Fate of Carbon-14 Trifluralin in Artificial Rumen Fluid and in Ruminant Animals

Tomasz Golab, R. J. Herberg, E. W. Day, A. P. Raun,
F. J. Holzer, and G. W. Probst

In time-rate studies, labeled ^{14}C-trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) was degraded in 11 hours in an artificial rumen fluid medium. In ruminant animals, 99% of the ingested radioactivity from labeled ^{14}C-trifluralin was recovered within 6 days in urine (17.8%) and feces (81.2%). Urine, feces, blood, and milk were examined for trifluralin and its possible metabolites. The milk and blood contained no radioactivity. Neither urine nor feces contained any trifluralin. The radioactivity was excreted mostly as nonidentifiable polar substances. The principal degradation products formed during the time course degradation in rumen fluid and in ruminant animals were reduction products of trifluralin—namely, N_2,N_2-dipropyl-3-nitro-5-trifluoromethyl-*o*-phenylenediamine and N_4,N_4-dipropyl-α,α,α-trifluorotoluene-3,4,5-triamine. Several other trace metabolites were formed, some of which could be identified.

The preemergent soil-incorporated herbicide, trifluralin (Alder *et al.*, 1960; Pieczarka *et al.*, 1961), has been registered for use on alfalfa and other trifluralin-tolerant crops which might be consumed as forage by livestock. Even though residues of trifluralin are negligible in forage crops, soil clinging to plant parts could be a source of ingested trifluralin. This possibility prompted an investigation on the fate of trifluralin in artificial rumen fluid and in ruminant animals.

MATERIALS AND METHODS

Labeled Trifluralin. Trifluralin used in artificial rumen experiments was labeled with ^{14}C in the trifluoromethyl group (specific radioactivity: 9.05 μc. per mg.), whereas that used in the ruminant animal experiments was a mixture of uniformly ring-labeled and trifluoromethyl-labeled trifluralins (specific radioactivity: 13.6 μc. per mg.). Degradation studies conducted in our laboratories, subsequent to this investigation, reveal that the purchased ^{14}C-labeled *p*-chlorobenzoic acid used in the synthesis of uniformly ring-labeled trifluralin in the Lilly Research Laboratories (Marshall *et al.*, 1966) was a mixture, determined to be 15% uniformly ring-labeled and 85% carboxyl-labeled *p*-chlorobenzoic acids, rather than a single molecular species. The radiochemical purity of the labeled compounds was greater than 99%, as determined by thin-layer chromatography (TLC). The ^{14}C uniformly ring-labeled trifluralin, referred to in a previous publication (Probst *et al.*, 1967), was also the mixture described.

Preparation of Artificial Rumen Fluid. Artificial rumen fluid is a two-component mixture of rumen fluid and artificial saliva solution. The rumen fluid, recovered from a steer about 30 minutes prior to laboratory use, was held in a water bath at 36° C. under CO_2 atmosphere. The artificial saliva solution of mineral salts, urea, and cellulose in water was prepared according to the formula of Hubbert *et al.* (1958). Cellulose powder served both as a substrate and as an adsorbent for labeled trifluralin. The labeled compound dissolved in acetone was added to the cellulose powder, then the mixture was agitated until the acetone evaporated. Trifluralin content was determined by measuring the radioactivity of the carbon dioxide evolved from the combustion of duplicate samples of the cellulose-trifluralin mixture, as described by Golab *et al.* (1967).

The artificial rumen medium was prepared by adding 450 ml. of saliva solution to a 1-liter aspirator bottle fitted with a side-arm. The mixture was saturated with carbon dioxide and warmed to 36° C. In succession an accurately weighed 3-gram sample of trifluralin-cellulose mixture was added, followed by 450 ml. of the warm rumen fluid. Both the solution and the flask head space were purged with carbon dioxide. The mixture contained 8.25 p.p.m. of trifluralin, equivalent to 1.71×10^7 disintegrations per minute (d.p.m.) of radioactivity.

After mixing the contents for 3 to 5 minutes, the initial 50-ml. aliquot was withdrawn, immediately frozen in a dry ice–alcohol bath, and retained in the frozen state until analyzed.

The remainder of the solution was placed in an incubator at 37° C. All subsequent 50-ml. aliquots were treated in a similar manner, and after each sample was withdrawn, the bottle was purged with carbon dioxide. After the initial withdrawal, subsequent aliquots were removed at ¼, ½, ¾, 1, 1½, 2, 2½, 3, 4, 5, 6, 7, 8, 9, 10, and 11 hours.

Extraction Procedure. Trifluralin and its degradation products were extracted from the rumen fluid by adding ethyl acetate directly to the flask containing the frozen sample. After thawing the mixture in warm water, it was transferred to a separatory funnel, shaken, and centrifuged. The ethyl acetate layer was recovered, and the aqueous layer was extracted an additional four times. The ethyl acetate extracts were combined and concentrated on a rotary evaporator to a 2-ml. volume. Aliquots of the concentrate were used for radiochemical, thin-layer, and gas chromatographic analyses.

The spent aqueous solution from ethyl acetate extractions was acidified with 0.5 ml. of concentrated hydrochloric acid, and again was extracted three times with ethyl acetate. The extracts were combined and

Greenfield Research Laboratories, Eli Lilly and Co., Greenfield, Ind. 46140

analyzed as the acidic extracts. Aliquots of the spent aqueous solution were combusted, and the residual radioactivity was determined as carbon dioxide.

Chromatographic Procedures. Conventional 20- × 20-cm. glass plates, coated with a 250-micron layer of silica gel GF (Brinkman No. 7730), were used in thin-layer chromatography. All plates were developed once with each solvent system in presaturated chambers, with the exception of the one-dimensional carbon tetrachloride system, in which the plates were developed twice in unsaturated chambers. The three one-dimensional chromatographic solvent systems were: carbon tetrachloride, benzene–ethylene chloride (1 to 1), and benzene–ethylene chloride (1 to 1) followed by *n*-hexane–methanol (97 to 3). For two-dimensional chromatography the solvent systems were benzene–ethylene chloride (1 to 1) in the first dimension and *n*-hexane–methanol (97 to 3) in the second dimension, and a special three-solvent system to resolve the more polar compounds consisting of benzene–ethyl acetate–acetic acid (60: 40: 1) in the first dimension and benzene–ethylene chloride (1 to 1) followed by *n*-hexane–methanol (97 to 3) in the second dimension.

The one-dimensional chromatoplates were used both for radioactivity measurements and for radioautography, whereas all two-dimensional plates were subjected to radioautography only. Two-dimensional chromatoplates were developed in the usual manner, with reference substances being cochromatographed with the sample. The reference substances were detected visually or with an ultraviolet source.

Analytical Measurements. The TLC systems, preparation of radioautographs, radioactivity counting procedures, and methods of gas chromatography employed in this investigation have been described (Golab *et al.*, 1967; Tepe and Scroggs, 1967).

Ruminant Animal Experiments. Cow Experiment. A lactating Holstein cow was fed trifluralin incorporated in the alfalfa ration at 1 and 1000 p.p.m. for 39 and 13 days, respectively. The feeding level of trifluralin used was in excess of any residue ever observed on forage crops. Samples of feces, urine, blood, and milk were collected at appropriate times during the feeding regimen. The samples were extracted, and the extracts were examined by gas chromatographic methods employing a Jarrell-Ash Model 28-730 instrument equipped with electron-capture and flame ionization detectors.

Goat Experiment. Two lactating goats, maintained in individual metal metabolism cages, were fed two parts alfalfa hay and one part complete mixed ration at a fixed rate during a pre-experimental period. At the conclusion of the pre-experimental period, one goat was fed unlabeled trifluralin at 1 p.p.m. incorporated in the ration for 11 days, labeled trifluralin for 1 day, and unlabeled trifluralin again for 14 days. A second goat, a control, received no trifluralin throughout the experimental period.

Labeled trifluralin was incorporated in the ration by spraying an acetone solution on 30-mesh alfalfa hay. The mixture was tumbled and the acetone removed by evaporation. The amount of trifluralin in the labeled ration was established by radioactive and chemical analyses to be 20.8 μc. or 1.53 mg. of trifluralin.

The milk, collected twice a day, was pooled daily. Urine, collected by means of a catheter anchored in the bladder, was allowed to accumulate for 24 hours in the glass bottle. Daily feces collections were placed in a large Waring blender with sufficient water to permit homogenization, and the total weight was determined. Daily blood samples (5 ml.) were obtained from the jugular vein and treated with oxalate.

Total radioactivity was determined in aliquots of milk, urine, feces, and blood from each daily excretion. The remainder of the daily sample collection was frozen for further analysis. Urine radioactivity was determined by diluting 1-ml. aliquots with 20 ml. of appropriate scintillation solution. Feces (1-gram aliquots), milk, and blood samples (1-ml. aliquots, respectively) were combusted. The carbon dioxide formed was adsorbed in ethanolamine–methylcellosolve solution and appropriately diluted in scintillation solution for counting. The recovery of radioactivity from feces, milk, and blood was checked by combusting samples containing added ^{14}C-stearyl alcohol. Radioactivity recovery values were greater than 96%. Counting efficiencies were determined by internal standardization with toluene-1-^{14}C.

Extraction Procedures for Urine and Feces. The urine sample, selected at the peak of radioactivity excretion, was extracted at neutral pH three times with chloroform; then the urine was adjusted to pH 1.0 and was extracted again three times with chloroform. The aqueous portion was refluxed for one-half hour and was extracted again three times with ethyl acetate. The extracts obtained under each condition were pooled, concentrated, and examined by chromatographic and radiochemical methods, as described for the artificial rumen fluid.

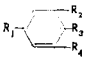
A 525-gram sample of the second-day fecal collection, suspended in water, was blended in an Omni-mixer with 500 ml. of methanol, filtered with the aid of Hyflo Super Cel, and the filter cake was washed with 500 ml. of methanol. An additional 500 ml. of 10% aqueous sodium chloride was added to the combined methanol-water solution, and the mixture was extracted five times with 500-ml. portions of methylene chloride. The methylene chloride extracts were combined, concentrated, and examined by the methods employed with the urine extracts.

RESULTS AND DISCUSSION

Artificial Rumen Fluid. Recovery of Radioactivity. From the artificial rumen fluid experiments, 98.5 to 98.8% of the original radioactivity was recovered in the ethyl acetate extracts and the spent aqueous fluid. The recovery values indicated that trifluralin was not degraded to radioactive gases such as carbon dioxide or methane. The amount of ethyl acetate extractable radioactivity decreased from 95.5 to 73.5% from initiation to the conclusion of the experiments. On the other hand, the radioactivity in the spent aqueous rumen fluid, determined as carbon dioxide by direct combustion, increased from 4.5 to 26.5%. These changes reflected a general rapid degradation of trifluralin into nonextractable products in rumen fluid.

Only the neutral ethyl acetate extracts obtained from rumen fluid were studied extensively, since preliminary observations revealed the acidic extract possessed simi-

Table I. Model Compounds



Experimental Number	Name	R_1	R_2	R_3	R_4
1	Trifluralin	CF_3	NO_2	$N—(C_3H_7)_2$	NO_2
2	α,α,α-Trifluoro-2,6-dinitro-*N*-propyl-*p*-toluidine	CF_3	NO_2	$N—C_3H_7$ (H)	NO_2
3	α,α,α-Trifluoro-2,6-dinitro-*p*-toluidine	CF_3	NO_2	$N—H_2$	NO_2
4	α,α,α-Trifluoro-5-nitrotoluene-3,4-diamine	CF_3	NO_2	$N—H_2$ (H)	NH_2
5	α,α,α-Trifluoro-5-nitro-N_4-propyl-toluene-3,4-diamine	CF_3	NO_2	$N—C_3H_7$ (H)	NH_2
7	α,α,α-Trifluoro-N_4,N_4-dipropyl-5-nitro-toluene-3,4-diamine	CF_3	NO_2	$N(C_3H_7)_2$	NH_2
9	α,α,α-Trifluoro-N_4,N_4-dipropyl-toluene-3,4,5-triamine	CF_3	NH_2	$N(C_3H_7)_2$	NH_2
12	α,α,α-Trifluoro-2,6-dinitro-*p*-cresol	CF_3	NO_2	OH	NO_2
15	4-(Dipropylamino)-3,5-dinitro-benzoic acid	COOH	NO_2	$N(C_3H_7)_2$	NO_2
26	α,α,α-Trifluorotoluene-3,4,5-triamine	CF_3	NH_2	NH_2	NH_2
31	α,α,α-Trifluoro-N_4-propyl-toluene-3,4,5-triamine (not presently available as a model compound)	CF_3	NH_2	$N—C_3H_7$ (H)	NH_2

lar characteristics. To facilitate the examination of the extracts, a series of model compounds shown in Table I was used as possible trifluralin metabolic products. The compounds of primary interest are Compound **7**, trifluralin with one nitro group reduced, and Compound **9**, trifluralin with both nitro groups reduced.

Mode of Degradation. The mode of trifluralin degradation and the formation of metabolic products are summarized graphically in Figure 1. The curves represent the average values obtained from a combination of radiochemical analysis of one-dimensional thin-layer chromatoplates and from gas-liquid chromatographic measurements. Trifluralin was degraded rapidly and appears to be converted initially to Compound **7**, which accumulates to a maximum in about 4 hours. Thereafter, the rate of conversion of Compound **7** to Compound **9** exceeds its formation from trifluralin. After 11 hours, the amount of trifluralin present was less than 1%, and after 20 hours, trifluralin was not detectable.

The amount of radioactivity remaining at the origin on thin-layer chromatoplates, the so-called polar compounds, increases continuously, indicating that the radioactivity of labeled trifluralin ultimately accumulates in this heterogenous mixture. At least a portion of the polar compounds must have Compound **7** as their precursor, since polar compounds markedly increased simultaneously with a decrease in the amount of Compound **7** in the reaction mixture.

Polar Products. Thin-layer chromatography in a variety of solvents failed to resolve this origin material into discrete, identifiable substances. Hydrolysis of the mixture failed to change its chromatographic behavior. However, strong reduction with tin and hydrochloric acid or with sodium hyposulfite ($Na_2S_2O_4$) in alkaline solution yields a mixture in which Compound **26**—namely, α,α,α-trifluorotoluene-3,4,5-triamine—is the predominant component. Formation of the aromatic triamine suggested that the polar mixture was formed as a result of aromatic amine condensation; for example: azo, azoxy, and hydrazo compounds. A similar condensation has been described by Bartha and Pramer (1967), in which the herbicide 3′,4′-dichloropropionanilide decomposes in soil to 3,4-dichloroaniline, which in turn is condensed to form 3,3′,4,4′-tetrachloroazobenzene. If polar compounds are formed by condensation reactions, as the evidence suggests, at least six different mono-, di-, and tri-substituted aromatic amines can contribute to a host of products typical of such a

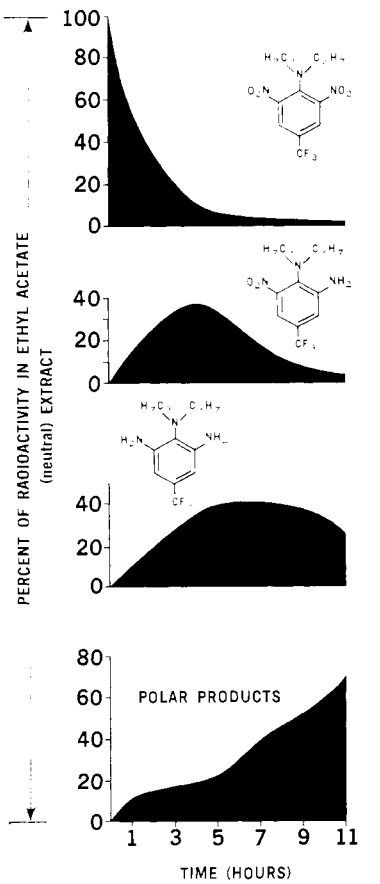


Figure 1. Rate of trifluralin degradation and formation of degradation products in artificial rumen fluid

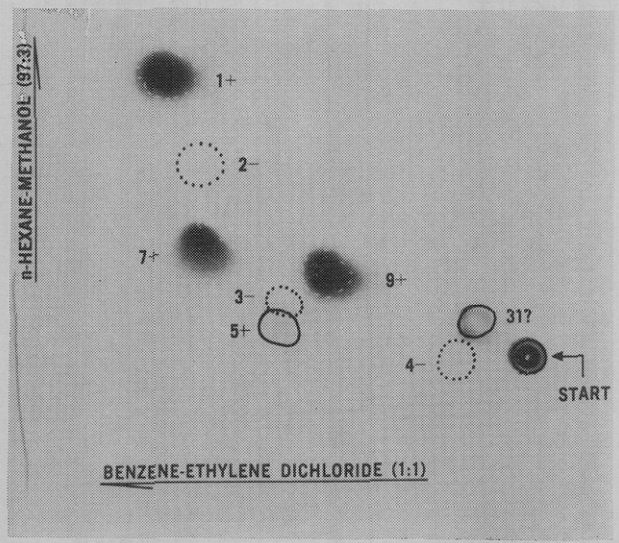


Figure 2. Thin-layer chromatoplate radioautograph (103-day exposure) of neutral ethyl acetate extract, showing trifluralin degradation after 2 hours' incubation in artificial rumen fluid. Radioactive detectable compounds are indicated by the symbol (+), nondetectable by (−)

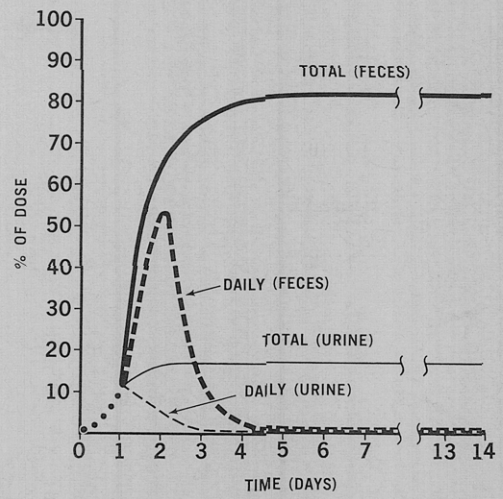


Figure 3. Excretion of radioactivity in goat urine and feces after ingestion of ^{14}C-labeled trifluralin

polar mixture. Resolution of this complex mixture is a separate subject not covered in this investigation.

Chromatographic Nature of Extracted Metabolites. The major metabolic products, Compounds **7** and **9,** were identified by both gas chromatography and radioautography of thin-layer chromatoplates. The minor metabolic products were detectable on radioautography only. A typical radioautograph of a thin-layer chromatoplate is shown in Figure 2. An extract prepared from artificial rumen fluid containing labeled trifluralin for 2 hours revealed the presence of trifluralin (Compound **1**) and Compounds **5, 7,** and **9.** Trace amounts of Compound **2** could be detected in extracts after the first hour of exposure. The unknown radioactive spot near the origin is speculated, based on its chromatographic behavior, to be Compound **31** (trifluralin with both nitro groups reduced and one propyl group removed), which is not available as a model compound.

Ruminant Animals. Fate of Labeled Trifluralin in Lactating Cow. The degradation pathway of trifluralin ingested by ruminant animals is very similar to that observed in the artificial bovine rumen fluid. Trace quantities of trifluralin and several metabolites were found only in the feces of a lactating Holstein cow after ingestion of trifluralin at 1000 p.p.m. The maximum levels of the various compounds found in the feces are as follows: 6.5 p.p.m. of trifluralin at six days, 2.8 p.p.m. of Compound **7,** 18 p.p.m. of Compound **9,** and traces (less than 0.01 p.p.m.) of Compounds **2** and **3.**

At the conclusion of the experiment, the animal was sacrificed, and the tissues were examined. There was no evidence of trifluralin or any of the seven related compounds in lean, liver, kidney, or heart tissue. Fat samples contained 0.03 p.p.m. of trifluralin and 0.06 p.p.m. of Compound **7.** The detection sensitivities by gas chromatographic techniques were: 5 to 10 p.p.b. for Compounds **1, 2,** and **3**; 20 to 30 p.p.b. for Compounds **5** and **7**; 0.10 to 0.25 p.p.m. for Compound **4**; and 1.0 to 1.5 p.p.m. for Compounds **9** and **26.**

Excretion and Recovery of Radioactivity in the Goat. These detection values for unlabeled trifluralin and its metabolites are inadequate for evaluating trifluralin which might be consumed in forage, and this prompted further investigation with labeled trifluralin, using the goat as the experimental ruminant animal. Ingestion of labeled trifluralin in the diet at 1 p.p.m. for one day permits a radioactivity detection level, expressed as trifluralin, of 1 to 4 p.p.b. above background radioactivity. The excretion of radioactivity in the urine and feces of the goat, as a function of time, is shown in Figure 3. The urine and feces contained 17.8 and 81.2%, respectively, of the administered radioactivity, resulting in a 99% recovery of the labeled material. The data was statistically analyzed by the method of Redman *et al.* (1965), in which a 90% certainty applies to statements concerning the presence of a radioactive residue. The analysis revealed radioactivity in the urine above normal level for three days after the labeled trifluralin was administered, radioactivity in the feces above normal level for six days after the labeled trifluralin was administered, and no radioactivity in the milk above the normal level at any time. The blood contained less than 2 p.p.b. radioactivity calculated as trifluralin. Tissue analyses of the sacrificed animal, performed on lean, liver, kidney, fat, small intestine, large intestine, and stomach, gave no indication of trifluralin residue or its metabolic products.

Chromatographic Nature of Metabolites in the Excreta of the Goat. The nature of the radioactivity present in urine and feces was examined. Extraction of urine removed approximately two thirds of the radioactivity present in the sample. In contrast, only one third of the radioactivity was extractable from feces. Trifluralin was not detectable in urine or feces. The principal metabolite found in both urine and feces extracts was Compound **9.** In the feces extract no other recognizable metabolites could be identified, with the possible exception of Compound **26.** On the other hand, the urine extract contained traces of Compounds **4, 5,**

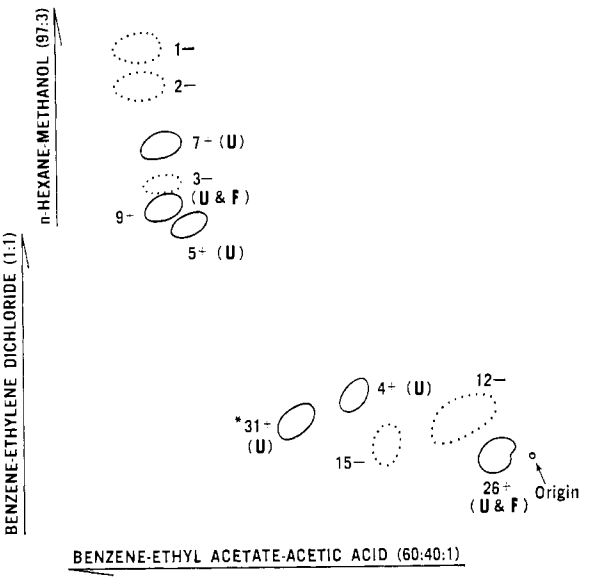


Figure 4. Replicate of typical TLC radioautograph of the neutral chloroform extract indicating metabolites present in goat urine (U) and feces (F). Radioactive detectable compounds are indicated by the symbol (+), nondetectable by (−)

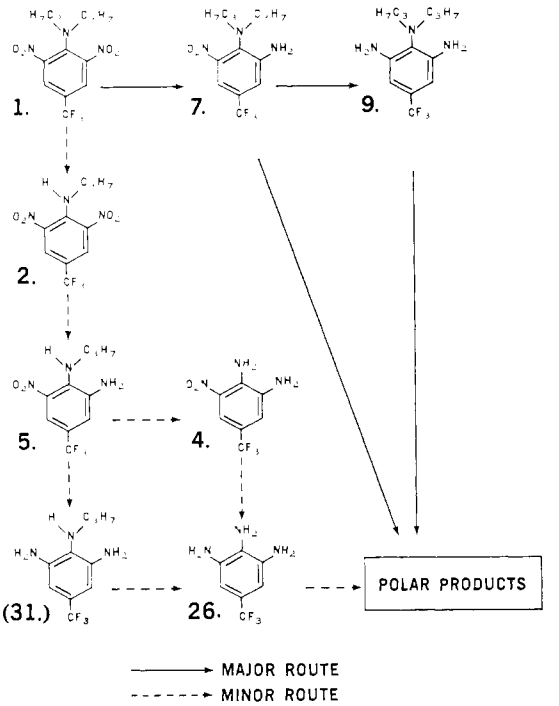


Figure 5. Postulated pathway of trifluralin degradation in artificial rumen fluid

7, 26, and possibly **31,** as determined by radioautography of thin-layer chromatoplates (Figure 4). The major portion of the extracted radioactivity from the urine and feces could not be identified. The so-called polar substances, located on the origin of thin-layer chromatoplates, constituted more than 90% of the radioactivity. Approximately 6.9% of the radioactivity extracted from the urine and 2.6% from the feces were identifiable with the reference model compounds.

Postulated Metabolic Pathway. The use of model compounds provides insight into the sequential steps involved in the metabolic degradation. A postulated pathway of trifluralin metabolism in artificial rumen fluid and ruminant animals is shown in Figure 5. Trifluralin is completely degraded in artificial rumen fluid in vitro within 20 hours with a continuous accumulation of extractable polar compounds and nonextractable products. The in vivo studies with ruminant animals further substantiate that these products constitute the ultimate degradation of trifluralin. This pathway is very similar to the pathway of trifluralin degradation in soil under water (Probst *et al.*, 1967). The distribution of radioactivity in urine and feces of the ruminant is similar to that found by Emmerson and Anderson (1966) on the oral administration of ^{14}C-trifluoromethyl-labeled trifluralin to rats. Metabolism in the rat was slightly different from that observed in the ruminant animal. Dealkylation products of trifluralin were isolated and identified in rat urine. The main route of metabolic degradation in rumen fluid and ruminant animals is the reduction of trifluralin nitro groups, yielding compounds which rapidly form the complex mixture of polar products.

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