.

Urine. The TRR in day 1−3 urine samples ranged from 290 to 390 ppm [^{14}C]-2,4-D equivalent. The major component in an aliquot of the day 3 urine that was subjected to HPLC analysis was found to be 2,4-D, accounting for 98% of the residue. Approximately 1.7% of the TRR present in the urine, eluting at 21 min, was shown by retention time comparison to be *p*-CPA). Radiolabeled *p*-CPA was a 2% impurity in the dosing material. Therefore, this residue was considered to be a carryover as an impurity rather than a metabolite from the radiolabeled 2,4-D.

Milk. A composite milk sample (0.202 ppm) was prepared from day 3 morning and evening milkings. Hexane partitioning to remove fats isolated only 7% of TRR (0.015 ppm). The ACN extract prior to mild acid hydrolysis (1 h of reaction time) contained 85% of the TRR (0.17 ppm). The major component of this ACN extract had a retention time corresponding to that of [^{14}C]-2,4-D (0.077 ppm, 38% of TRR), with two additional significant peaks at approximately 18 and 22 min, accounting for 0.031 ppm (15% of TRR) and 0.053 ppm (26% of TRR), respectively. After 1 h of mild acid hydrolysis, the ^{14}C components eluting at 18 and 22 min were diminished and additional [^{14}C]-2,4-D (47% of TRR) and [^{14}C]-2,4-DCP (5.0% of TRR) were released. The mild acid hydrolysis procedure was repeated for 4 h. Figure 3 shows the before hydrolysis profile and Figure 4 the profile after hydrolysis of the 18−22-min components. The major component, [^{14}C]-2,4-D, accounted for 0.085 ppm (42% of TRR) after the 4-h mild acid hydrolysis. An additional minor component (0.010 ppm, 6.9% of TRR) observed in the chromatogram of Figure 4 had a retention time similar to that of *p*-CPA eluting at 21 min. As with the *p*-CPA identified in the urine, this residue was considered to be a carryover as an impurity from the radiolabeled test material rather than a metabolite.

In a separate isolation another subsample of milk was extracted as above and hydrolyzed for 2 h. The major ^{14}C component was isolated by TLC and derivatized with diazomethane for analysis by GC-MS along with a derivatized 2,4-D standard. The resulting mass spectrum for the derivatized isolated residue and that for

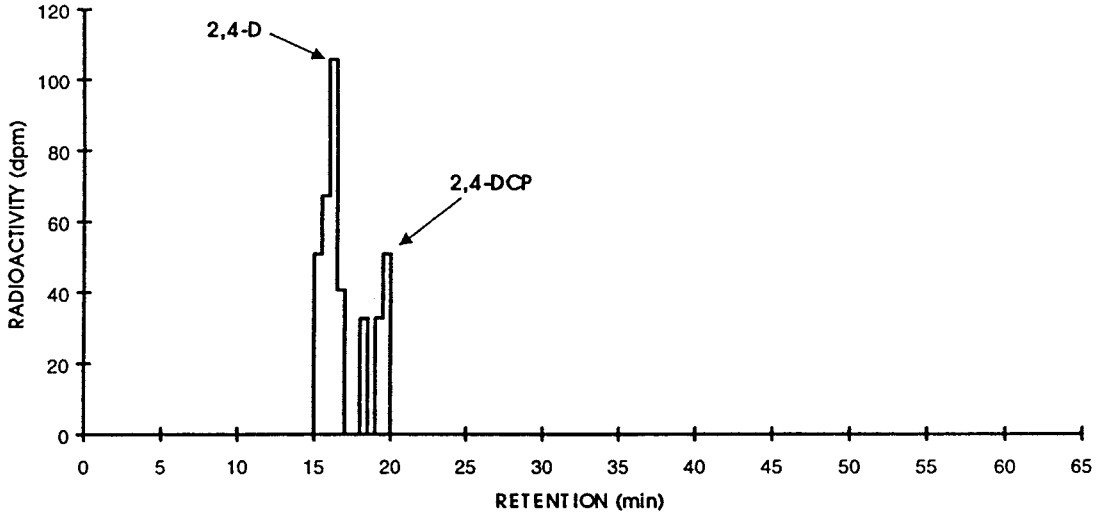

Figure 2. HPLC radiochromatogram of day 7 composite egg ether-extractable residues.

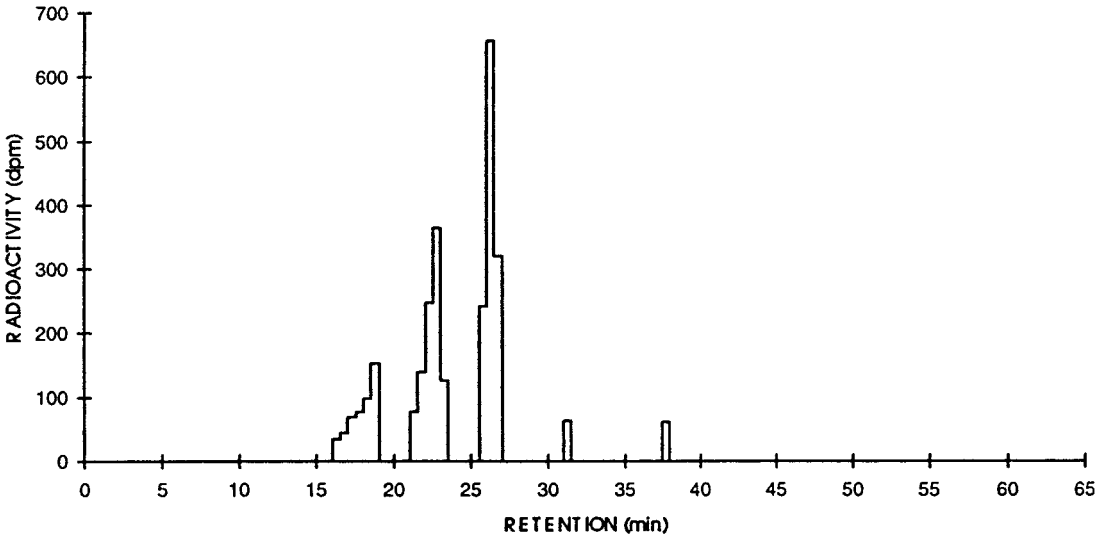

Figure 3. HPLC radiochromatogram of the ACN-soluble day 3 composite milk residues prior to 4-h acid hydrolysis.

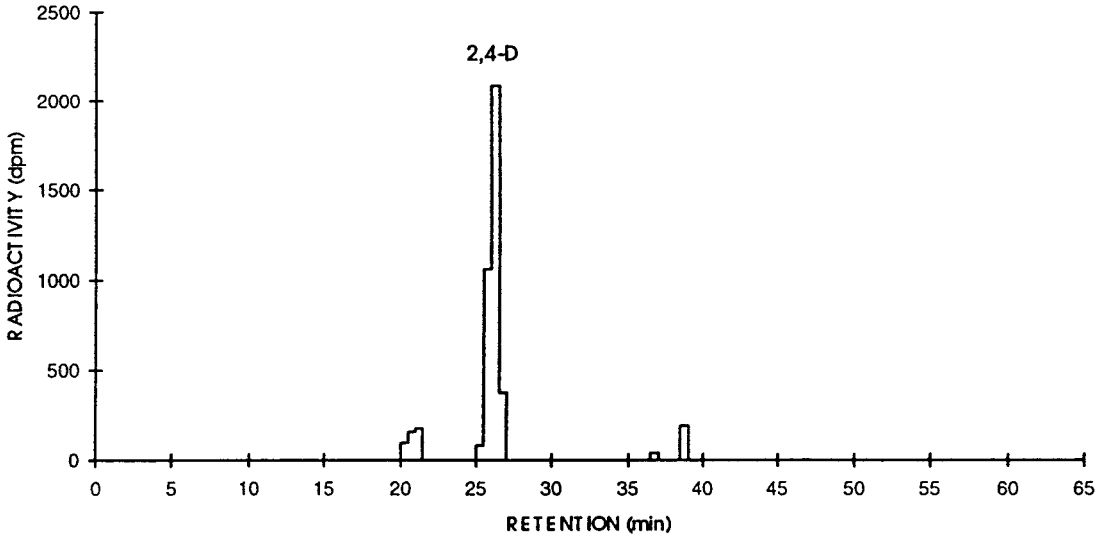

Figure 4. HPLC radiochromatogram of the ACN-soluble day 3 composite milk residues following 4-h acid hydrolysis.

the derivatized standard were identical with the molecular ion for the 2,4-D methyl ester at *m/z* 234 and a base peak at *m/z* 199 (Figure 5). The typical isotopic patterns for mono- and dichlorinated fragments were also observed. The stability of [^{14}C]-2,4-D during hydrolysis was demonstrated by hydrolyzing the standard material in 2 N HCl for 4 h. Approximately 90% of the [^{14}C]-2,4-D was recovered after the hydrolysis.

Liver. The total radioactive residue level in liver was 0.224 ppm. Pancreatin enzyme was utilized to fully

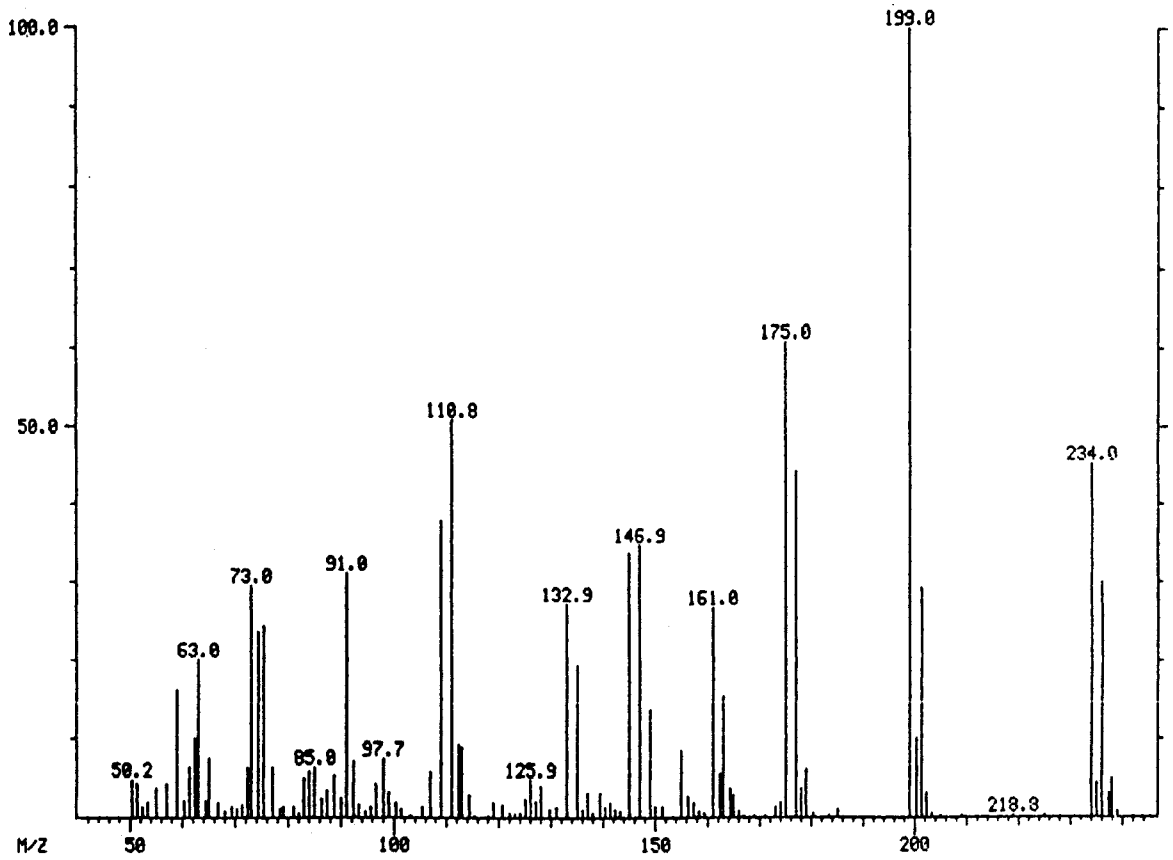

Figure 5. EI mass spectrum of the methyl ester of 2,4-D isolated from milk.

disrupt the matrix and release trapped residues. After the enzyme digestion, 0.204 ppm of the radioactive residues (91% of TRR) was extractable with ACN. HPLC after cleanup revealed a peak corresponding to 2,4-D (0.046 ppm, 21% of TRR). This residue assignment was supported by TLC analysis of the liver extract. Two less polar components were observed, one (NP1, 15% of TRR; 0.033 ppm) eluting at 38 min and the other (NP2, 18% of TRR; 0.040 ppm) at 40 min.

Kidney. The total kidney residue level was 1.44 ppm. Initial acidic aqueous ACN extract released ~1.33 ppm of the ^{14}C residue (92% of TRR). After cleanup by organic partitioning ($CHCl_3$) of neutralized concentrated aqueous ACN extract, 89% of the TRR (1.28 ppm) remained. HPLC analysis of this extract revealed 2,4-D (0.76 ppm, 53% of TRR) and three major nonpolar metabolites (eluting at 38, 40, and 42 min), which accounted for a total of 0.15 ppm (10% of TRR), 0.31 ppm (22% of TRR), and 0.059 ppm (4.1% of TRR), respectively.

Treatment of the remainder of the tissue with 0.5 NaOH did not release radioactive residues. Therefore, the extracted tissue was solubilized in HCl and partitioned with ether. The ether extract (0.030 ppm, 2% of TRR) was analyzed by HPLC, revealing 2,4-D (0.013 ppm, 1% of TRR) and an unknown with a retention time of ~40 min (0.007 ppm, 0.5% of TRR).

The identity of 2,4-D in kidney tissue was confirmed by GC-MS analysis of the isolate tentatively identified as 2,4-D by HPLC after diazomethane derivatization alongside a 2,4-D methyl ester standard. The mass spectrum for both the derivatized residue and the 2,4-D methyl ester standard clearly shows the molecular ion peak (*m*/*z* 234) corresponding to the methyl ester of 2,4-D and a base peak of *m*/*z* 199 corresponding to the loss of Cl. Other significant fragment peaks at *m*/*z* 175 and 161 were present in the GC-MS results for both standard and derivatized isolate.

The three nonpolar metabolites (NP1, NP2, and NP3 with HPLC retention times of 38, 40, and 42 min, respectively) were isolated from the aqueous ACN extract of kidney using HPLC. GC-MS analysis was attempted on the isolated NP1, NP2, and NP3. Neither a GC peak nor a spectrum of NP1 and NP3 could be obtained by GC-MS analysis. It should be noted that both NP1 and NP2 had HPLC retention times similar to those of two low-level impurities in the test material. Analysis on the second nonpolar isolate (NP2) gave a GC retention time that was consistent with that of DCA standard. Comparison of the mass spectrum of DCA standard with the mass spectrum of the isolated NP2 showed that the molecular ion for both DCA and NP2 was 176 and that the same ions with *m*/*z* >100 could be seen at similar intensities. Although the GC retention time and mass spectrum of NP2 suggested NP2 was DCA, an explanation for the differing HPLC retention times for NP2 and DCA standard was not available. Possibly NP2 is a positional isomer of DCA.

The chemical nature of each of the three isolated nonpolar residues NP1, NP2, and NP3 was investigated using sodium bicarbonate partitioning of the isolated metabolite. Prior to this investigation, partitioning of aqueous bicarbonate solutions of [^{14}C]DCP and [^{14}C]-2,4-D with ether revealed that [^{14}C]DCP was almost completely extracted into ether after only one partition, whereas >90% of [^{14}C]-2,4-D remained in the aqueous phase after five partitions. NP3 was the most readily extractable of the three nonpolar residues with >78% extracted with only four partitions. NP2 was partitioned 16 times, extracting 55% of the total radioactive residue.

Table 3. Distribution of Identified Residues in Laying Hens and Lactating Goats Following Dosing with [^{14}C]-2,4-D

matrix	total ^{14}C, ppm	2,4-D, % TRR	2,4-DCP, % TRR	NP1, % TRR	NP2,[a] % TRR	NP3, % TRR	*p*-CPA,[b] % TRR	acid releasable 2,4-D residues, % TRR	base releasable 2,4-D residues, % TRR
hen									
eggs	0.018	23[e]	7.3[e]						
fat	0.027								25[e]
liver	0.030	18[e]	4.4[e]						
kidney	0.714	1.4						57[e]	17[e]
goat									
urine	320	98					1.7		
milk	0.202	38[e]	5.0	1.0			6.9	9[d]	
liver	0.224	21[e]		15	18	5.4			
kidney	1.44	54[e]		10	22	4.1		1[d]	
fat	0.088	45[e]	2.3	3.4	14				
muscle	0.037	38[e]		2.7	24				

[a] The GC-MS profile for NP2 matched that for 2,4-dichloroanisole, whereas the HPLC retention times for NP2 and 2,4-dichloroanisole differed. Results indicate that these compounds are similar but not the same. [b] *p*-CPA is an impurity in the original test material and is not considered to be a metabolite of 2,4-D. [c] This fraction also contained 1.7% of TRR (0.002 ppm) of base labile 2,4-DCP. [d] Acid labile conjugate. [e] Identity confirmed by coelution with nonlabeled standards, second chromatographic system, or MS analysis.

The partitioning pattern for NP1 from aqueous bicarbonate was similar to that for NP2 and required 10 partitions to extract 95% of the total radioactivity into the ether. These results demonstrated that the three nonpolar residues were more acidic than DCP but not as acidic as 2,4-D. This suggests that the residues could be phenols with strong electron-withdrawing groups, such as a hydroxyl, attached to the ring. Derivatization of NP2 with diazomethane resulted in a 5-min extension of the HPLC retention time of the compound relative to the nonderivatized isolate, supporting the hypothesis of the presence of an acidic hydroxyl group on the compound.

Fat. Omental and perirenal fat were composited. The TRR in fat was 0.088 ppm. The initial ether extract, containing 0.016 ppm (18% of TRR), was concentrated and partitioned between hexane and ACN. Subsequent base hydrolysis of the hexane fraction followed by a cleanup step resulted in a fraction containing 6% of the TRR (0.005 ppm). HPLC analysis of the fraction revealed three peaks, each containing 0.001 ppm (1.1% of the TRR), eluting at 24−25, 33−35, and 35.5−36.6 min. These components were not identified.

The fat tissue remaining after the ether extraction was then extracted with ACN, resulting in the solubilization of 56% (0.049 ppm) of TRR. After the ACN extract had been partitioned against hexane, 42% of the TRR (0.037 ppm) remained in the ACN, whereas 14% of the TRR was transferred to the hexane. HPLC analysis of the ACN extract after partitioning indicated the presence of primarily 2,4-D (0.026 ppm, 29.5% of TRR) and two nonpolar components eluting at approximately 38 and 40 min, NP1 (2.3% of TRR) and NP2 (10% of TRR), respectively. The component eluting at 24−25 min was identified as 2,4-D by retention time comparison and by TLC analysis using a 2,4-D standard.

After base hydrolysis and partitioning cleanup, the radiolabeled residue contained in the hexane phase from the partitioning of the ACN extract was analyzed by HPLC. This analysis revealed that the base releasable residues displayed the same profile seen in the ACN extract, indicating the presence of primarily 2,4-D (0.005 ppm, 5.7% of TRR) and two nonpolar components eluting at approximately 38 and 40 min, NP1 (1.1% of TRR) and NP2 (2.3% of TRR), respectively. The combined total of 2,4-D, NP1, and NP2 isolated in the ACN extract of fat was 35, 3.4, and 12% of the TRR, respectively.

HPLC analysis of the concentrated ether phase from the partitioning of the base hydrolysis of post-ether/ACN-extracted tissue pellet, containing 0.011 ppm or 12% of the TRR, resolved three components eluting at 25.5−26.5, 28, and 40 min. The ^{14}C residue eluting at 25.5−26.5 min was identified as 2,4-D (0.009 ppm, 10% of the TRR), whereas the residue eluting at 28 min was tentatively identified as 2,4-DCP (0.002 ppm, 2.3% of the TRR).

Muscle. Muscle tissues (longissimus, dorsi, semimembranous, and triceps) were composited for analysis. The TRR found for the muscle tissue was 0.037 ppm. HPLC analysis following ACN extraction, concentration, and acidic partitioning with ether demonstrated that 2,4-D was the largest component, accounting for 38% (0.014 ppm) of the TRR. Unknown metabolites were noted with retention times of approximately 38 and 40 min, NP1 (2.7% of TRR) and NP2 (24% of TRR), respectively. The presence of 2,4-D in muscle was verified by a TLC R_f value identical to that of a standard.

DISCUSSION

In both laying hens and lactating goats >90% of the administered dose was accounted for in the excreta or urine and feces, whereas <0.1% of the total dose was accounted for in each of the edible tissues, eggs, and milk. These data demonstrated that 2,4-D was rapidly eliminated from farm animals and is consistent with findings in earlier reports (*1*, *2*). 2,4-D and conjugates of 2,4-D were found to comprise significant portions of the ^{14}C residues found in edible tissues, eggs, and milk (Table 3).

The three nonpolar ^{14}C residues observed in the goat study were isolated from kidney tissue. Sodium bicarbonate partitioning demonstrated that these residues were more acidic than 2,4-DCP but not as acidic as 2,4-D. 2,4-D, 2,4-DCP, and nonpolar residues were identified by HPLC following the cleanup of a mild basic extraction of the three nonpolar residues. Minor radiolabeled impurities seen in the HPLC analysis in the radiolabeled test material elute at the same times as the residues isolated from the various goat tissues. If the nonpolar residues were from the test material, then the actual mass of the nonpolar residues present in the tissues would be much lower than calculated from the specific activity of the 2,4-D used for dosing. The impurity would be present at the specific activity of the

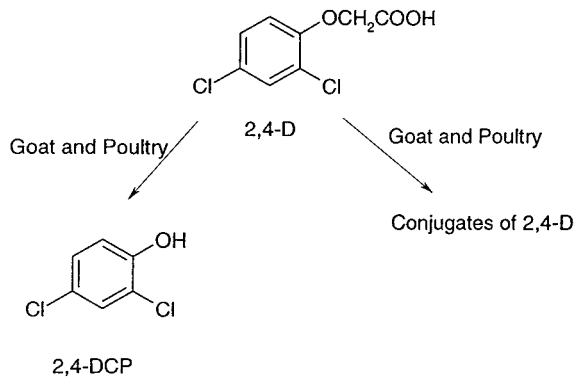

Figure 6. Metabolic pathway of 2,4-D administered to laying hens and lactating goat.

original [^{14}C]-2,4-D before isotopic dilution, therefore explaining the challenge of obtaining meaningful MS analysis of NP1 and NP3 when they were isolated from the tissues.

The metabolic fate of [^{14}C]-2,4-D administered orally to laying hens and a lactating goat resulted in either conjugation or metabolism to [^{14}C]-2,4-DCP (Figure 6). The metabolic fate of 2,4-D for the laying hen and the lactating goat is consistent with that described by Clark et al. (*3*) with the exception that in this work evidence for the formation of conjugates was clearly demonstrated.

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