Absorption, Excretion, and Metabolism of Nitrofen by a Sheep

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The herbicide nitrofen (2,4-dichlorophenyl 4-nitrophenyl ether) was ^{14}C labeled and administered orally to a sheep at 40 mg/kg. Ninety-nine hours after treatment, 76.2% of the applied dose was accounted for in the excreta. Nitrofen was found in feces and blood, but not in urine. At the 100-h slaughter, radiocarbon levels were highest in fat (23.1–24.1 ppm); the liver, thyroid, and mammary gland had levels of 2–3 ppm; and the adrenal gland, kidney, lung, muscle, skin, and spleen had levels of 1–2 ppm. The most predominant metabolism products were 2,4-dichlorophenyl 4-aminophenyl ether, 2,4-dichloro-5-hydroxyphenyl 4-nitrodiphenyl ether, 2,4-dichlorophenol, 2-chlorophenyl 4-nitrophenyl ether, and conjugates. No evidence of rearrangement of the chlorines was seen.

Nitrofen (2,4-dichlorophenyl 4-nitrophenyl ether) (Figure 1, compound 1), a herbicide, is widely used for pre- and postemergence control of annual grasses and broadleaf weeds in rice paddies and in fields containing other grain and hay crops. Previous studies (Nakagawa and Crosby, 1974) have shown that in aqueous suspensions, nitrofen is photodecomposed by sunlight through cleavage of the ether linkage to form 2,4-dichlorophenol and 4-nitrophenol as the major degradative products. *N*-[2-Chloro-4-(4-chlorophenoxy)phenyl]formamide, 4,4′-bis(2,4-dichlorophenoxy)azabenzene, 4-nitro-1,2-benzenediol, and the phenolic diphenyl ethers were also detected in small amounts. About 10% of the initial nitrofen remained after 4 weeks of sunlight exposure.

In studies by Gutenmann and Lisk (1967), nitrofen was metabolized by cattle to the amine, 2,4-dichlorophenyl 4-aminophenyl ether. However, neither the parent compound nor the amine was detected in the urine, milk, or feces of a lactating dairy cow treated with nitrofen at 5 ppm for 4 days. Although these studies indicate that nitrofen is biodegradable in mammals and photodecomposed when exposed in aqueous suspensions, the widespread use of nitrofen necessitates an evaluation of its metabolism and excretion by livestock and of residues in the blood and tissues. This study reports the metabolic fate of radiolabeled ^{14}C-labeled nitrofen administered orally to a sheep.

MATERIALS AND METHODS

Chemicals. Nitrofen-^{14}C (0.45 mCi/mmol, uniformly labeled in the dichlorobenzene ring) was obtained from New England Nuclear (Boston, Mass.). The purity of the compound was determined by thin-layer chromatography (TLC) developed in a solvent mixture of hexane and acetic acid (94:4 v/v). The radiochemical purity was >99%. Six nitrofen derivatives were used for TLC comparisons with ^{14}C-labeled metabolites that occurred in blood, urine, and feces. The structure, chemical name, and source of the compounds are shown in Figure 1.

Treatment and Sample Collection. A 22-kg Delaine ewe was shorn and placed in a metabolism stall 24 h before treatment. A Foley retention catheter, No. 16, was inserted for the collection of urine samples. The sheep was dosed orally with a gelatin capsule containing a small amount of crushed grain and 0.883 g of ^{14}C-labeled nitrofen (1.39 mCi), equivalent to 40 mg/kg of body weight. Alfalfa hay, grain, and water were available ad libitum.

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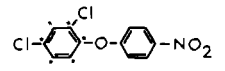
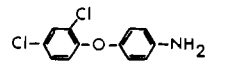
Blood, urine, and feces (if available) were collected every 0.5 h for the first 4 h, then hourly through 9 h, and at 4-h intervals for the remainder of the test. Samples of feces were mixed thoroughly, lyophilized, and subsampled for subsequent quantitation by combustion analysis or extraction and TLC analysis. Urine and blood samples were also subsampled before being quick-frozen and held at −70 °C for later analysis. The sheep was slaughtered 100 h after treatment. Selected tissue samples and organs were collected, weighed, and frozen for subsequent analysis.

Chromatography. After treatment, the radiochemical compositions were determined by TLC, autoradiography, and subsequent liquid scintillation counting (LSC) of the indicated areas from the gel plates. Both one- and two-dimensional systems were used to separate nitrofen and metabolites in urine, blood, and feces. Brinkman 0.25-mm silica gel F_{254} precoated chromatoplates were used in all analyses. Plates were developed in the first system, Skelly B–ethyl acetate–acetonitrile (11:5:1). This system separated all six standards, but the more polar compounds remained at or near the origin. The second system, acetonitrile–water (80:20), was used to separate polar and apolar compound, but it placed all of the known standards in about the same location, that is, near the front at an R_f of 0.82 to 0.92. The standards were applied to each plate as side markers and located after development of the plate in both TLC systems by quenching of fluorescence under short-wavelength UV light. The radioactive areas from the samples were located by autoradiography.

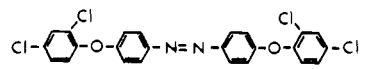
Analytical Procedures. The lyophilized feces were pressed into 100-mg pellets and combusted in a Packard 305 oxidizer. The resulting trapped $^{14}CO_2$ was quantitated by LSC (Beckman LS-150 spectrometer); both external and internal standards were used. Other subsamples of feces (500 mg) were extracted in Soxhlets for 10 or more cycles with either chloroform or methanol. The extracted powder was dried, combusted, and quantitated for comparison with original radioactivity. The extracts were diluted to volume, and an aliquot was used for quantitation directly by LSC. Radioactive areas on developed TLC plates were located by radioautography (Gaevert blue x-ray film) and quantitated by scraping the appropriate gel regions into scintillation vials for LSC. Tissues (100 mg wet weight) were analyzed by combustion. Blood (100 μL) was combusted on small cotton balls, and the resulting $^{14}CO_2$ was counted by LSC. Total radiocarbon in urine was determined by pipetting 1 mL or less (depending on the activity) into 15 mL of Insta-gel (Packard) and counting directly by LSC. Urine and blood were extracted (undiluted or diluted with water or buffer) with chloroform. Samples of 15-h and 23-h urine were treated with

1. 

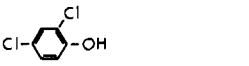
2,4-dichlorophenyl 4-nitrophenyl ether - NITROFEN

2. 

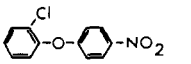
2,4-dichlorophenyl 4-aminophenyl ether - AMINO-NITROFEN

3. 

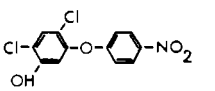
4,4′-bis(2,4-dichlorophenoxy)azobenzene - AZO-NITROFEN

4. 

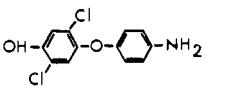
2,4-dichlorophenol - PHENOL

5. 

2-chlorophenyl 4-nitrophenyl ether - 2-CHLORO ETHER

6. 

2,4-dichloro-5-hydroxyphenyl 4-nitrophenyl ether - 5-HYDROXY ETHER

7. 

2,5-dichloro-4-hydroxyphenyl 4-aminophenyl ether 2,5-DICHLORO ETHER

Figure 1. Sources of compounds: 1, New England Nuclear (Boston, Mass.); 2, 6, and 7, Rohm and Haas (Briston, Pa.); 3 and 5, Dr. D. G. Crosby (University of California, Davis, Calif.); and 4, Eastman Chemical (Rochester, N.Y.).

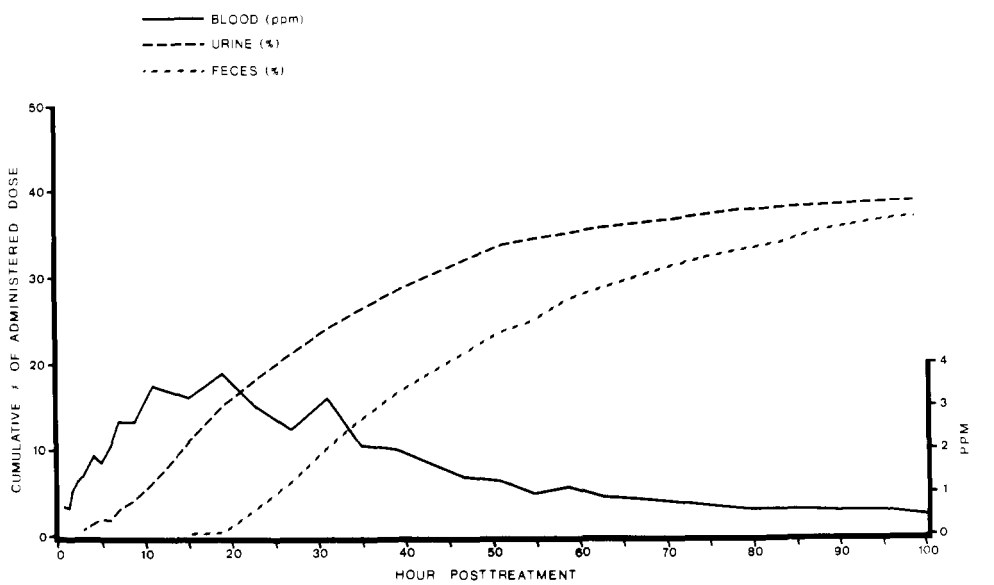


Figure 2. Radioactive materials found in urine, feces, and blood of a sheep treated orally with ^{14}C-labeled nitrofen.

glucuronidases (Sigma Chemical Co.) in acetate buffer [0.2 MO by the procedure described by Chamberlain and Hopkins (1973)]. A sample of each was also boiled for 0.5 h with 3 N HCl. The resulting material was then either chromatographed by one- or two-dimensional chromatography or extracted with chloroform before chromatography.

RESULTS

The level of radioactive material in the blood was highest 19 h after treatment, but the blood levels plateaued at 11 to 31 h. The final blood sample at 100 h had less activity than the 30-min posttreatment blood sample (Figure 2). The greatest urinary excretion rate was during the 11- to 19-h posttreatment collection period. Ninety-nine hours after treatment, 39% of the applied dose had been recovered, and less than 0.3% of the applied dose was being excreted during a 4-h collection period (Figure 2). The peak of radioactive material excreted in the feces was during the 39- to 47-h posttreatment collection, and 37.2% of the applied dose was recovered in the feces. A total of 76.2% of the applied dose was recovered in the urine and feces. The omental and renal fats contained the highest total radiocarbon of the 22 tissues analyzed (Table I). The level of radiocarbon in the gut was still relatively high at the 100-h slaughter; this finding indicated that further absorption was possible. The liver, thyroid, and mammary

Table I. Radioactive Material Found in Tissues of a Sheep Treated with ^{14}C-Labeled Nitrofen[a]

Tissue	ppm
Fat (kidney)	23.08
Fat (omental)	24.11
Gut	3.94
Liver	2.91
Mammary gland	2.06
Thyroid gland	2.66

[a] Brain, gallbladder contents, medulla brain stem, muscle (backstrap), pancreas, pituitary gland, rumen fluid, tubes and ovaries had residues of 0.47 to 0.89 ppm. Adrenal gland, gallbladder, heart, kidney, lung, muscle (ham), skin, and spleen had residues of 1.02 to 1.87 ppm.

Table II. Metabolites Found in Whole Urine or Extracts of Urine of a Sheep Treated with ^{14}C-Nitrogen (Average of Six Samples from 5 to 71 h); Skelly B-Ethyl Acetate-Acetonitrile System (11:5:1)

Material designation	Av % in whole urine	Av % in chloroform extracts
Origin	94.3	30.4
Unknown A	1.3	9.1
Unknown B	1.0	7.4
Unknown C	0.7	8.6
Aminonitrofen	1.1	31.5
5-Hydroxy ether	0.6	5.7
Phenol	0.3	3.2
2-Chloro ether + nitrofen	0.3	2.9
Azonitrofen	0.1	0.8
Unknown D	0.3	0.3

gland had levels of 2–3 ppm, and the adrenal gland, kidney, lung, muscle, skin, and spleen had levels of 1–2 ppm. The rest of the tissue had detectable levels, but all were below 1 ppm of total radioactive material.

Chromatography of whole urine (Table II) in the first system left an average 94.3% of the total radioactivity at or near the origin, which indicated a high percentage of compounds such as glucuronides and sulfates that would not migrate in this system. Nine distinct spots could be seen on most exposed x-ray films, and the most distinct spot chromatographed as aminonitrofen; however, no definitive trends were noticed from hour to hour on chromatography of the whole urine. The second system moved from 61 to 66% of the radioactivity to locations (R_f 0.67 to 0.97) on the chromatograms for apolar compounds. The separation of the polar and apolar compounds was good with most of the polar compounds (27 to 33%, depending on the sample hour) occurring as a streak between R_f 0.23 and 0.54. Small definite spots of radioactive material occurred at R_f 0.13 (1.3 to 2.3%) and at R_f 0.60 (2.8 to 4.1%). Nitrofen and all the known metabolites chromatographed from R_f 0.82 to R_f 0.92 in this system.

Only 2–5.5% of the radioactivity in the urine from samples taken at various times after treatment was extractable with chloroform. TLC analysis of the chloroform extracts (Table II) of the urine in the first system revealed the presence of at least nine metabolites, but only five spots chromatographed with the available standards: aminonitrofen, 5-hydroxy ether, the phenol combination with 2-chloro ether, and nitrofen and azonitrofen. The aminonitrofen was the most predominant material found in the extracts. The 2,5-dichloro ether standard can be confused with unknown B, but close examination shows that the 2,5-dichloro ether standard consistently appeared slightly below the unknown B in the first system. The amount of aminonitrofen was low (21%) at 5 h, reached a maximum of 54% at 15 h, and then declined to 24% by 57 h. During the same time period the unknown A decreased from 21 to 6%. Other compounds varied but showed no definitive trends related to time after treatment. Acidification of the urine did not change the composition of the metabolites in the urine did not change the composition of the metabolites in the urine extracts, but it did increase the amounts of radioactive materials extractable to 5.4–10.1%.

When the urine was treated with β-10 glucuronidase, about 25% of the radiocarbon material was removed by extraction (Table III). The addition of water or buffer had litte effect. Extractability increased when glucuronidase containing a small quantity of sulfatase (H-1 glucuronidase) or pure sulfatase was used. The results

Table III. Effect on Extraction Efficiency of Various Treatments of the 15-h Urine of the Sheep (Chloroform Extraction)

Treatment	% of radioactivity: Water phase	% of radioactivity: Chloroform phase
Water diluted but no incubation	96.4	3.6
19-h incubation at 36.5 °C, diluted with water	95.1	4.9
19-h incubation at 36.5 °C, diluted with buffer	93.7	6.3
19-h incubation at 36.5 °C, diluted with buffer and β-10 glucuronidase	75.5	24.5
19-h incubation at 36.5 °C, diluted with buffer and H-1 glucuronidase (contains some sulfatase)	24.0	76.0
19-h incubation at 36.5 °C, diluted with buffer and sulfatase	29.6	70.4
30-min boiling with 3 N HCl	83.3	16.7

Table IV. Metabolites Found in Chloroform Extracts of the Feces or of the Blood of a Sheep Treated with ^{14}C-Labeled Nitrofen; Skelly B-Ethyl Acetate-Acetonitrile System (11:5:1)

Material designation	Av % in feces extracts	Av % in blood extracts
Origin	16.0	12.3
Unknown A	5.0	3.0
Unknown B	7.7	13.4
Unknown C	4.3	3.6
Aminonitrofen	20.7	20.0
5-Hydroxy ether	2.4	7.0
Phenol	1.3	6.2
2-Chloro ether	3.5	0.6
Nitrofen	29.1	33.9
Azonitrofen	1.1	
Unknown D	7.6	
Unknown E	1.3	

indicate that large amounts of the conjugates were sulfate conjugates. Note that the amount extractable after treatment with β-10 glucuronidase added to that extractable with the sulfatase treatment did not equal that extractable after treatment with H-1 glucuronidase. The small amount of sulfatase in the latter probably was not sufficient to take care of the large amount of the sulfates found in the 15-h urine. Boiling with HCl liberated some radioactive material, which indicated the possible existence of glycine conjugates with nitrofen metabolites. When these extracts were chromatographed, the urine samples treated with sulfates showed a decline in the amount of the aminonitrofen and also of the 5-hydroxy ether. With some treatments larger quantities of unknown B appeared. The data on these results are available on microfilm (see Supplementary Material Available paragraph).

In the preparation of methanol or chloroform extracts of feces, chloroform extracted 47–86% of the radioactive materials, and methanol extracted 45–76%. The higher percentage of extractable materials was found in the early-hour samples whereas the least percentage was found in the 87-h samples. The total amount of radioactive material in the original samples and that in the extracts plus that in the extracted powder were always compared. The recoveries were generally in good agreement with the original; only 6 of the 20 extractions showed more than a 20% deviation from the original.

When the chloroform extracts of the freeze-dried feces were chromatographed, the predominant radiocarbon

material was nitrofen (Table IV). The percentage of nitrofen varied from 43% to 23 h to 16% at 33 h after treatment, and during the same time period, the amount of aminonitrofen increased from 14 to 25%. The other metabolites of nitrofen showed no consistent trends with time after treatment.

The results of extracting blood of the sheep with chloroform (Table IV) show that the predominant radiocarbon material in the blood extracts was nitrofen; however, the quantity of aminonitrofen was also fairly large. In the chloroform extracts of blood, the only really noticeable change with time after treatment was the increase in nitrofen from 31% at 4 h to 43% at 19 h and the decline to 28% by 31 h.

DISCUSSION

^{14}C-labeled nitrofen was rapidly absorbed as shown by the low residues in the rumen and high levels in the fat and blood. Approximately 30% of the total radiocarbon excreted in the feces was unmetabolized nitrofen. This amount represents about 11% of the total dose administered.

When untreated urine was chromatographed in the apolar system, large amounts of radioactive material remained at the origin though they moved well in the polar system; this finding suggests that the metabolites might be conjugates. When the urine was treated with several enzymes to disrupt the conjugates, much larger quantities of radioactive materials migrated in the apolar system. The most effective enzyme was sulfatase, and the results indicate the conjugation of several of the standards with sulfate. Of the known materials, aminonitrofen and 5-hydroxy ether most commonly conjugated with sulfate. Unknowns A and C also readily conjugated with sulfate. Unknown B was more likely to form a conjugate with glucuronic acid. Boiling with 3 N HCl is usually considered a good test for the presence of glycine conjugates, and the results indicated such conjugates with the 5-hydroxy ether and with unknown B and also probably some glycine conjugates with the phenol. In contrast to the work of Gutenmann and Lisk (1967) on the cow, we were able to establish the existence of nitrofen in the feces of the sheep.

The primary metabolites of nitrofen in the sheep are the same as those formed photochemically (Nakagawa and Crosby, 1974). In our study, only those compounds retaining the ^{14}C-labeled dichlorobenzene ring could be identified, and a comparison of our work with the work of Nakagawa and Crosby shows that, in both studies where known standards were available, the same compounds were identified. The only compound we did not have as a standard that Nakagawa and Crosby identified was 4-chlorocatechol. Possibly, unknowns A, B, or C may represent this compound. Unknowns D and E are probably polymers because they appear high on the chromatogram above but near the locus of 4,4′-bis(2,4-dichlorophenoxy)azobenzene, a polymer standard. Nakagawa and Crosby also state that unidentified polymeric material derived from 2,4-dichlorophenyl *p*-aminophenyl ether appeared during sunlight irradiation.

The forming of conjugates during metabolism of nitrofen is not restricted to sheep, Honeycutt and Adler (1975) also reported the presence of conjugates with lignin of plants. Wargo et al. (1975) found that the ^{14}C label from nitrofen was incorporated into the starch of cereal grains and proposed that the ring structure must be opened and incorporated into glucose and eventually into starch. We noted that even after enzymatic treatment, some radioactive materials did not chromatograph with the first system but did in the second system. These rather polar materials could not be identified, but likely the sheep synthesized a variety of polar compounds from fragments of the nitrofen molecule. We could find no positive evidence for the rearrangement of the chlorines.

Supplementary Material Available: Results of two-dimensional chromatography (2 pages). Ordering information is given on any current masthead page.

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Received for review December 23, 1976. Accepted May 24, 1977. This paper reflects the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the USDA nor does it imply registration under FIFRA as amended. Also, mention of a commercial or a proprietary product in this paper does not constitute an endorsement by the USDA.