DCHBA glucuronide and 2,5-dichlorophenol (Table IV). The phenol was apparently generated by decarboxylation of DCHBA.

The appearance of DCHBA as a metabolite in feces may have been the result of O-demethylation of dicamba within the digestive tract or it may have resulted from limited biliary excretion of the metabolite. Earlier work in these laboratories has shown, however, that O-demethylation of dicamba does not occur in ovine rumen fluid in vitro (Ivie et al., 1974).

Results in studies on the fate of [^{14}C]dicamba in the rat have shown that the compound is excreted primarily in the urine in the form of free dicamba, with lesser amounts of dicamba glucuronide (Tye and Engel, 1967). It was not established whether or not DCHBA was a dicamba metabolite in the rat. Thus, dicamba metabolism by ruminants may differ from that of laboratory rodents in at least two ways: The conjugation of dicamba with glucuronic acid in rats but not in cows and O-methylation with subsequent glucuronide conjugation or decarboxylation in cows but possibly not in rats.

Our studies with [^{14}C]dicamba in the cow are more definitive than, but are certainly consistent with, an earlier report on the fate of unlabeled dicamba in ruminants (St. John and Lisk, 1969). These workers showed that oral treatment of lactating dairy cattle with dicamba resulted in rapid elimination of unmetabolized dicamba in the urine and no detectable dicamba residue in milk. Our work confirms these earlier observations, but also indicates that dicamba is, in fact, metabolized to a limited extent in ruminants and that although dicamba itself is not secreted into milk, its metabolite, DCHBA, does have the potential for appearance in milk at trace levels.

On the basis of results in the current studies, exposure of livestock to low levels of dicamba through contaminated forage, feed, or water likely will be followed by rapid elimination from the body with little or no retention of residues by edible tissues. The secretion of appreciable levels of residues into the milk of exposed animals seems highly unlikely.

ACKNOWLEDGMENT

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In Vitro Rumen Metabolism of ^{14}C-Labeled Oxamyl and Selected Metabolites of Oxamyl

Irvin J. Belasco and John Harvey, Jr.*

The metabolism of radiolabeled oxamyl, methyl *N',N'*-dimethyl-*N*-[(methylcarbamoyl)oxy]-1-thiooxamimidate-*1*-^{14}*C*, by rumen microorganisms, in vitro, was rapid. Essentially all (99%) of the oxamyl had been metabolized within 6 h. The major metabolites after 24 h of incubation were methyl *N*-hydroxy-*N',N'*-dimethyl-1-thiooxamimidate and *N,N*-dimethyl-1-cyanoformamide (DMCF), which accounted for 80% of the residual radioactivity. Separately, the in vitro rumen metabolism of metabolite A, the glucose conjugate of methyl *N*-hydroxy-*N',N'*-dimethyl-1-thiooxamimidate, yielded DMCF. In turn, DMCF was found to metabolize to *N,N*-dimethyloxamide, *N,N*-dimethyloxamic acid, and *N*-methyloxamic acid. All the metabolites isolated and characterized, except for *N,N*-dimethyloxamide, were identical with those previously demonstrated in the rat.

Oxamyl, the generic name for methyl *N',N'*-dimethyl-*N*-[(methylcarbamoyl)oxy]-1-thiooxamimidate, is the active ingredient in Du Pont's Vydate oxamyl insecticide/nematicide. It was formerly known as DPX-1410.

Information relative to the metabolic fate of oxamyl in a variety of crop plants, as well as in the rat, and the characterization of the metabolites found has been reported by Harvey et al. (1978) and by Harvey and Han (1978a). Also, the degradation of oxamyl in water and its decomposition and movement in soil under laboratory and field conditions have been reported (Harvey and Han, 1978b).

The findings reported in this study describe the metabolism of oxamyl, *N,N*-dimethyl-1-cyanoformamide, and the glucose conjugate of methyl *N*-hydroxy-*N',N'*-dimethyl-1-thiooxamimidate (metabolite A) in the presence of rumen microorganisms.

E. I. du Pont de Nemours & Co., Inc., Biochemicals Department, Experimental Station, Wilmington, Delaware 19898.

0021-8561/80/1428-0689$01.00/0

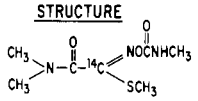
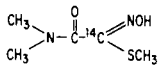
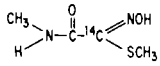
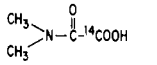
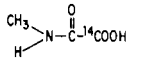
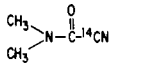
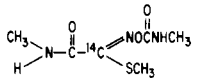
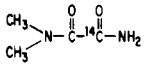
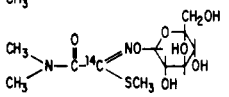
COMPOUND	NAME	STRUCTURE
OXAMYL	METHYL N',N'-DIMETHYL-N[(METHYLCARBAMOYL) OXY]-1-THIOOXAMIMIDATE	
(I)	OXIMINO METABOLITE METHYL N-HYDROXY-N',N'-DIMETHYL-1-THIOOXAMIMIDATE	
(II)	METHYL N-HYDROXY-N'-METHYL-1-THIOOXAMIMIDATE	
(III)	N,N-DIMETHYLOXAMIC ACID	
(IV)	N-METHYLOXAMIC ACID	
(V)	DMCF N,N-DIMETHYL-1-CYANOFORMAMIDE	
(VI)	METHYL N'-METHYL-N-[(METHYLCARBAMOYL)OXY]-1-THIOOXAMIMIDATE	
(VII)	N,N-DIMETHYLOXAMIDE	
METABOLITE A	GLUCOSE CONJUGATE OF I	

Figure 1. Names and structures of oxamyl and related compounds.

Table I. Composition of Nutrient Salt Solutions

	g/L
nutrient solution A	
$NaHCO_3$	10.2
NaH_2PO_4	7.23
Na_2HPO_4	6.06
KCl	1.45
NaCl	1.45
$MgCO_3$	0.17
Na_2SO_4	0.83
nutrient solution B	
$CaCl_2$	0.34
$FeCl_3$	0.49

EXPERIMENTAL SECTION

Chemicals. [1-^{14}C]oxamyl with a radiopurity of >99% was synthesized as described by Harvey et al. (1978). The synthesis of ^{14}C-labeled metabolite A and [^{14}C]dimethylcyanoformamide (DMCF), both with radiopurities of >99%, has been reported by Harvey and Han (1978a). The preparation of reference compounds (Figure 1) for comparison with oxamyl metabolites was also described earlier (Harvey et al., 1978).

In Vitro Rumen Procedure. The rumen fluid was taken from a rumen fistulated Holstein cow (University of Delaware), which had been maintained on a corn-silage and grain ration with access to pasture. The rumen sample was removed before the morning feeding and centrifuged at approximately 700 rpm for 3–5 min to remove the coarser feed particles. Fifty-milliliter aliquots of rumen fluid were transferred to nine Erlenmeyer flasks (250 mL) which contained 1.0 g of cellulose, 0.1 g of starch, 0.1 g of dextrose, 80 mg of urea, 10 mL of nutrient solution A (Table I), and 5 mL of nutrient solution B (Table I).

The contents of three flasks were supplemented with 10 mL of 0.1% oxamyl aqueous solution having 31.8 μCi of radioactivity. Similarly, the contents of three other flasks were supplemented with 10 mL of 0.15% DMCF aqueous solution having 2.08 μCi of radioactivity. Only a limited quantity of radiolabeled metabolite A was available (1.61 μCi). Consequently, this compound was dissolved in 10 mL of water and used as the substrate in a single flask. The remaining two flasks served as controls.

The solutions in the flasks were incubated at 38 ± 0.1 °C in a constant temperature bath while purging with nitrogen at a flow rate of 10–20 mL/minute to maintain anaerobic conditions. The exit gas from each flask was passed through individual scrubbers containing 1 N NaOH to trap any $^{14}CO_2$ or volatile radioactive organic compounds that may have formed. One flask from each of the oxamyl and DMCF treatments was removed after 1, 6, and 24 h. The single flask with metabolite A was incubated for 24 h. The contents of all flasks were frozen at −20 °C for brief periods until analyzed to prevent further metabolism.

Sample Analysis (Oxamyl and DMCF Incubations). Twenty-five-milliliter aliquots from each flask were centrifuged at 10 000 rpm for 10 min. The supernatants were decanted and the residues were washed two times with 10 mL of water. The water washes were combined with the respective supernatant solutions which were exhaustively extracted with ethyl acetate. The extracts were reduced in volume under vacuum at 50 °C to 2 mL, taking care not to evaporate the extract to dryness due to volatility of one of the metabolites, DMCF. The washed residues, consisting mainly of microbial cells and solid nutrients, were air-dried and assayed for their ^{14}C content by a standard combustion procedure, using a Packard Model 306 Tri-Carb sample oxidizer, followed by scintillation counting with a Searle Isocap 300 scintillation counter.

The caustic in the traps was radioassayed before and after treatment with a $BaCl_2$ solution to determine the extent of $^{14}CO_2$ production and the presence of other ^{14}C organic compounds.

Thin-Layer Chromatography. A known volume (usually 100 μL) of each of the concentrated ethyl acetate extract fractions was applied as a streak on a Brinkmann silica gel 60-254 TLC plate, co-spotted with standards of oxamyl and other known metabolites (I, II, V, VI, Figure 1). After development for 15 cm in ethyl acetate, the plates were radioscanned on a Varian Aerograph/Berthold Model 6000-2 automatic/integrating TLC radioscanner and radioautographed, using Kodak X-ray film, Type SB-5. Subsequently, the radioactive bands of silica gel were removed from the plates for radioassay by scintillation counting. Comparable radioactive bands of silica gel were removed from duplicate plates and were exhaustively extracted with ethyl acetate for combination gas chromatography/mass spectrometry (GLC/MS) analyses.

The mass spectra were obtained on a Du Pont Model 21-492 double-focusing mass spectrometer. Gas chromatography was carried out on a 2 ft × 2 mm i.d. glass column packed with 10% Carbowax 20M on 80–100 mesh Chromasorb WAW. The column was programmed from 80 to 220 °C at 8 °C min^{-1}. The retention time of *N,N*-dimethyloxamide was 14 min.

In a similar manner, 100–200 μL of each of the various aqueous fractions, after reduction to 2 mL under vacuum at 70 °C, was applied as a streak on cellulose TLC plates (Brinkmann, Celplate 22, 100-μm thickness), co-spotted with mono- and dimethyloxamic acids (III, IV) and methyl *N*-hydroxy-*N'*,*N'*-dimethyl-1-thiooxamimidate (I). The plates were developed in methanol/acetic acid (4:1, v/v) and were radioscanned and radioautographed as previously described prior to removal of radioactive bands of cellulose for radioassay and for metabolite extraction.

Gas Chromatography. Both DMCF and *N,N*-dimethyloxamide present in the ethyl acetate extracts were also determined by gas chromatography, using a F&M

Table II. Recovery of Radioactivity at Various Time Intervals

treatment	time of exposure, h	radioactivity (μCi) recovered in rumen fluid supernatant	rumen fluid solids[a]	gas scrubbers	total recov, %
oxamyl	0	30.50			
oxamyl	1	30.35	0.10	0.00	100
oxamyl	6	29.15	0.22	0.10	97
oxamyl	24	25.58	0.24	1.68	90
DMCF	0	2.08			
DMCF	1	2.03	0.00	0.00	98
DMCF	6	1.99	0.04	0.02	99
DMCF	24	1.50	0.04	0.27	87
metabolite A	0	1.61			
metabolite A	24	1.49		0.01	93

[a] After centrifugation and washing with water.

Model 810 gas chromatograph with a flame ionization detector and a 4:1 effluent splitter. The splitter permitted the collection of 80% of the flow from the column into vials containing scintillation solution for radioassay, with the remaining 20% going to the detector. A 2-m column (7 mm o.d. × 4 mm i.d.) packed with 10% Carbowax 20M on Chromosorb W AW was used for the chromatographic separation. The detector and injection port temperatures were 250 °C. The column temperature was programmed at 6 °C/min from an initial temperature of 150 °C to a final temperature of 250 °C. The respective retention times for DMCF and *N,N*-dimethyloxamide were 4.1 and 21.4 min, with a helium flow rate of 60 mL/min.

The injection port temperature was reduced to 100 °C during DMCF analyses to minimize the thermal degradation of residual oxamyl present in some of the extracts to DMCF.

Sample Analysis (Metabolite A Incubation). The entire incubation mixture was centrifuged to precipitate solids, which were in turn washed with several milliliters of water. The combined aqueous phases were lyophilized, and the powdery residue was washed with 50-mL portions of ethyl acetate (three times), methanol (three times), and water (one time). The radioactivity in all fractions, including the melted sublimate from the lyophilization, was determined by scintillation counting. The incubation solids were combusted for residual radioactivity.

Much of the radioactivity was recovered from the sublimate, and this radioactive material was extracted into three 150-mL portions of ethyl acetate. The extracts were combined, and a 7-mL aliquot was concentrated to 0.3 mL for injection on a 2.8 mm i.d. × 1000 mm high-pressure LC column of Porasil A (Waters Associates). The column was equilibrated with distilled-in-glass ethyl acetate at a flow rate of 0.50 mL/min.

The ethyl acetate extract of the freeze-dried residue was concentrated and examined by thin-layer chromatography on silica gel developed with ethyl acetate as described previously. The methanol extract was purified by gel filtration chromatography on Sephadex LH-20/methanol, and the major ^{14}C peak was characterized further by chromatography on Porasil A/THF, Permaphase AAX, and Aminex A-6 [Ca^{2+}] under the conditions described previously by Harvey et al. (1978) for identification of oxamyl metabolites.

RESULTS AND DISCUSSION

Oxamyl has been shown to be stable in aqueous solution (less than 5% decomposition in 24 h) at pH 7 and lower (Harvey and Han, 1978). Thus, little chemical hydrolysis would be expected to occur in the normal range of bovine rumen fluid contents (pH 5.8–7.5) (Dukes, 1955).

The total radioactivity of the supernatant solutions from all treatments decreased with time of incubation (Table II). This suggested (1) losses due to radiolabeled volatiles and/or (2) the reincorporation of metabolic carbon-14 into microbial cellular materials. Both of these factors participated in this apparent loss of radioactivity from the incubating systems (Table II). The radioactivity of the caustic in the gas scrubbers was not precipitable with an excess of barium chloride, demonstrating the absence of $^{14}CO_2$. The volatile losses were presumed to be due to DMCF, which was confirmed by subsequent analysis.

Table III. Distribution of Metabolites during in Vitro Rumen Incubation with [^{14}C]Oxamyl

compd	distribution of radioactivity, % 1 h	6 h	24 h
oxamyl	58.8	1.2	1.1
I	14.0	42.5	66.9
II	ND	1.9	1.4
III	0.4	0.9	4.6
IV	0.2	0.2	1.6
V	26.6	51.8	12.8
VI	ND[a]	0.7	1.2
VII	ND	0.8	10.4
	100.0	100.0	100.0

[a] Not detectable.

Evidence for microbial reincorporation of metabolic carbon-14 was the findings showing increasing levels of radioactivity in the washed microbial residues with increasing time of incubation (Table II) in the presence of both oxamyl and DMCF.

Comparison of the thin-layer chromatograms of the ethyl acetate extracts and the residual aqueous fractions of the in vitro rumen samples taken at various time intervals revealed the rapid metabolism of oxamyl by rumen microorganisms to I, II, III, IV, V, and VI, plus another metabolite, which was subsequently identified and confirmed by GC/MS as *N,N*-dimethyloxamide (VII) (Figure 2). The major metabolites of oxamyl after 1 h of incubation were methyl *N*-hydroxy-*N'*,*N'*-dimethyl-1-thiooxamimidate (I) and DMCF (V), accounting for 14 and 27% of the total radioactivity, respectively (Table III). Residual oxamyl accounted for 59% of the ^{14}C activity. After 6 h, the amount of residual oxamyl decreased to only 1.2% of the total radioactivity, while I and V accounted for 43 and 52%, respectively. At the end of the experimental period (24 h), the residual oxamyl was present at a level of approximately 1% of the total radioactivity, while I and V accounted for 67 and 13%, respectively. At this point, the concentration of dimethyloxamic acid (III) and dimethyloxamide (VII) accounted for 5 and 10% of the total ^{14}C activity, respectively. The minor metabolites, methyl *N'*-methyl-*N*-[(methylcarbamoyl)oxy]-1-thiooxam-

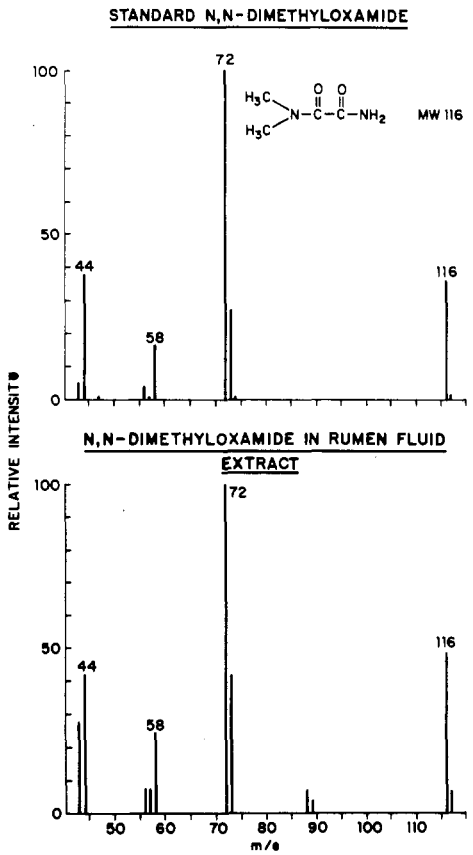


Figure 2. Mass spectra of standard *N,N*-dimethyloxamide and that found in rumen fluid extract.

Table IV. Distribution of Metabolites during in Vitro Rumen Incubation with [^{14}C]Dimethylcyanoformamide (DMCF)

compd	distribution of radioactivity, μCi		
	1 h	6 h	24 h
V (DMCF)	0.83	1.05	0.16
III	ND[a]	ND	0.06
IV	ND	ND	0.07
VII	ND	ND	0.02

[a] Not detectable.

imidate (VI), methyl *N*-hydroxy-*N'*-methyl-1-thiooxamimidate (II), and *N*-methyloxamic acid (IV), all increased in concentration over the 24-h incubation period, but each accounted for only 1–2% of the total radioactivity.

The low specific radioactivity of DMCF (V) used as a substrate in this experiment limited the ability to isolate its metabolites in the complex rumen system. Nevertheless, TLC and radioassay data (Table IV) demonstrated the biodegradation of DMCF to dimethyloxamide (VII), dimethyloxamic acid (III), and monomethyl oxamic acid (IV).

Due to the volatile character of DMCF, it was initially believed that results obtained by radioassay of TLC plate scrapings of this material, after 4–7 days of X-ray film exposure, would be markedly reduced. As a result, a radio-GLC technique was also used to analyze for DMCF. As shown in Table V, the radio-GLC values for DMCF were in excellent agreement with those obtained by TLC, thus indicating that DMCF was absorbed to the silica gel strongly enough to prevent losses.

Table V. Comparison of TLC and GLC Procedures for Determining ^{14}C-Labeled DMCF

in vitro treatment	exposure, h	total radioactivity, μCi	
		TLC	GLC
oxamyl	1	5.10	4.74
oxamyl	6	10.32	10.02
oxamyl	24	1.63	1.60

Metabolite A, a major metabolite of oxamyl in plants (Harvey et al., 1978), was largely unchanged during incubation with rat liver microsomes and was metabolized only slowly in vivo by the rat (Harvey and Han, 1978a). However, when the aqueous supernatant from the rumen fluid incubation of metabolite A was freeze-dried, 67% of the carbon-14 volatilized with the water and was recovered as [^{14}C]DMCF in the sublimate. In addition, about half of the radioactivity in the ethyl acetate of the freeze-dried residue was also identified by TLC as [^{14}C]DMCF for a total conversion of 70%. The remainder of the radioactivity in the ethyl acetate was polar and remained at the origin of the TLC plate. Gel filtration chromatography of the methanol extract of the freeze-dried residue showed that a wide range of compounds were present but that most (63%) eluted in a single band with a retention time near that of metabolite A. Further chromatography on Porasil/THF, however, showed that only a trace of metabolite A (<1%) remained unchanged. Chromatography on Permaphase AAX anion exchanger showed that most of the radioactivity was either nonionic or very weakly acidic (less acidic than compound III), and Aminex A-6 [Ca^{2+}] separated the fraction into a number of components, none of which were present in large enough quantity for further identification.

CONCLUSIONS

This study has demonstrated that oxamyl and its metabolites are extensively metabolized by rumen microorganisms in vitro by the same pathway previously reported in rat liver microsome and in vivo rat experiments. Biodegradation in the rumen is apparently so rapid and extensive that much of the oxamyl taken in the diet of a ruminant would be expected to break down before adsorption into the body tissues of a ruminant animal.

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