Fate of Diflubenzuron in Cattle and Sheep

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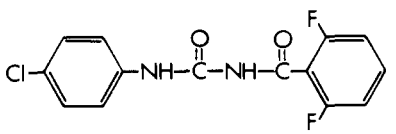
The fate of a radiolabeled preparation of the insect growth regulator diflubenzuron (Dimilin, TH-6040, *N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide) was determined after oral administration to a lactating cow and castrate male sheep, in vitro incubation with ovine and bovine digestive tract fluids, and dermal application to cattle. Orally administered diflubenzuron was absorbed, extensively metabolized, and almost totally excreted by cattle and sheep. Only very small amounts of carbon-14 were secreted into the milk of a cow fed [^{14}C]diflubenzuron. Studies with bile-duct cannulated sheep showed that bile is of greater importance than urine in the elimination of diflubenzuron metabolites from the body of ruminants. Major metabolites of diflubenzuron excreted by the cow and sheep resulted from hydroxylation on the difluorobenzoyl and chlorophenyl rings and by cleavage between the carbonyl and amide groups to give metabolites that were excreted either free or as conjugates. Diflubenzuron was not metabolized when incubated with digestive tract fluids from these ruminants. When applied dermally to cattle held outdoors in an unprotected pasture, diflubenzuron residues disappeared rapidly, but the compound was not chemically degraded or absorbed through the skin to any detectable degree. The major hydroxylated diflubenzuron metabolite in cow milk when fed to white rats was rapidly and quantitatively excreted with little further biotransformation.

The insect growth regulator diflubenzuron (Dimilin, TH-6040, *N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide) is very toxic to the larval stages of a



number of insect species and is highly selective due to its unique mode of action. Diflubenzuron reduces chitin deposition into insect cuticle, which disrupts normal moulting and development processes (Ishaaya and Casida, 1974; Mulder and Gijswijt, 1973; Post et al., 1974). The compound has shown excellent potential for controlling the larval stages of mosquitoes (Mulla et al., 1974; Schaeffer et al., 1975) and other Diptera (Miller, 1974; Miller et al., 1975; Wright, 1974; Wright and Harris, 1976; Wright and Spates, 1976), certain Lepidoptera (Granett and Dunbar, 1975; Tamaki and Turner, 1974), and Coleoptera (Moore and Taft, 1975; Neal, 1974).

The high degree of toxicity exhibited by diflubenzuron toward many destructive insects and its extremely low mammalian toxicity (Ferrell, 1977) indicate that the compound may be extensively used for insect control. A thorough evaluation of the environmental fate of diflubenzuron is therefore needed. Studies by Metcalf et al. (1975) showed that diflubenzuron was moderately stable in a model ecosystem, but the compound was not highly concentrated through food chains or by absorption from water. Diflubenzuron was not metabolized by the salt marsh caterpillar *Estigmene acrea* (Metcalf et al., 1975), the boll weevil *Anthonomous grandis* (Still and Leopold, 1975), or *Pieris brassicae* larvae (Verloop and Ferrell, 1977). Diflubenzuron was degraded only to a very limited extent by sheep liver microsomes (Metcalf et al., 1975), but was extensively metabolized after oral administration to laboratory rats (Verloop and Ferrell, 1977).

The investigations reported here were initiated in an effort to provide additional information on the fate of diflubenzuron in nontarget species. Radiolabeled diflubenzuron was orally or dermally administered to cattle and sheep for an evaluation of the compound's metabolic and residual behavior. In a companion report (Ivie and Wright, 1978), the fate of [^{14}C]diflubenzuron in the stablefly (*Stomoxys calcitrans*) and housefly (*Musca domestica*) is considered. Certain aspects of both of these studies have previously been reported in preliminary form (Ivie, 1977).

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MATERIALS AND METHODS

Chemicals. [^{14}C]Diflubenzuron was supplied by Thompson-Hayward Chemical Co., Kansas City, Kans. The radiochemical was either technical crystalline material or was formulated as a 25% wettable powder (WP, 2–5 μm particle size). Both samples were uniformly and essentially equally labeled in the two rings. The difluorobenzoyl ring contained 51.1% of the total radioactivity and the chlorophenyl ring 48.9%. The sp act. was 17.4 mCi/mM. The radiochemical purity, as determined by thin-layer chromatographic (TLC) analysis followed by radioautography and liquid scintillation counting (lsc) was ≥99.0% in both samples. Unlabeled preparations of diflubenzuron and certain compounds considered as likely degradation products were also supplied by Thompson-Hayward. These compounds included 4-chlorophenylurea, 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 4-chloroaniline, *N*-[[(4-chloro-2-hydroxyphenyl)amino]carbonyl]-2,6-difluorobenzamide, and *N*-[[(4-chloro-3-hydroxyphenyl)amino]carbonyl]-2,6-difluorobenzamide. The two hydroxylated diflubenzuron analogues were designated 4-chloro-2-hydroxydiflubenzuron and 4-chloro-3-hydroxydiflubenzuron. In addition, the followng compounds were obtained commercially: 4-chloroacetanilide and 4-chlorophenol (Aldrich, Milwaukee, Wis.), 4-chloro-*N*-methylaniline (Calbiochem, San Diego, Calif.), and 4-chloro-*N*,*N*-dimethylaniline (Adams Chemical Co., Round Lake, Ill.).

Lactating Cow. A 360-kg lactating Jersey cow obtained from a local dairy was placed in a metabolism stall and catheterized (Foley retention catheter, size 28FR) to allow separate collection of urine and feces. The animal was provided water and coastal bermudagrass hay ad libitum and was fed a commercial dairy ration twice daily. Although milk production had averaged about 10 kg daily, production dropped to about 7 kg daily during the course of the study. This drop was probably caused by stress on the animal as a result of unfamiliar surroundings, constant retention within the stanchion, and catheterization.

For treatment, the [^{14}C]diflubenzuron 25 WP formulation (in water) was diluted with unlabeled diflubenzuron

25 WP such that the final treatment mixture contained 3.6 g of diflubenzuron active ingredient and a total of 0.65 mCi of radiocarbon, or a sp act. of 400 dpm/μg of diflubenzuron. The treatment mixture, as a slurry in 500 mL of water, was administered as a single oral dose to the animal via a stomach tube and was equivalent to 10 mg of diflubenzuron/kg of body weight.

After treatment, total urine and feces samples were collected at 24-h intervals, and the animal was machine milked every 12 h. Aliquots of fresh milk and urine (0.2 mL) were assayed for radiocarbon by lsc. Parts of the remaining samples were frozen for later analysis. Feces samples were mixed thoroughly, and small parts (0.5–1.0 g) were removed and air-dried for quantitation of radiocarbon residues by oxygen combustion and lsc. Parts of the remaining samples were frozen for subsequent study. Seven days after treatment, the cow was killed, and numerous tissue samples were collected and frozen after small samples (<1 g) were retained for combustion analysis.

Sheep. Four mixed breed castrate male sheep (28–42 kg) from a flock maintained at this laboratory were held in metabolism stanchions that allowed separate collection of urine and feces without catheterization. For measurement of the elimination of carbon-14 in the bile, the common bile duct of two sheep was surgically cannulated at least 7 days before [^{14}C]diflubenzuron treatment. One cannulated and one intact sheep were treated orally with [^{14}C]diflubenzuron 25 WP by the same procedure used to treat the cow. The treatment level was 10 mg of diflubenzuron/kg of body weight; the sp act. of the preparation was 570 dpm/μg of diflubenzuron. For easier isolation of larger quantities of [^{14}C]diflubenzuron metabolites, the other two sheep (one bile cannulated and one intact) were treated orally with [^{14}C]diflubenzuron 25 WP at 500 mg/kg (sp act. 10 dpm/μg).

Total urine, bile, and feces samples were collected at 24-h intervals after treatment. Aliquots of whole urine and bile were radioassayed by lsc, and feces samples were soaked several hours in water and then slurried, and parts were analyzed by combustion analysis. The rest of all samples was frozen for later analysis. After 4 days, the two sheep treated at 10 mg/kg were killed, and tissue samples were collected for combustion analysis.

Ruminant Digestive Tract Fluids. Two pairs of mixed breed ewes (approximately 40 kg each) were housed outdoors in small pens. One pair was fed a high-energy sheep ration (Ivie et al., 1974) plus alfalfa hay. The other pair was fed an all roughage diet consisting solely of Coastal bermudagrass hay. Both pairs of sheep were provided water and mineral supplements free choice.

After 4 weeks on their respective diets, the sheep were killed and fluids from the rumen, abomasum, and small intestine were collected from each animal by squeezing the gut contents through several layers of cheesecloth. The like fluids from the two sheep in each pair were pooled, and these fluids were quickly pipetted into 40-mL screw cap centrifuge tubes containing 3.1 μg deposits of technical grade [^{14}C]diflubenzuron (obtained by evaporation of an acetone solution of the radiochemical). The amount of digestive tract fluids added to each tube (10 mL) was such that the [^{14}C]diflubenzuron concentration was 1×10^{-6} M. The tubes were quickly flushed with carbon dioxide, tightly capped, and then incubated for 24 h in a shaking water bath at 39–40 °C. Controls were also run in which the enzymatic activity of the fluids had been previously destroyed by boiling for 30 min.

Additional studies with ovine digestive tract fluids were conducted in an identical manner, except that the sheep were from grazing flocks maintained at this laboratory and received no special diet. In addition, [^{14}C]diflubenzuron was incubated with rumen fluid obtained by stomach tube from a 450-kg steer. The incubation and analysis procedures were identical with those used for sheep.

Dermal Studies. In studies designed to evaluate the persistence of diflubenzuron on the hair and skin of cattle under environmental conditions, three Black Angus steers (350–400 kg each) were stanchioned and 20 × 20 cm areas were marked off on each side of the animals near the top of the back just behind the shoulders. The hair immediately surrounding the treatment area was closely clipped, but hair on the treatment area itself was not disturbed. The 400 cm^2 areas were then treated with either a 1% (active ingredient) water suspension of diflubenzuron 25 WP formulation or a 1% light mineral oil (Lubinol, Purepac Corp., Elizabeth, N.J.) suspension of an oil-based, water-immiscible diflubenzuron formulation. The diflubenzuron in both formulations was 2–5 μm particle size, and both were fortified with [^{14}C]diflubenzuron 25 WP as a tracer. Specific activity of the diflubenzuron in each treatment solution was 700 dpm/μg. The [^{14}C]diflubenzuron was applied with a small hand pump sprayer (such as those used with household cleaners, etc.) that was adjusted to emit a solid stream to avoid mist loss. The areas were treated to saturation but not runoff, and the liquid was worked into the hair by rubbing with the sprayer head. The amount of radiocarbon applied to each of the treatment areas was determined by weigh-back of the treatment solution. One of the treatment areas of each steer was treated with the WP formulation and the other area was treated with the oil-based formulation. In each case, the applied [^{14}C]diflubenzuron was equivalent to a level of about 0.125 mg/cm^2 of hide. The total volume of liquid applied to the 400 cm^2 treatment areas was approximately 5 mL in each case. After treatment, the animals were transferred to a 2-ha pasture that was not protected from the elements other than by afternoon shading. At 1, 2, and 4 weeks, one animal was killed, and the treated skin patches were removed for analysis of residual radiocarbon. In addition, a number of tissue samples were taken from each animal for combustion analysis.

In a second study, designed to determine whether diflubenzuron is absorbed through the skin after dermal exposure, a 525-kg cow was stanchioned indoors, catheterized, and two 20 × 20 cm areas were marked off and treated as before, except that both treatment areas were treated with the [^{14}C]diflubenzuron 25 WP formulation. During the first 3 days after treatment, total urine and feces samples were collected at 24-h intervals and were analyzed for radiocarbon residues. At the end of this time, the cow was not killed, but the treated areas were clipped closely and the exposed skin was cleaned thoroughly with tissue paper moistened with acetone. The radiocarbon on the hair and paper was then quantitated, extracted, and analyzed by TLC.

Extraction and Analysis. Urine and bile samples (up to 20 mL) were adjusted to pH 2.0 with 1 N HCl, then were extracted four times with equal volumes of ethyl acetate. Centrifugation was used where required to break emulsions. Aliquots of both the aqueous and the organic phases were assayed for radiocarbon content by lsc, then the organic phase was dried over sodium sulfate and concentrated under reduced pressure and finally by a gentle stream of nitrogen for TLC analysis. In some samples, radiocarbon remaining in the extracted aqueous phase of the urine or bile was subjected to enzyme hydrolysis in

attempts to cleave possible conjugates. After adjustment of the extracted urine or bile to pH 4.5, 5-mL aliquots were incubated with β-glucuronidase–arylsulfatase (from *Helix pomatia*, Calbiochem, San Diego, Calif.). The enzyme activity in each sample was equivalent to about 50 000 Fishman units and ≤25 000 Whitehead units. In an effort to determine the extent of nonenzymatic conversion to organic extractable products, samples were also run in which the enzyme activity was first destroyed by heat (100 °C, 20 min). After incubation of the samples at 37–38 °C for 24 h, they were adjusted to pH 2.0 and extracted four times with ethyl acetate. The extracts were then concentrated for TLC analysis.

Whole milk (100 mL) was acidified to pH 2.0 and extracted with ethyl acetate as was the urine and bile. The combined ethyl acetate extracts were stripped of solvent, and the oily residue was then partitioned between acetonitrile and hexane. The hexane phase was back-extracted once with acetonitrile and the combined acetonitrile fractions were concentrated for TLC.

Feces samples (10 g) were briefly homogenized in 10 mL of water with a Willems Polytron homogenizer and were then adjusted to pH 2.0. They were subsequently extracted four times with 20-mL volumes of ethyl acetate by Polytron homogenization and then centrifuged to break the resulting emulsions. The combined organic phase was then analyzed by TLC. Radiocarbon remaining in the water–residue slurry was quantitated by oxygen combustion.

Digestive tract fluids were acidified to pH 2.0 and extracted five times with equal volumes of ethyl acetate. The extracts were then analyzed by TLC. Hide samples from the dermal studies were rinsed thoroughly with acetone, and the acetone rinses were analyzed by TLC. In some samples, hexane–acetonitrile partitions were required to remove interfering oils from the hide rinses. In such samples, only very low levels of radiocarbon partitioned into the hexane–oil fraction, and this carbon-14 fraction was not further studied.

The radiocarbon content of tissues, feces, hair, and skin was determined by combustion under 1 atm of oxygen. The combustion gases were bubbled through a carbon dioxide trap solution consisting of equal parts of 2-aminoethanol and 2-methoxyethanol. The trap solution was then assayed for radiocarbon by lsc. Appropriate corrections were made for combustion efficiency and quench.

Metabolite Resolution and Identification. Diflubenzuron metabolites or degradation products in extracts of milk, urine, bile, feces, digestive tract fluids, and hide were resolved by two-dimensional TLC. The extracts were applied as a spot or short band to one corner of 20 × 20 cm precoated silica gel chromatoplates (0.25 mm gel thickness, with fluorescent indicator, Brinkman Silplate F-22). The plates were developed in benzene–ether (5:1) in the first direction, then in hexane–ethyl acetate–methanol (2:2:1) in the second direction (solvent systems 1 and 2, respectively). The plates were exposed to Kodak No Screen Medical x-ray film for 4–7 days, then the radioactive areas of the plates were scraped and quantitated by lsc. Metabolite characterizations were made by comparing TLC behavior of radioactive components in the extracts with the available unlabeled compounds of known structure. The metabolite standards were spotted on TLC plates as a mixture with the ^{14}C metabolites, and after two-dimensional development, the plates were analyzed for coincidence of metabolites and standards. The standards were visualized by viewing the plates under short-wavelength ultraviolet light. Coincidence of standard and metabolite constituted tentative metabolite characterization. Where cochromatography with unlabeled standards was the only method of metabolite characterization, the identifications were confirmed in four additional solvent systems. These were benzene–ethanol (9:1), benzene–ethanol–acetic acid (93:7:2), chloroform–ethanol–acetic acid (85:10:5), and benzene–dioxane–acetic acid (90:30:1).

Preparative TLC (2.0 mm silica gel precoated plates, Brinkman) or preparative GLC was used to isolate metabolites in sufficient quantity for spectral studies. The chromatograph was a Varian Model 2800 gas chromatograph with a thermal conductivity detector and fitted with a 10 mm (i.d.) × 2.1 m aluminum column packed with 3% SE 30 on Chromosorb W. The column oven temperature was 170 °C, and the helium flow rate was 150 mL/min. Components eluting from the column were trapped by condensation in glass capillary tubes.

Electron impact mass spectral analyses (70 eV) were accomplished using a Varian-MAT CH-7 magnetic scan spectrometer. Samples were analyzed either by direct insertion probe analysis or by GLC–mass spectrometry. The 2 mm × 1.8 m glass column was packed with 3% SE 30 on Varaport 30. Helium flow through the column was maintained at 50 mL/min. When quantities of purified metabolites were large enough, the products were also examined by nuclear magnetic resonance (NMR) spectroscopy. The NMR studies were conducted in deuterated acetone, Me_2SO, or chloroform, mostly on a JEOL Model JNM-MH 100 instrument, but some were done in the Varian NMR Applications Laboratory (Palo Alto, Calif.) using a 300-mHz superconducting NMR system. Chemical shifts are reported as parts per million downfield from tetramethylsilane.

Fate of *N*-[[(4-Chlorophenyl)amino]carbonyl]-2,6-difluoro-3-hydroxybenzamide in White Rats. The major hydroxylated metabolite in the milk of the [^{14}C]-diflubenzuron-treated cow was isolated from the cow urine and feces by preparative TLC. The metabolite, dissolved in Me_2SO, was then administered to two lightly etherized 275-g adult female Sprague-Dawley rats by stomach tube. Each rat received 4.1 mg of the ^{14}C metabolite, a dose equivalent to 15 mg/kilogram of body weight. The treated rats were held in a common metabolism cage and total urine and feces samples were collected daily for 3 days after treatment. The rats were then killed, and tissue samples were analyzed for radiocarbon content by oxygen combustion. Radiocarbon excreted through the urine and feces of the rats was quantitated by lsc and combustion analysis. The nature of the excreted radioactivity was investigated by two-dimensional TLC of urine and feces extracts.

RESULTS

Radiocarbon Excretion and Tissue Residues. Radioactive residues were rapidly excreted by the cow and sheep after oral treatment with [^{14}C]diflubenzuron (Tables I–III). The cow eliminated about 85% of the administered ^{14}C in the feces and about 15% in urine during the 7-day posttreatment period. Sheep treated with [^{14}C]diflubenzuron at 10 mg/kg eliminated a considerably larger proportion of the ^{14}C in the urine than did the cow, but the bile was the major route of radiocarbon elimination from the bile-duct cannulated sheep (Table II). Sheep treated with 500 mg/kg of [^{14}C]diflubenzuron as a single oral dose eliminated a much smaller proportion of the ^{14}C in urine and bile (Table III), which was probably due to reduced absorption from the gastrointestinal tract when given at this exaggerated dose level.

Table I. Radiocarbon Elimination by a Lactating Cow after Oral Treatment with [^{14}C]Diflubenzuron (10 mg/kg of body weight)

Days after treatment	Radiocarbon eliminated (cumulative % of dose)		
	Milk	Urine	Feces
1	0.1	4.9	16.9
2	0.2	12.5	53.9
3	0.2	15.1	76.8
4	0.2	16.0	85.2
5	0.2	16.3	86.9
6	0.2	16.4	87.3
7	0.2	16.4	87.7

Table II. Radiocarbon Elimination by Sheep after Oral Treatment with [^{14}C]Diflubenzuron (10 mg/kg of body weight)

Days after treatment	Radiocarbon eliminated (cumulative % of dose)		
	Urine	Bile	Feces
	Bile-Duct Cannulated Sheep		
1	15.1	22.2	10.4
2	21.5	33.3	30.6
3	23.1	35.3	31.8
4	23.8	36.4	32.2
	Intact Sheep		
1	19.4		12.8
2	35.9		29.6
3	40.4		40.5
4	41.1		42.4

Table III. Radiocarbon Elimination by Sheep after Oral Treatment with [^{14}C]Diflubenzuron (500 mg/kg of body weight)

Days after treatment	Radiocarbon eliminated (cumulative % of dose)		
	Urine	Bile	Feces
	Bile-Duct Cannulated Sheep		
1	2.3	2.8	
2	4.4	4.5	
3	6.1	5.1	
4	7.1	5.2	73.8[a]
	Intact Sheep		
1	4.4		
2	7.9		
3	9.4		
4	9.8		78.7[a]

[a] Combined 1-4 day feces sample.

Only 0.2% of the ^{14}C activity given the cow was secreted into the milk (Table I). Maximum milk residues of 0.8 ppm of diflubenzuron equivalents were observed in the milk samples collected 24 h after dosing, but radiocarbon in milk had dropped to undetectable levels (<0.1 ppm diflubenzuron equivalents) by 3 days after treatment. There was little tendency toward retention of ^{14}C in the body tissues of the cow or sheep. Analysis of tissue samples collected 7 days (cow) and 4 days (sheep) after treatment revealed that only the liver contained appreciable radiocarbon residues, ranging from 2.3–3.6 ppm diflubenzuron equivalents (Table IV). Kidney samples from the bile-duct cannulated sheep contained low levels of radiocarbon, but the kidney from the cow and intact sheep did not have detectable residues. Because such a large proportion of the dose was voided in the feces of the cow, the residues detected in the skin of the cow (Table IV) might have been a result of surface contamination that occurred while the animal lay down in the stanchion. Tissues from the two sheep treated with 500 mg of [^{14}C]diflubenzuron/kg of body weight were not analyzed.

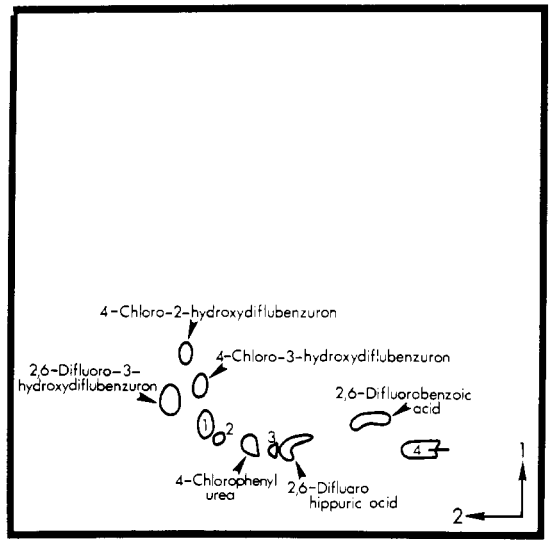

Figure 1. Representation of the TLC behavior of metabolites excreted in urine of sheep and a lactating cow after oral treatment with [^{14}C]diflubenzuron. Metabolites designated by numbers are uncharacterized products. Plates were developed in benzene–ether (5:1) in the first direction followed by hexane–ethyl acetate–methanol (2:2:1) in the second direction.

Metabolite Resolution and Characterization. *Urine.* Most of the radiocarbon in acidified samples of cow and sheep urine was extracted into ethyl acetate. Two-dimensional TLC of the urine extracts resolved eight radioactive fractions from cow urine and nine from sheep urine (Figure 1, Table V). Six of the urine metabolites were characterized by TLC studies or by spectral analysis as will be discussed later. The major metabolites in the urine of sheep were 2,6-difluorobenzoic acid and its glycine conjugate, 2,6-difluorohippuric acid. These two metabolites accounted for almost one-half of the ^{14}C in the sheep urine (Table V). The benzoic acid and hippuric acid metabolites were also present in significant quantity in the urine of the cow, but the major metabolite in cow urine resulted from hydroxylation at the 3 position of the 2,6-difluorobenzoyl ring. This metabolite, designated 2,6-

Table IV. Radiocarbon Residues in Tissues of Sheep and a Lactating Cow after Treatment with [^{14}C]Diflubenzuron (10 mg/kg of body weight)

Animal[b]	ppm diflubenzuron equivalents[a]					
	Brain	Liver	Kidney	Muscle	Fat[c]	Skin
Cow	<0.1	2.9	<0.1	<0.1	<0.4	0.8
Sheep, bile-duct cannulated	<0.05	3.60	0.40	<0.05	<0.2	
Sheep, intact	<0.05	2.30	<0.05	<0.05	<0.2	

[a] The following tissues from the cow contained <0.1 ppm diflubenzuron equivalents: heart, tongue, pancreas, spleen, lung, udder, adrenal, ovary, pituitary, abomasum, omasum, small intestine, large intestine, rumen, and gall bladder. [b] Tissues collected 7 days (cow) and 4 days (sheep) after treatment. [c] Smaller samples analyzed by oxygen combustion account for reduced sensitivity limits.

Table V. Metabolites in Urine from Sheep and a Lactating Cow after Oral Treatment with [^{14}C]Diflubenzuron (10 mg/kg of body weight)[a]

Metabolite	Percent		
	Sheep, bile-duct cannulated	Sheep, intact	Cow
2,6-Difluoro-3-hydroxydiflubenzuron[b]	1.2	1.4	45.0
4-Chloro-2-hydroxydiflubenzuron[b]	0.3	0.2	1.6
4-Chloro-3-hydroxydiflubenzuron[b]	0.4	0	3.7
4-Chlorophenylurea	0	0	0.6
2,6-Difluorobenzoic acid	15.1	26.7	6.0
2,6-Difluorohippuric acid	30.2	22.3	6.9
Unknown 1	2.6	2.1	5.6
Unknown 2	0.2	0.3	0
Unknown 3	0.4	0.4	0
Unknown 4	20.7	12.7	13.0
Water soluble	28.9	33.9	17.6

[a] Samples collected 1 day after treatment. [b] Trivial names for *N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluoro-3-hydroxybenzamide; *N*-[[(4-chloro-2-hydroxyphenyl)amino]carbonyl]2,6-difluorobenzamide; and *N*-[[(4-chloro-3-hydroxyphenyl)amino]carbonyl]-2,6-difluorobenzamide.

Table VI. Metabolites in Feces from Sheep and a Lactating Cow after Oral Treatment with [^{14}C]Diflubenzuron (10 mg/kg of body weight)[a]

Metabolite	Percent		
	Sheep bile-duct cannulated	Sheep, intact	Cow
Diflubenzuron	97.7	40.0	42.5
2,6-Difluoro-3-hydroxydiflubenzuron[b]	0	0.4	17.6
4-Chloro-2-hydroxydiflubenzuron[b]	0	0.8	0.6
4-Chloro-3-hydroxydiflubenzuron[b]	0	0.4	0.7
Unknown 1[c]	0	1.2	1.1
Unknown 5[d]	0	4.8	5.3
Unknown 6[d]	0	0	2.1
Unknown 7[d]	0	0	0.8
Unextractable	2.3	52.4	29.3

[a] Samples collected 2 days after treatment. [b] See Table V, footnote *b*, for full chemical names. [c] Chromatographically the same as unknown 1 from urine (Figure 1). [d] TLC R_f values in solvent systems 1 and 2, respectively, are as follows: unknown 5 (0.0, 0.0–0.45 streak), unknown 6 (0.10, 0.0), unknown 7 (0.13, 0.45).

difluoro-3-hydroxydiflubenzuron, accounted for 45% of the total radiocarbon in the 24-h sample of cow urine (Table V). Metabolites hydroxylated on the 4-chlorophenyl ring, 4-chloro-2-hydroxydiflubenzuron and 4-chloro-3-hydroxydiflubenzuron, were seen in extracts of urine from the sheep and cow, and very low levels of 4-chlorophenylurea were seen in the urine of the cow but not of the sheep (Table V). No trace of unmetabolized diflubenzuron was seen in any urine sample. Unknown 1 in urine (Figure 1) cochromatographed with 4-chloroacetanilide in solvent systems 1 and 2, but additional TLC studies indicated that these products were not the same.

Table V shows metabolite distribution in only those urine samples collected 1 day after [^{14}C]diflubenzuron treatment, but the metabolites in the 2- and 3-day samples were qualitatively almost identical with those indicated in Table V. In sheep urine, the relative distribution of metabolites did not change appreciably with time after treatment, but in cow urine, the tendency was toward greater quantities of more polar metabolites with time after dosing. Thus, 3 days after treatment, 2,6-difluoro-3-hydroxydiflubenzuron comprised only 20% of the urine radiocarbon, but unknown 4 (origin, Figure 1) contained almost one-half of the radiocarbon in the sample. The percentage of radiocarbon remaining in the aqueous phase after ethyl acetate extraction of both cow and sheep urine did not change appreciably among samples and ranged between 25–34% for sheep urine and 15–18% for cow urine.

Incubation of water-soluble metabolites in cow and sheep urine with β-glucuronidase–arylsulfatase indicated that appreciable quantities of these products were conjugates. Ethyl acetate extraction of these samples after incubation with active enzymes resulted in partitioning of 52–70% of the radioactivity into the organic phase upon extraction. Analysis of similar samples incubated with boiled enzyme preparations showed only 0–23% conversion of the radioactivity into ethyl acetate extractable products, indicating that about half of the urine water-soluble radiocarbon was in the form of glucuronide or sulfate conjugates. It may be that the extent of conjugation of diflubenzuron metabolites was even greater than that indicated by the enzyme studies because sulfate ester conjugates, if present, may have been hydrolyzed under the acidic conditions of the original ethyl acetate extraction of acidified whole urine (Roy and Trudinger, 1970). TLC examination of the enzyme hydrolysis products did little to reveal their identity. As many as seven components were observed in the extracts of the enzyme-treated urine water solubles from cow and sheep, but only one was tentatively identified by two-dimensional TLC. This product, 2,6-difluoro-3-hydroxydiflubenzuron, was a minor component (<5%) in enzyme-treated sheep urine samples but was not seen in the enzyme-treated cow urine samples.

Feces. TLC analysis showed that eight radioactive components were present in extracts of cow feces, six in the feces of intact sheep, and only one in the feces of the bile-duct cannulated sheep (Table VI). The major radioactive component in all feces extracts was identified as unmetabolized diflubenzuron. Extracts from cow feces contained appreciable quantities of 2,6-difluoro-3-hydroxydiflubenzuron, but feces samples from the intact sheep contained only trace quantities of this metabolite (Table VI). Feces from both the cow and intact sheep

contained very low amounts of 4-chloro-2-hydroxydiflubenzuron and its 3-hydroxy isomer. Unextractable radiocarbon accounted for about 30% of the total radioactivity in cow feces, about 52% of the total in feces from the intact sheep, but only about 2% of the total in feces from the bile cannulated sheep (Table VI). TLC revealed that extracts of feces from the bile cannulated animals contained only one radioactive component—unmetabolized diflubenzuron.

The water–residue slurry remaining after ethyl acetate extraction of cow and sheep feces samples contained as much as one-half of the sample radiocarbon (Table VI). Soxhlet extraction of these fractions with methanol (after lyophilization) or boiling in 1 N HCl and subsequent extraction with ethyl acetate recovered as much as one-fourth of the radioactivity present. However, TLC analysis of these extracts did not result in satisfactory resolution of the metabolites because of the low levels of radioactivity in the samples and high levels of interfering materials that caused the TLC plates to streak badly.

Bile. More than one-third of the ^{14}C given as an oral 10 mg/kg of [^{14}C]diflubenzuron dose appeared in the bile of a bile-duct cannulated sheep, and TLC studies showed the presence of at least eight radioactive components in the bile extracts. Three of these were identified as the same hydroxydiflubenzuron isomers seen in the urine and feces of the cow and sheep, but these metabolites collectively comprised <5% of the total radiocarbon in the bile. Most of the biliary radiocarbon was quite polar—from 30–50% was not extracted from the aqueous phase, and of the radiocarbon that was extracted, >80% remained at or streaked from the origin. Incubation of the bile water-soluble metabolites with β-glucuronidase–arylsulfatase converted 40–50% of the ^{14}C into organic extractable products. Two-dimensional TLC resolved the extracts into at least eight radioactive components, none of which were identified by TLC comparisons with the available compounds of known structure.

Milk. Only very low radiocarbon residues appeared in the milk of a cow after [^{14}C]diflubenzuron treatment, but >80% of this was extracted with ethyl acetate. Seven radioactive components in the ethyl acetate extract were resolved by two-dimensional TLC in solvent systems 1 and 2 (Figure 2). The major radioactive component of the milk extract was chromatographically identical with diflubenzuron, and the two major metabolites were identified as 2,6-difluorobenzamide and 2,6-difluoro-3-hydroxydiflubenzuron. The milk metabolite with the lowest R_f in solvent systems 1 and 2 (Figure 2) appeared to be 2,6-difluorohippuric acid. However, confirmatory TLC studies in the four additional solvent systems revealed that this fraction was a mixture of the hippuric acid, and an unidentified compound (Figure 2). It may be that unknown 8 in the milk is 4-chlorophenylurea, but cochromatography studies were not conclusive due to the exceedingly low levels of this metabolite. Less that 20% of the radiocarbon in the milk remained in the aqueous phase after solvent extraction, and the amounts were too low to allow further study.

Spectral Evidence for Metabolite Identification. Of the eight radioactive components identified in the excreta of the [^{14}C]diflubenzuron-treated cow and sheep, four were identified on the basis of both cochromatography (TLC) with authentic standards and spectral analysis. Two were identified by spectral evidence alone because standards were not available, and two were identified only by cochromatography with the known compounds. 4-Chlorophenylurea was detected only in cow urine and 2,6-difluorobenzamide only in cow milk, and both metabolites were present in amounts too low to permit spectral analysis. Metabolites isolated in sufficient quantities for spectral studies were obtained by large-scale extraction and preparative isolation procedures either from the cow urine or feces, or from the excreta of sheep treated with [^{14}C]diflubenzuron at 500 mg/kg.

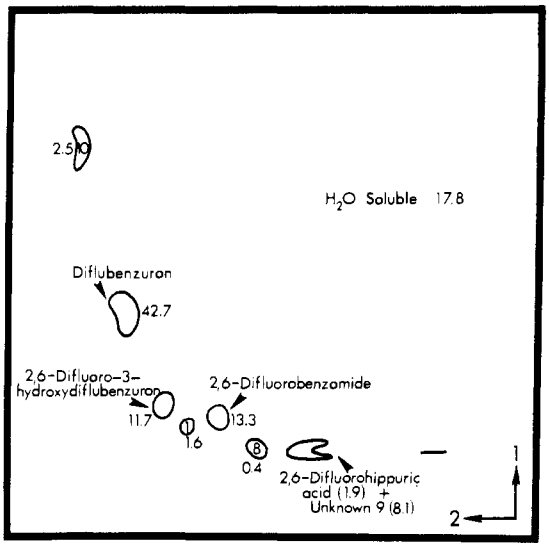

Figure 2. The TLC separation of metabolites in milk of a lactating cow after oral treatment with [^{14}C]diflubenzuron at 10 mg/kg of body weight. Numbers outside the circles indicate the percentage of the total radiocarbon in the sample as indicated metabolites. Numbers inside the circles designate uncharacterized metabolites. The plate was developed in benzene–ether (5:1) in the first direction followed by hexane–ethyl acetate–methanol (2:2:1) in the second direction.

Diflubenzuron. The unmetabolized parent compound appeared in milk and feces but not in urine of the treated animals. There were insufficient quantities in milk for spectral studies, but diflubenzuron was isolated in milligram amounts by preparative TLC from the feces of both cow and sheep. The isolated diflubenzuron (crystallized once from boiling ether) gave identical mass spectral and NMR data as did authentic diflubenzuron. The mass spectrum of diflubenzuron shows diagnostic ions at m/e 310 (M^+) 153 (chlorophenyl isocyanate) and the base peak at 141 (difluorobenzoyl).

2,6-Difluoro-3-hydroxydiflubenzuron. This metabolite was isolated by preparative TLC in milligram quantities from cow urine and feces and sheep bile. The product was crystallized from ether–hexane and analyzed by direct insertion probe mass spectrometry. The molecular ion at m/e 326 indicated that the metabolite was a hydroxylated diflubenzuron derivative. Strong ions at m/e 157 (difluorohydroxybenzoyl) and 153 (base peak, chlorophenyl isocyanate) and the absence of the difluorobenzoyl ion at m/e 141 placed the hydroxyl group in the difluorobenzoyl ring. High-resolution NMR (Me_2SO-d_6) gave conclusive evidence that the hydroxylation was at the 3 position of the 2,6-difluorobenzoyl ring, and thus that the metabolite was *N*-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluoro-3-hydroxybenzamide. The high-resolution NMR spectra of the metabolite and of diflubenzuron are shown in Figure 3. In the spectrum of diflubenzuron, the AA′ protons of the chlorophenyl ring overlap the triplet of triplets due to H_D being coupled to the two H_C protons (J = 8.5 Hz) and then to the two ^{19}F nuclei (J = 6.5 Hz). The H_C protons are coupled about equally to the adjacent ^{19}F nuclei and the H_D proton and quite weakly to the other H_C and the ^{19}F in the para position, which leads to the simple triplet

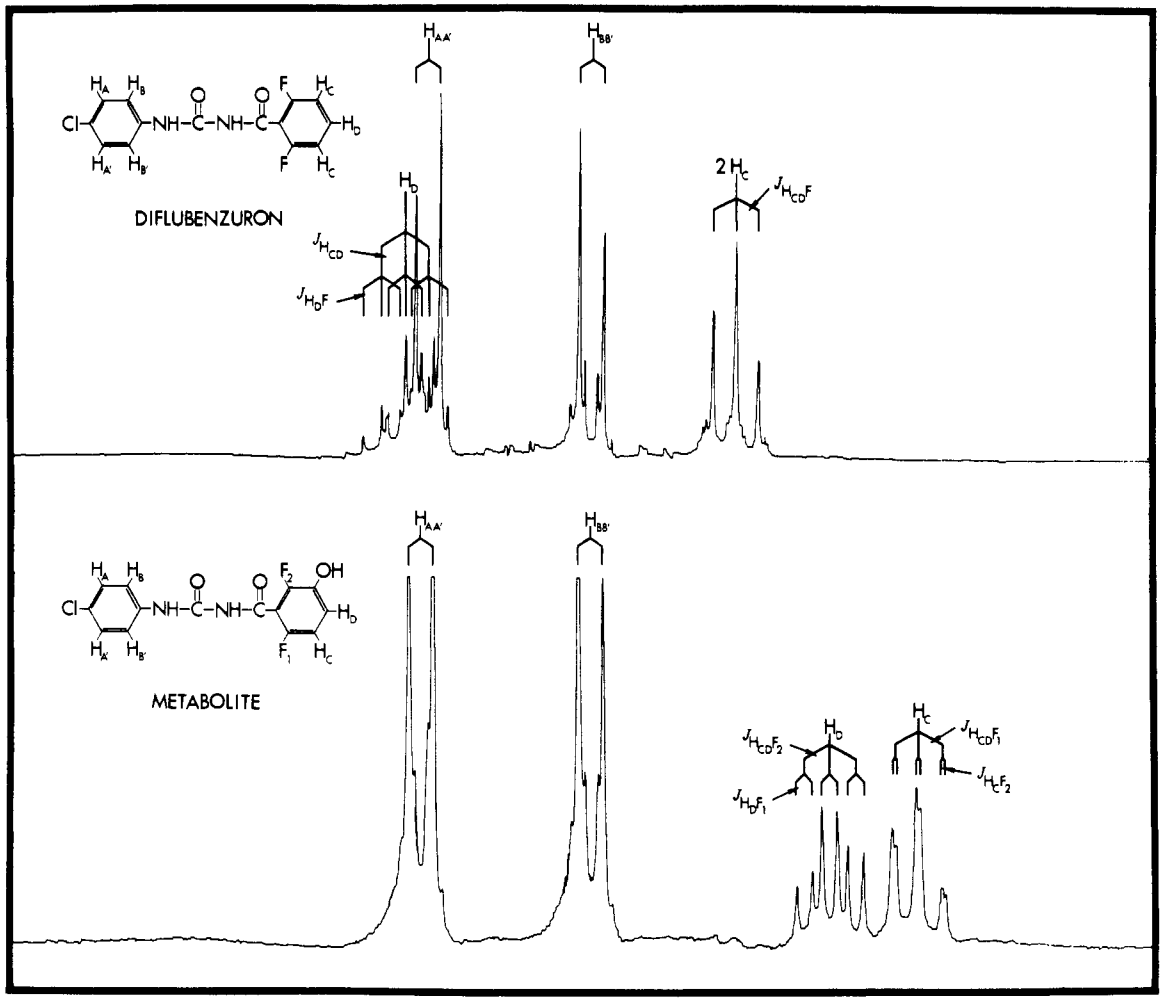

Figure 3. High-resolution NMR of the aromatic protons of diflubenzuron and its major hydroxylated metabolite in the cow and sheep. Spectra recorded in Me_2SO-d_6 at 75 °C to reduce viscosity of the solvent and minimize hydrogen bonding in the metabolite.

observed (J = 8.5 Hz). In the metabolite, the hydroxyl group shifts the aromatic proton resonances upfield and increases the coupling of H_D to $^{19}F_2$. H_D is coupled about equally to H_C and $^{19}F_2$, leading to a triplet (J = 9.0 Hz) which is split into a triplet of doublets by coupling with $^{19}F_1$ (J = 5.5 Hz). H_C is coupled about equally to the adjacent $^{19}F_1$ and the H_D proton, leading to a triplet (J = 9.0 Hz) which in this case is split by small coupling to the p-$^{19}F_2$ (J = 1.5 Hz).

4-Chloro-2-hydroxy- or 4-Chloro-3-hydroxydiflubenzuron. These two metabolites were isolated by preparative TLC from sheep bile and were crystallized from ether–hexane. Their identities as indicated by TLC with the authentic standards were confirmed by direct insertion probe mass spectral analysis. The mass spectra of the two isomers were essentially identical, with major ions at m/e 326 (M^+), 169 (chlorohydroxyphenyl isocyanate), 158 (protonated difluorobenzamide), 157 (difluorobenzamide), 141 (base peak, difluorobenzoyl), and 113 (difluorophenyl). Neither of these metabolites was obtained in sufficient quantity for NMR studies.

2,6-Difluorobenzoic Acid. This metabolite, tentatively identified by TLC cochromatography with an authentic standard, was isolated from cow and sheep urine in sufficient quantities for confirmation of structure by GLC–mass spectrometry. The metabolite was reacted with diazomethane to generate its methyl ester, then was injected onto a column at 100 °C. The eluting component gave retention time and mass spectrum identical with those of authentic methyl 2,6-difluorobenzoate. Major ions were at m/e 172 (M^+), 141 (base peak, difluorobenzoyl), and 113 (difluorophenyl).

2,6-Difluorohippuric Acid. Milligram quantities of this metabolite were isolated from sheep urine by preparative TLC, but all efforts at crystallization were unsuccessful. Efforts to analyze the metabolite by GLC–mass spectrometry were likewise unsuccessful, but the compound reacted with diazomethane to give a single product which passed through the gas chromatograph (175 °C column, retention time 2.1 min). Examination of the mass spectrum of the methylated metabolite indicated it was the glycine conjugate of 2,6-difluorobenzoic acid. Major ions were as follows: m/e 229 (M^+), 197 (M – OCH_3, H), 170 (M – CO_2CH_3), 141 (base peak, difluorobenzoyl), and 113 (difluorophenyl).

About 10 mg of the isolated metabolite was subsequently methylated and purified by preparative GLC. Analysis by NMR confirmed the metabolite as 2,6-difluorohippuric acid. NMR signals ($CDCl_3$) at 3.82 (s, 3 H, OCH_3), 4.27–4.32 (d, 2H, $NHCH_2CO_2CH_3$), 6.50–6.90 (br, 1 H, NH), 6.94–7.10 (t, 2 H, m-difluorobenzoyl), and 7.30–7.60 (m, 1 H, p-difluorobenzoyl).

Attempted GLC–mass spectral analysis of additional metabolites from cow and sheep excreta was not successful. Preparative isolation procedures likewise failed to provide any of the uncharacterized metabolites in sufficient quantity or purity for definitive spectral analysis.

Stability of Diflubenzuron in Digestive Tract Fluids. [^{14}C]Diflubenzuron was not degraded to any significant extent when incubated in vitro with digestive

tract fluids of sheep and cattle. Radiocarbon in these samples was readily extractable (>99% partitioned into ethyl acetate in all samples), and TLC showed that unaltered diflubenzuron comprised ≥99% of the radiocarbon in all extracts. A very minor radioactive component of lower TLC R_f than diflubenzuron was detected in the extracts of certain samples of sheep digestive tract fluids, but attempts to reproduce these findings using fluids obtained from additional sheep were not successful. This product was also detected in some incubations in which the fluids had been previously boiled; thus, the unidentified compound likely arose through nonenzymatic reactions.

Fate of 2,6-Difluoro-3-hydroxydiflubenzuron in Rats. The major hydroxylated diflubenzuron metabolite occurring in milk was rapidly excreted after oral administration to laboratory rats. About 23% of the administered ^{14}C was excreted in the urine within 3 days after treatment, and >85% of the urinary excretion occurred during the first day after treatment. Seventy-one percent of the ^{14}C was eliminated through the feces of the treated rats. Radiocarbon elimination in the feces was almost complete within 2 days after treatment, and less than 2% of the administered ^{14}C was recovered in the 3-day feces sample.

Analysis of tissues collected when the animals were killed 3 days after treatment confirmed that excretion of radiocarbon was essentially complete. Samples of brain, liver, kidney, muscle, and omental fat from each animal contained no detectable radiocarbon residues.

Ethyl acetate extraction of acidified samples of urine and feces gave >95% partitioning of radiocarbon into the organic phase from each sample. Two-dimensional TLC of the extracts indicated that the metabolite underwent little additional degradation in the rat before excretion. In the feces, >90% of the radiocarbon was recovered as unmetabolized 2,6-difluoro-3-hydroxydiflubenzuron. None of the four other radioactive components in the feces extracts cochromatographed with any of the available compounds of known structure. TLC analysis of the radioactive components in the urine extracts revealed more evidence of metabolism than was seen in feces. About 76% of the radiocarbon in the extracts of urine was unaltered 2,6-difluoro-3-hydroxydiflubenzuron, but at least five additional radioactive components were resolved from the mixture. However, none of these cochromatographed with any of the available metabolite standards, and no further attempts were made to determine their chemical nature.

Fate of [^{14}C]Diflubenzuron Applied Dermally to Cattle. [^{14}C]Diflubenzuron residues were rapidly dissipated after the insecticide was applied dermally to cattle that were subsequently held in an unprotected pasture. Both WP and oil-based formulations were lost from the treated areas very rapidly—<4% of the applied radiocarbon remained after 1 week and only 0.1% of the applied dose remained after 4 weeks (Table VII). TLC examination of acetone rinses from the treated areas showed that diflubenzuron was the only detectable radioactive component in all samples. Analysis of numerous tissues taken when the animals were killed showed none to have detectable radiocarbon residues, other than hair and skin samples collected at or adjacent to the treated areas. There were no significant differences in persistence or residue patterns between the WP and oil based formulations. Although the radiochemical used to spike both formulations was in wettable powder form, the particle size of the [^{14}C]diflubenzuron was the same as that of both formulations (2–5 μm) and the extremely minute amounts of WP formulation accompanying the radiochemical likely did not significantly alter the properties of the oil based–oil carrier mixture applied to the animals. The 4-week study was conducted during late summer, a period of mostly sunny and hot weather. A total of about 2 cm of rain was recorded during the study period, but rain clearly was not the cause of the rapid dissipation of diflubenzuron residue from the animals. The first rain did not fall until 1 day after the 1-week animal was killed for analysis.

Studies with a stanchioned, catheterized cow indicated that diflubenzuron is not absorbed through the skin to any significant extent after dermal spraying of the insecticide. During a 3-day period after application of [^{14}C]diflubenzuron WP formulation to the cow, no detectable residues were excreted in the urine. On the basis of studies involving oral dosing of cattle and sheep with diflubenzuron, radiocarbon excretion in the urine would have been expected if diflubenzuron had been absorbed through the skin. Although 2.1% of the applied radiocarbon was detected in the feces, this almost certainly represents contamination by rub-off or other external transfer processes. The feces from the animal were allowed to remain where deposited until collected at 24-h intervals, and some contamination was probably inevitable.

After 3 days, 68% of the radioactivity applied to the stanchioned animal was recovered by clipping and extracting the treated hair and thoroughly washing the exposed skin with acetone. TLC of these fractions showed that diflubenzuron was the only detectable radioactive component.

DISCUSSION

The studies reported here indicate that oral treatment of sheep and cattle with diflubenzuron is followed by absorption of the compound through the gastrointestinal tract, metabolism, and elimination of residues through the urine, feces, and to a very limited extent, milk. The fact that only a trace of unmetabolized diflubenzuron was secreted into milk and none was seen in urine or bile of the treated animals indicates that the absorbed diflubenzuron is almost completely metabolized to more polar products before excretion. Intact diflubenzuron was eliminated in the feces of orally dosed cattle and sheep, but considerable quantities of metabolites and unextractable radiocarbon were also detected in feces of all but the bile-duct cannulated animals. The radiocarbon eliminated in the feces of bile-duct cannulated sheep was almost totally extracted and consisted only of unmetabolized diflubenzuron. These observations, and the studies indicating that diflubenzuron is not metabolized in vitro by ruminant digestive tract fluids, seem conclusive evidence that this insecticide is not degraded within the digestive tract of ruminants to any significant degree. It follows that the levels of diflubenzuron metabolites detected in feces of ruminants are a direct indication of the extent of biliary excretion. Thus, even though no studies with bile-duct cannulated cattle were made, the data in Table VI indicate that >50% of the radiocarbon eliminated in the feces of a cow (that as metabolites or unextracted radiocarbon) likely came from biliary excretion. Estimates of biliary excretion based on the levels of diflubenzuron metabolites in feces are, if in error, likely to be low because of enterohepatic circulation.

Sheep and cattle qualitatively metabolized diflubenzuron almost identically, but there were significant differences in the relative amounts of the metabolites generated. The major identified metabolic transformation in the cow was hydroxylation at the 3 position of the 2,6-difluorobenzoyl ring, but in sheep the major identified

Table VII. Persistence of [^{14}C]Diflubenzuron on the Hair and Skin after Dermal Application to Cattle Held in an Unprotected Pasture

Weeks after treatment	Residual radiocarbon, % of applied	Diflubenzuron residue, ppm	
		Hair	Skin
	Wettable powder formulation		
1	3.8	85.1	0.4
2	1.7	19.7	0.1
4	0.1	2.9	<0.1
	Oil-based formulation		
1	3.5	128.0	0.4
2	0.7	19.5	0.1
4	0.1	3.8	<0.1

metabolites arose through cleavage of the amide bond at the benzoyl carbon to give 2,6-difluorobenzoic acid, which was excreted in the urine either free or conjugated with glycine. Both sheep and cow metabolized diflubenzuron by hydroxylation at both the 2 and 3 positions of the chlorophenyl ring and at the 3 position of the difluorobenzoyl ring. It was not determined whether hydroxylation at the 4 position of the difluorobenzoyl ring also occurred. On the basis of chromatographic behavior in relation to the three hydroxydiflubenzuron isomers that were identified, unknown 1 (Figures 1 and 2) may be 2,6-difluoro-4-hydroxydiflubenzuron. However, attempts at isolating unknown 1 in pure form were not successful, and mass spectral analysis of the preparation that was obtained did not give interpretable results.

Although the 4-chlorophenyl ring was radiocarbon labeled at essentially the same specific activity as the difluorobenzoyl ring, these studies for the most part did not elucidate the fate of this half of the cleaved diflubenzuron molecule. 4-Chlorophenyl urea was the single such metabolite identified, and it was detected only in very low amounts.

Some of the diflubenzuron metabolic pathways identified in sheep and cattle have been previously reported. Metcalf et al. (1975) found that diflubenzuron was quite resistant to in vitro degradation by sheep liver microsomes, but obtained chromatographic evidence for its metabolism to trace quantities of 2,6-difluorobenzoic acid, 2,6-difluorobenzamide, 4-chlorophenylurea, 4-chloroacetanilide, and 4-chloroaniline and its *N,N*-dimethyl derivative. Some of these products were also seen in alga, snail, mosquito, and mosquitofish populations in model ecosystem studies with [^{14}C]diflubenzuron (Metcalf et al., 1975). In the current studies, the 4-chloroaniline derived metabolites generated by sheep liver microsomes were not seen as sheep metabolites in vivo. Studies of the metabolism of diflubenzuron in rats have been reported in preliminary form (Verloop and Ferrell, 1977). Rats degrade diflubenzuron both by hydrolysis to 4-chlorophenylurea and 2,6-difluorobenzoic acid and by ring hydroxylation. 4-Chloro-2-hydroxy- and 4-chloro-3-hydroxydiflubenzuron were major metabolites in the rat, as was a metabolite hydroxylated at either the 3 or 4 position of the difluorobenzoyl ring. It may be that this latter rat metabolite is 2,6-difluoro-3-hydroxydiflubenzuron, since cattle show clear preference for hydroxylation at this position, and the metabolite is seen in sheep as well.

Diflubenzuron and its metabolites showed little tendency toward secretion into milk or retention within the body of ruminants, as evidenced by minimal tissue residues 4–7 days after treatment. Treatment of rats with the major hydroxylated diflubenzuron milk metabolite likewise showed that this compound is rapidly excreted by other mammals. Based on these studies, it seems likely that the use of diflubenzuron for insect control will not result in appreciable contamination of meat or milk of livestock that may be exposed to low residues of the compound.

Experiments in which [^{14}C]diflubenzuron was applied dermally to cattle were made because recent studies from this laboratory have shown that the dermally applied compound inhibits reproduction of biting flies feeding on the treated animals (Wright and Harris, 1976; Kunz et al., 1977). [^{14}C]Diflubenzuron sprayed onto cattle held outdoors in an unprotected pasture rapidly disappeared from the hair and skin, but this loss was not due to absorption or chemical degradation. Rub-off or other weathering factors, including licking by the animals themselves, likely accounted for this rapid loss.

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