conjugates of 2,4,5-T and their methyl esters will assist pesticide chemists in their efforts to further elucidate the plant metabolism of 2,4,5-T and permit the evaluation of the biological activity of the amino acid conjugates. In additional work from this laboratory we will report identification of 2,4,5-T amino acid conjugates in plant tissue cultures.

ACKNOWLEDGMENT

Appreciation is expressed to Steven Loerch for technical assistance and to Jerry Porter and the Department of Food Science for the mass spectra.

LITERATURE CITED

Andreae, W. A., Good, N. E., *Plant Physiol.* **30**, 380 (1955).
Andreae, W. A., Good, N. E., *Plant Physiol.* **32**, 556 (1957).
Arjmand, M., Hamilton, R. H., Mumma, R. O., *J. Agric. Food Chem.*, submitted for publication (1978).
Burke, J. A., Gaul, J. A., Ed., "Pesticide Analytical Manual", Vol. 1, U.S. Department of Health, Education, and Welfare, 1968, Sec. 221, 12C.
Feung, C. S., Hamilton, R. H., Witham, F. H., *J. Agric. Food Chem.* **19**, 475 (1971).
Feung, C. S., Hamilton, R. H., Witham, F. H., Mumma, R. O., *Plant Physiol.* **50**, 80 (1972).
Feung, C. S., Hamilton, R. H., Mumma, R. O., *J. Agric. Food Chem.* **21**, 632 (1973a).
Feung, C. S., Hamilton, R. H., Mumma, R. O., *J. Agric. Food Chem.* **21**, 637 (1973b).
Feung, C. S., Mumma, R. O., Hamilton, R. H., *J. Agric. Food Chem.* **23**, 373 (1975).
Feung, C. S., Hamilton, R. H., Mumma, R. O., *Plant Physiol.* **58**, 666 (1976).
Good, N. E., *Can. J. Chem.* **34**, 1356 (1956).
Good, N. E., Andreae, W. A., *Plant Physiol.* **31**, 231 (1956).
Hamilton, R. H., Hurter, J., Hall, J. K., Ercegovich, C. D., *J. Agric. Food Chem.* **19**, 480 (1971).
Hill, C. M., Towns, M. B., Senter, G., *J. Am. Chem. Soc.* **71**, 257 (1949).
Hutzinger, O., Kosuge, T., *Biochemistry* **7**, 601 (1968).
Klämbt, H. D., *Planta* **57**, 339 (1961).
Row, U. U., Sanford, W. W., Hitchcock, A. W., *Contrib. Boyce Thomps. Inst.* **21**, 1 (1961).
Tillberg, E., *Physiol. Plant* **31**, 271 (1974).
Wood, J. W., Fontaine, T. D., *J. Org. Chem.* **17**, 891 (1952).

Received for review December 7, 1977. Accepted March 6, 1978. Authorized for publication as Paper No. 5402 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Supported in part by Northeastern Regional Research Project NE-53 and Regional Research Funds.

Metabolism of Oxamyl and Selected Metabolites in the Rat

John Harvey, Jr.* and Jerry C-Y. Han

The metabolism of oxamyl and of its principal plant metabolites, methyl *N'*,*N'*-dimethyl-*N*-[1-glucosyl)oxyl]-1-thiooxamimidate (metabolite A) and *N*,*N*-dimethyl-1-cyanoformamide (DMCF), was investigated by incubation with rat liver microsomes and by oral administration to preconditioned rats. Oxamyl was degraded by two major pathways: hydrolysis to the oximino compound (I), or enzymatic conversion via DMCF to *N*,*N*-dimethyloxamic acid (III). Conjugates of I, III, and the monomethyl derivatives II, IV in urine and feces were the major (>70%) elimination products. No oxamyl or other organosoluble metabolites were detected in urine, feces, or tissues. The incorporation of carbon-14 into normal amino acids accounted for most (>50%) of the radioactivity retained in tissues. Metabolite A was somewhat resistant to degradation, 30% of the dose being eliminated unchanged while the remaining ^{14}C was converted to the same conjugates obtained from oxamyl. DMCF was degraded and eliminated mainly as conjugates of III and IV.

This paper is the third in a series to describe the metabolism and biodegradation of oxamyl, which is the active material in Du Pont's Vydate Insecticide/Nematicide. The first paper (Harvey et al., 1978) showed that oxamyl in plants hydrolyzes first to the corresponding oximino compound (I) which in turn is conjugated with glucose to form methyl *N'*,*N'*-dimethyl-*N*-[(1-glucosyl)oxy]-1-thiooxamimidate, designated metabolite A. Additional glucose units may be added to the original glucose of the conjugate, and partial demethylation of the dimethylcarbamoyl group may also occur. In some fruits, metabolism to *N*,*N*-dimethyl-1-cyanoformamide (DMCF) was shown to occur. In soils (Harvey and Han, 1978) hydrolysis to the oximino compound followed by extensive conversion of the carbon-14 to $^{14}CO_2$ was reported. The present paper describes the fate and interrelationships of oxamyl and its principal metabolites in the rat.

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

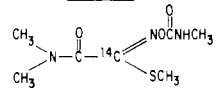
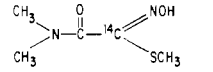
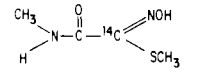
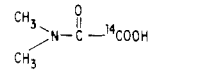
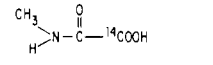
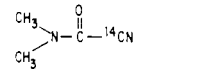
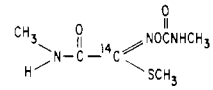
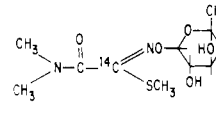
EXPERIMENTAL SECTION

Radiolabeled Materials. *[^{14}C]Oxamyl.* The synthesis of [^{14}C]oxamyl was described earlier (Harvey et al., 1978). *[^{14}C]Oximino compound* (I) is an intermediate in the synthesis of [^{14}C]oxamyl, or may be obtained readily by mild alkaline hydrolysis of [^{14}C]oxamyl.

^{14}C Metabolite A. The thallium salt of I was formed by addition of 68 mg of thallous ethoxide to 2 mL of benzene solution containing 44 mg of [^{14}C]oximino compound (I). After stirring for 30 min, 113 mg of freshly recrystallized tetraacetyl-α-D-bromoglucose (Sigma Chemical) was added, and the mixture was stirred overnight. After filtration, the filtrate was evaporated under a slow stream of nitrogen to give the acetylated glucose derivative as an oil. Hydrolysis of the acetyl groups was accomplished by addition

0021-8561/78/1426-0902$01.00/0 © 1978 American Chemical Society

Table I. Names and Structures of Compounds Related to Oxamyl

COMPOUND	NAME	STUCTURE
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	
METABOLITE A	GLUCOSE CONJUGATE OF I	

of 4.5 mL of methanol saturated with anhydrous ammonia and stirring for 4 h. Evaporation of the solvent under nitrogen gave a brown oily residue which was dissolved in 1.5 mL of methanol.

A 9 mm × 150 mm glass chromatographic column was packed to a depth of 115 mm with Davidson Code 62 silica gel. Enough silica gel to fill the rest of the column was treated as uniformly as possible with the methanolic solution of crude product. After evaporation of the solvent, the treated gel was packed on the top of the column, and the column was washed with ethyl acetate until no more radioactivity was detected in the effluent.

The product was then eluted with acetonitrile, purified by chromatography on Sephadex LH 20/methanol, and analyzed by HPLC on Porasil A/tetrahydrofuran as described by Harvey et al. (1978) to obtain 18.3 mg of ^{14}C metabolite A, sp act. 3.83 μCi/mg, radiochemical purity >99%.

[^{14}C]DMCF. Sodium [^{14}C]cyanide (.03 mmol, 250 μCi) (New England Nuclear) and dimethylcarbamoyl chloride (0.03 mmol) dissolved in 250 μL of anhydrous dimethylformamide were mixed in a 0.6-mL Microflex reaction vial (Kontes) with Teflon-lined screw cap. After 24 h at room temperature with occasional shaking, the mixture was diluted to 5 mL with ethyl acetate and filtered. The product was purified and analyzed by liquid chromatography (LC) on Porasil A equilibrated with ethyl acetate. The final product was diluted with unlabeled DMCF to obtain 15.2 mg with a sp act. of 10 μCi/mg, radiochemical purity >99%.

Reference Compounds. The preparation of reference compounds (Table I) was described earlier (Harvey et al., 1978).

EQUIPMENT AND METHODS

Liquid scintillation counting (LSC), combustion analysis (CA) of solid samples, thin-layer chromatography (TLC), and high-performance liquid chromatography (LC) were all carried out as described by Harvey et al. (1978).

Incubation with Liver Microsomes. Livers from freshly killed Charles River-CD rats were homogenized in 0.2 M Tris buffer (pH 7.3) (Sigma Chemical Co.) for 4 min at 0–4 °C with a Teflon Elvehjem homogenizer to yield a 20% (w/v) homogenate, on a fresh liver weight basis. The homogenate was centrifuged at 15 000*g* for 10 min at 0–4 °C. The supernatant microsomal plus soluble fraction was used as the liver enzyme source. A typical reaction mixture in a 50-mL test tube contained: 8 mL of liver microsomal fraction (20% w/v), 8 mL of 0.2 M Tris buffer (pH 7.3), 8 mL of NADPH (2 mg/mL) in buffer solution, and 2 mL of aqueous sample solution containing the radiolabeled test material.

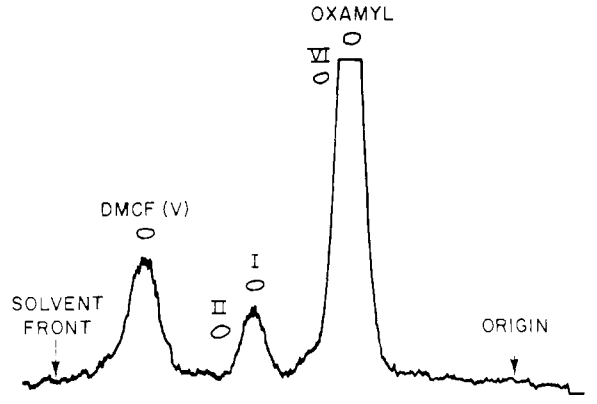


Figure 1. TLC of ethyl acetate extract from oxamyl incubation (1 mg) with liver microsomes (solvent, ethyl acetate).

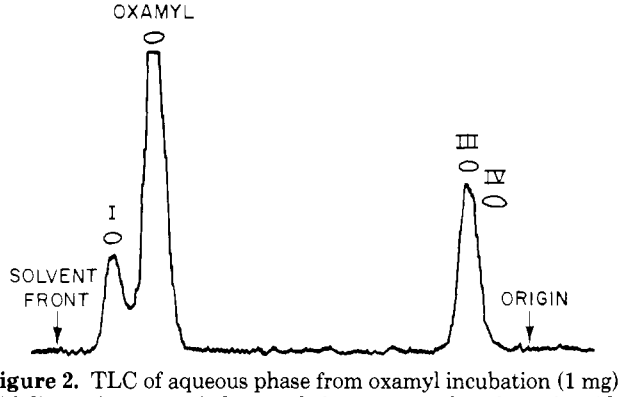


Figure 2. TLC of aqueous phase from oxamyl incubation (1 mg) with liver microsomes (solvent, ethyl acetate–methanol–acetic acid, 100:50:1).

The following ^{14}C-labeled compounds at three dose levels were used: oxamyl (0.3, 1, and 2 mg), oximino compound (I) (0.3, 1, and 5 mg), DMCF (V) (0.3, 1, and 5 mg), and metabolite A (0.3, 1, and 5 mg). A control test system which contained 1 mg of the ^{14}C-labeled compound, but lacked the liver microsomal fraction, was included in each test.

All test tubes with their contents were incubated for 2 h at 37 °C. The reaction mixtures were extracted three times with 15-mL portions of ethyl acetate. Aliquots of both phases were radioassayed (LSC) and analyzed by TLC on silica gel 60F-254, 250 μm plates (Brinkmann, Inc.) developed with ethyl acetate together with the reference materials shown in Table I. Aliquots of the aqueous phases from the oxamyl, oximino compound, and DMCF incubations were also analyzed on silica gel TLC plates developed in ethyl acetate–methanol–acetic acid solution (100:50:1, v/v/v). Representative TLC scans of the ethyl acetate and aqueous phases from an oxamyl incubation are shown in Figures 1 and 2. When the whole ethyl acetate fraction from the incubation of oxamyl was chromatographed, followed by rechromatography of the eluted bands containing oxamyl and I (Figure 1), small amounts

Table II. Distribution of Radioactivity after Treatment of Rats with [^{14}C]Oxamyl and Metabolites (Calculated as Percent of Original Treatment)

	Treatment			
	Oxamyl			
	Rat A	Rat B	Metabolite A	DMCF
External fractions				
Pre-furnace gas trap	0.00	0.24	0.10	0.28
Post-furnace gas trap	0.00	0.06	0.00	0.00
Subtotal	(0.00)	(0.30)	(0.10)	(0.28)
Urine, 0-24 h	47.3	36.2	56.2	50.6
Urine, 24-48 h	9.36	9.14	5.57	10.0
Urine, 48-72 h	4.55	3.06	1.70	3.74
Subtotal	(61.2)	(48.4)	(63.5)	(64.3)
Feces, 0-24 h	3.21	16.96	0.68	2.80
Feces, 24-48 h	2.41	4.11	3.97	1.40
Feces, 48-72 h	0.80	1.93	0.61	0.65
Subtotal	(6.42)	(23.00)	(5.26)	(4.84)
Total eliminated	67.6	71.7	68.9	69.4
Body fractions				
Hide (skin and hair)	6.98	12.55	4.16	4.88
Carcass	6.34	4.18	4.18	5.07
Gastrointestinal tract	4.76	1.32	1.67	1.83
Liver	1.58	0.20	1.43	2.69
Blood	1.55	2.13	1.57	1.65
Kidneys	0.19	0.27	0.25	0.41
Testes	0.13	0.09	0.11	0.08
Lungs	0.11	0.16	0.22	0.37
Heart	0.11	0.14	0.08	0.17
Muscle	0.11		0.11	0.15
Spleen	0.05	0.06	0.06	0.12
Brain	0.03	0.36	0.02	0.05
Fat	0.03	0.08	0.01	0.02
Subtotal	(22.0)	(21.5)	(13.9)	(17.5)
Total recovery	89.6	93.2	82.8	86.9

of the corresponding monomethyl compounds were observed and determined.

The aqueous solutions from the incubations with ^{14}C metabolite A were evaporated to dryness on a rotary evaporator, and the residue was transferred quantitatively to methanol. Nonradioactive contaminants in the methanolic solutions were removed by gel filtration on Sephadex LH-20 using methanol as carrier. The radioactive portion in the effluent was transferred to chromatographic grade tetrahydrofuran (THF) and analyzed on Porasil A equilibrated with THF containing 0.5% water as described by Harvey et al. (1978), Figure 3.

Treatment of Rats with Oxamyl. A male Charles River-CD rat (rat A), weighing 263 g, was given a diet composed of ground Purina Laboratory Chow to which had been added 1% corn oil and 50 ppm nonradiolabeled oxamyl. Thirty-two days later, when the animal weighed 393 g, he was given by intragastric intubation 2 mL of peanut oil containing 1.0 mg (3.74 μCi) of [^{14}C]oxamyl and immediately placed in a glass metabolism unit (Stanford Glassblowing Labs, Inc.) through which 500 mL min^{-1} of dried and carbon dioxide-free air was drawn. The effluent air from the chamber was first scrubbed with 4 N aqueous sodium hydroxide to remove respiratory carbon dioxide and then conducted through a 3 ft long × 1 in. diameter quartz tube, the 6-in middle section of which had been packed with cupric oxide and heated to 700 °C by an electric furnace. Its purpose was to oxidize to carbon dioxide any volatile organic compounds not trapped in the first sodium hydroxide trap. Any carbon dioxide so generated was collected by scrubbing in a second sodium hydroxide trap.

Urine, feces, and the contents of the traps were collected at 24-h intervals. After 72-h, the animal (wt, 394 g) was lightly anesthetized with chloroform; blood was removed by syringe and needle from the heart. The animal was killed with an overdose of chloroform and the following organs and tissues were surgically removed: brain, lungs, heart, liver, spleen, kidneys, testes, gastrointestinal tract, and portions of muscle and fat. The remainder of the carcass, after removal of the hide, was ground repeatedly in a small meat grinder to obtain a uniform mixture.

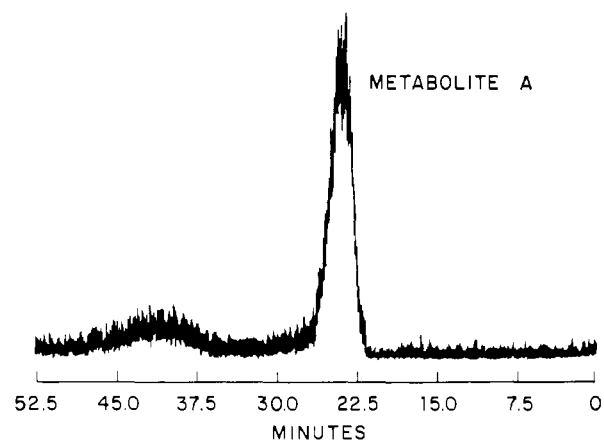


Figure 3. LC of radioactivity in aqueous phase after incubation of ^{14}C metabolite A (1 mg) [Porasil A/THF (0.5% H_2O)].

A second Charles River-CD rat (Rat B), weighing 143 g, was preconditioned for 18 days on a diet containing 150 ppm unlabeled oxamyl. This animal (weight 225 g) was treated like the first with 1.03 mg (5.6 μCi) of [^{14}C]oxamyl and immediately placed in the metabolism unit. Fractions were collected, and after the 72-h test period, this animal (250 g) was killed and dissected as previously described.

Total ^{14}C content of gas trap solutions and urine was determined by LSC. Aliquots of feces, tissues, and organs

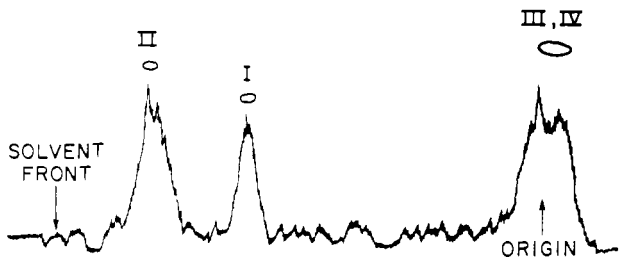


Figure 4. TLC of 24-h urine after treatment with methanolic hydrochloric acid (solvent, ethyl acetate).

were analyzed by combustion (CA) after freeze-drying. Recovery of radioactivity is shown in Table II for all treatments.

Urinary Metabolites. Aliquots of urine were cochromatographed with nonradioactive oxamyl (R_f 0.27) and I (R_f 0.47) on silica gel GF TLC plates (Analtech) developed with ethyl acetate. Further characterization of urinary elimination products was obtained by the use of two gel permeation columns in sequence to separate and purify metabolites on the basis of molecular size. The first was a 12.5 mm × 1000 mm Sephadex G-15 (Pharmacia Fine Chemicals, Inc.) column equilibrated with water at 3.0 mL min^{-1}. After evaporation of water from metabolite-containing fractions, and transfer of the radioactive material to methanol, a 9 mm × 1000 mm column of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.) equilibrated with distilled-in-glass methanol (Burdick and Jackson Laboratories, Inc.) was used for further resolution.

A 2.8 mm × 1000 mm column packed with XE-255 strong anion-exchange resin on <37 μm glass beads as described by Kirkland (1970) and equilibrated with 0.004 M phosphate buffer, pH 5.3 at 0.50 mL min^{-1}, was used to determine if radioactive materials were anionic.

Additional urine aliquots were partitioned with ethyl acetate to extract any organosoluble compounds which might be present. Because treatment of a portion of the 0–24 h urine with β-glucuronidase–aryl sulfatase (C. F. Boehringer & Soehne) did not liberate any ethyl acetate extractable radioactivity from the aqueous phase, degradation by acid hydrolysis was then attempted.

The aqueous phases were freeze-dried individually without loss of radioactivity from the residue. Aliquots of the residues were dissolved in anhydrous methanolic HCl reagent (Supelco, Inc.) and incubated at 36 °C for 24 h. The reagent was evaporated in a stream of nitrogen and the dry residue redissolved in methanol. Portions of these solutions were applied to silica gel TLC plates, 60-F-254 (Brinkmann, Inc.) along with reference solutions of I–IV which had been exposed to the same reagent. One TLC plate was developed for 15 cm with ethyl acetate; the other with ethyl acetate–chloroform 1:1 (v/v). Radioactivity was located as previously described. A typical radioscan is shown in Figure 4 with the positions of the reference compounds marked. The amount of radioactivity in each radioactive band above the origin was determined by adding the silica gel in each band directly to scintillation cocktail and counting.

In order to characterize the polar ^{14}C materials, remaining after methanolic HCl treatment, the silica gel at the origin of the TLC plates was scraped off and eluted with hot 50% aqueous methanol (90% recovery). After evaporation of the solvent on a rotary evaporator, the dry residues were treated with 5 mL of BCl_3–2-chloroethanol reagent (Supelco, Inc.) and incubated at 37 °C for 24 h. Following evaporation of the reagent in a stream of nitrogen, the derivatized compounds were dissolved in 10 mL of ethyl acetate and washed with 5 mL of water to remove

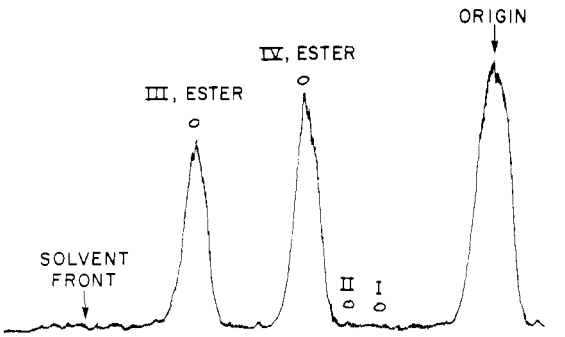


Figure 5. TLC of polar fraction from urine after treatment with BCl_3–$ClCH_2CH_2OH$ (solvent, ethyl acetate–chloroform, 1:1).

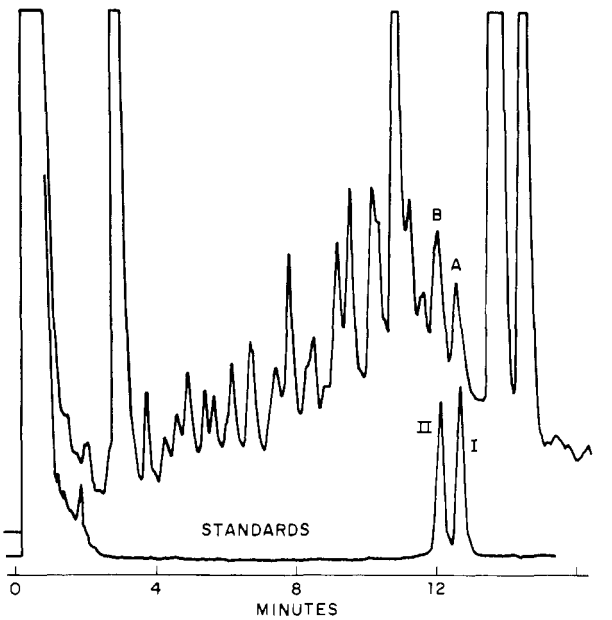


Figure 6. GC of 24-h urine after treatment with methanolic hydrochloric acid.

inorganic contaminants. The aqueous phase was reextracted twice with 10-mL portions of ethyl acetate. The organic phases were combined and concentrated to 2 mL. Aliquots were streaked on silica gel TLC plates (Brinkmann) together with reference spots of III and IV, derivatized in the same manner. The plates were developed in three solvent systems: ethyl acetate, ethyl acetate–chloroform 1:1 (v/v), or chloroform–methanol 9:1 (v/v). A typical radioscan of these derivatives is shown in Figure 5. In all three solvent systems the spots for the β-chloroethyl esters of III and IV coincided with the two mobile radioactive unknowns.

The methanolic HCl hydrolysate of the urine was also analyzed with a F&M Model 810 gas chromatograph equipped with a flame ionization detector using a glass column (6 ft × 0.25 in.) containing 30% OV-101 on 80–100 mesh Chromosorb W. Column temperature was programmed from 100 to 250 °C at 15 °C/min. The chromatograph was equipped with a 10 to 1 splitter, which permitted trapping of 90% of the effluent gas in scintillation cocktail for radioassay. Samples of I and II were chromatographed as standards. As shown in Figure 6, there was agreement between the retention times of the standards and peaks designated A and B. The radioactivity in each peak of the chromatogram in Figure 6 was determined by scintillation counting. Peaks A and B accounted for 18 and 23% of the injected radioactivity, respectively, while no other peak contained as much as 1% of the radioactivity injected.

Table III. Oxamyl Metabolites in Urine and Feces after Treatment with [^{14}C]Oxamyl (as Percent Total Radioactivity Present)

Conjugates of	0-24 h		24-48 h		48-72 h		Total	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
I	18	20	5	5	1	1	14.5	12.6
II	23	21	26	24	28	29	23.9	22.2
III	21	16	13	19	15	15	18.0	16.5
IV	22	18	22	25	32	35	22.6	20.7
							79.0	72.0

Urine samples were also reacted directly with BCl_3–$ClCH_2CH_2OH$ reagent without prior treatment with methanolic HCl. These mixtures were analyzed by TLC as described. The results of all these analyses are shown in Table III.

Feces Metabolites. Approximately 1 g of each of the three freeze-dried feces collections was mixed with 50 mL of distilled water and extracted with ultrasonic vibration for 10 min. After centrifugation, the supernatant liquid was decanted, and the extraction repeated twice more. The efficiency of extraction was 81 to 88%. The feces extracts were partitioned with ethyl acetate, and the aqueous phases were freeze-dried without loss of radioactivity from the residue. The residues from the feces extracts were hydrolyzed by methanolic HCl and by BCl_3–$ClCH_2CH_2OH$ and analyzed by TLC as described for the urine. The results are shown in Table III.

Tissue Residues. Samples of coagulated rat blood, freeze-dried liver, and skin and hair were extracted with ethyl acetate. No radioactivity (<1%) was removed by the ethyl acetate.

Because radioactivity from administered [^{14}C]oxamyl has previously been found incorporated into natural products (Harvey et al., 1978; Harvey and Han, 1978), it seemed logical to hydrolyze the protein of selected tissues and investigate the liberated amino acids for ^{14}C content. Two grams of coagulated blood (6.95×10^4 dpm) and 4 g of chopped rat skin and hair (7.82×10^4 dpm) were used for amino acid analysis. Each sample was placed in a round-bottom flask and subjected to protein hydrolysis with 100 mL of 6 N HCl for 24 h under reflux conditions. The hydrolyzed mixture was centrifuged and the supernatant was evaporated with a rotary evaporator at 60 °C to remove water and most of the hydrochloric acid. Twenty milliliters of distilled water was added and the solution quantitatively transferred to an ion-exchange column containing ca. 20 mL of 100–200 mesh Dowex 50W-X8 (H^+ form) resin to isolate amino acids. The solution was passed through the ion-exchange column at 0.5 mL/min, and the column was washed with 600 mL of distilled water at a rapid flow rate (ca. 10 mL/min). After all water had passed through the resin, the amino acids were eluted with 120 mL of 1 N NH_4OH at 0.5 mL/min. The eluate was collected in a round-bottom flask and the solvent removed with a vacuum rotary evaporator at 60 °C. The amino acid mixture was converted to the *O*-*n*-butyl-*N*-trifluoroacetyl derivatives as described by Lamkin and Gehrke (1965) and Gehrke et al. (1968). Acetone solutions of the derivatives were used for both GC and GC–MS analysis. The samples were injected into a Microtek 220 gas chromatograph equipped with flame ionization detector, using a glass column (6 ft × 0.25 in.) containing 3% OV-17 on 80–100 mesh Chromosorb W (HP). Column temperature was programmed from 80 to 225 °C at a rate of 7.5 °C/min. The gas chromatograph was equipped with a splitter, which permitted trapping of 65% of the effluent gas in a scintillation cocktail for radioassay. A typical gas chromatographic scan of the derivatized amino acids is shown in Figure 7. Gas chromatographic–mass spectral confirmation of the above amino acids was also obtained.

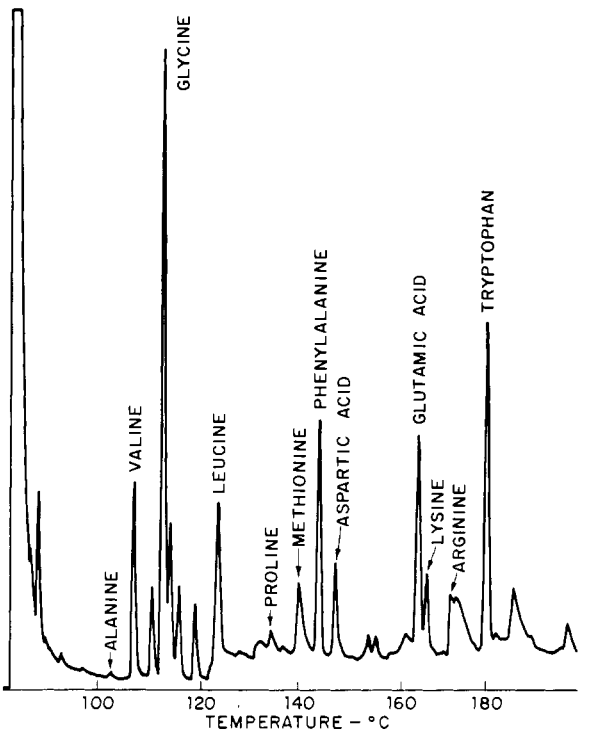


Figure 7. GC of *O*-butyl-*N*-TFA derivative of amino acids from rat skin and hair.

Table IV. Distribution of Radioactivity in Amino Acids of Selected Tissues from [^{14}C]Oxamyl-Treated Rats

	Percentage of ^{14}C in sample
Skin and Hair	
Alanine and valine	5.1
Glycine	9.1
Leucine	5.4
Proline and methionine	3.2
Phenylalanine and aspartic acid	10.2
Glutamic acid, lysine, and arginine	10.2
Tryptophan	8.0
	51.2
Blood	
Alanine, valine, and glycine	7.3
Leucine	9.4
Proline, methionine, and aspartic acid	5.1
Glutamic acid and lysine	11.8
Arginine and tryptophan	9.3
	42.9

Repeated injections of the derivatized samples were made, and the amino acid peaks were trapped in separate vials of scintillation cocktail for counting. The results are shown in Table IV.

Treatment of a Rat with Metabolite A. A male Charles River-CD rat, weighing 250 g, was given a water supply containing 1540 ppm of nonradiolabeled metabolite

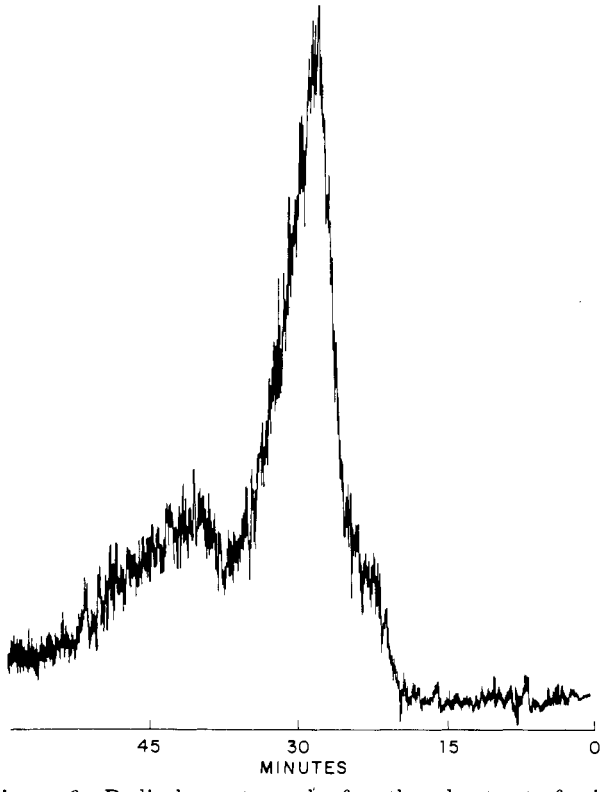


Figure 8. Radiochromatograph of methanol extract of urine (Sephadex LH20/methanol).

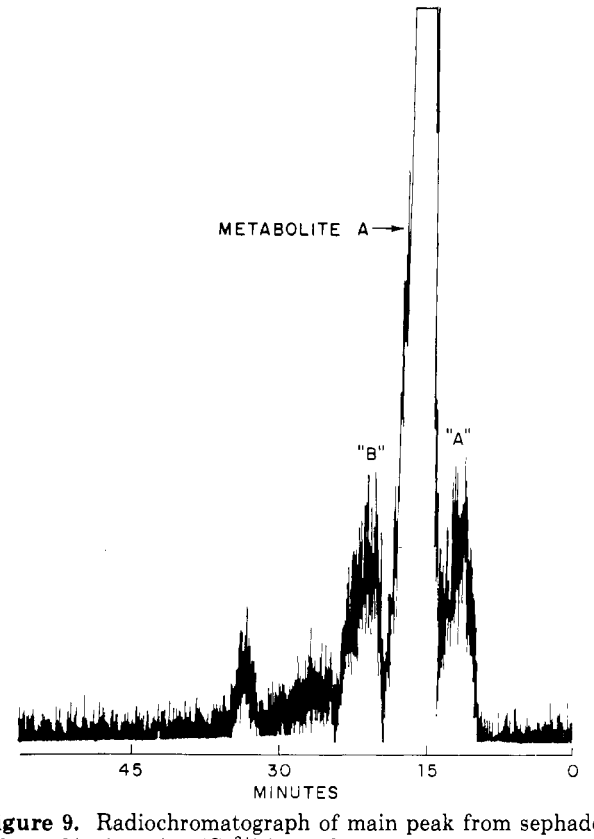


Figure 9. Radiochromatograph of main peak from sephadex column [Aminex A-6 (Ca^{2+})/water].

A. Eight days later, when the animal weighed 277 g, he was given by intragastric intubation 4 mL of an aqueous solution containing 0.67 mg (3.77 μCi) of ^{14}C metabolite A and immediately placed in the metabolism unit. This ^{14}C metabolite A had been isolated from field-grown alfalfa treated with [^{14}C]oxamyl (Harvey et al., 1978). Fractions were collected and after the 72-h test period, this animal (307 g) was killed and dissected as previously described (Table II).

Urinary Metabolites. The rat urine samples were combined and freeze-dried without loss of radioactivity. The resulting powdery residue was extracted exhaustively in turn with ethyl acetate, and then with methanol. The insoluble residue after the extraction was resuspended in water. Because of the low amount of radioactivity in the ethyl acetate extracts (<1%) and the aqueous suspension (2%), these fractions were not investigated further.

The methanol extracts after concentration were chromatographed in aliquots on a 9 mm × 1000 mm column of Sephadex LH-20 equilibrated with distilled-in-glass methanol at 1.6 mL min^{-1}.

A single major peak eluting 24–35 min after injection was obtained that accounted for 64% of the radioactivity in the urine (Figure 8). Because this time coincided with the retention time of the dosing material (metabolite A), the identification of this material was carried out in the following manner. The eluate was concentrated to remove most of the methanol and the residue was dissolved in water. Aliquots were chromatographed on a 6 mm × 500 mm column of Aminex A-6 (BioRad Laboratories) in the Ca^{2+} form, equilibrated with water at 0.50 mL min^{-1}. This procedure was reported earlier for purification of metabolite A in difficult extracts (Harvey et al., 1978). Three radioactive peaks were observed, the major one (70%) coinciding with the retention time of metabolite A (Figure 9). This material was pure enough that after evaporation of the water, the radioactivity could be transferred to tetrahydrofuran (THF) and chromatographed by adsorption on the 2.8 mm × 1000 mm Porasil A column equilibrated with THF (0.5% water) as described earlier. All radioactivity in the extract chromatographed as metabolite A. This identification was confirmed by conversion of the fraction to the trimethylsilyl derivative and analysis by gas chromatography–mass spectrometry (Harvey et al., 1978).

Of the two minor peaks eluted off Aminex A-6 (Ca^{2+}) by water, one (designated A, Figure 9) accounted for 13% of the radioactivity in the major peak off Sephadex LH-20 while the other (designated B, Figure 9) accounted for 11%. Characterization of A and B was achieved by individually subjecting these fractions to anhydrous methanolic HCl hydrolysis, followed by reaction of the unhydrolyzed polar fraction with BCl_3–$ClCH_2CH_2OH$. The remaining radioactivity (36% of total in the urine) separated from the major peak on Sephadex LH-20 (Figure 8) was also characterized by the same procedure.

Tissue Residue. The freeze-dried liver (4.1 g) and a 5-g sample of the freeze-dried homogenized carcass were each submitted to 16 h of continuous extraction with methanol in a Soxhlet extractor. The resulting extracts were concentrated and analyzed by TLC for the presence of I and II. The tissue after extraction was treated with Pronase (CalBiochem) to hydrolyze all protein into its constituent amino acids (Nomoto et al., 1960). The radioactivity solubilized by Pronase treatment was characterized by chromatography on Bio-Gel P-2. The amino acids were isolated as a fraction by adsorption on 50–100 mesh Dowex 50W-X8 (H^+ form) cation-exchange resin as previously described. Low specific activities of the amino acids precluded identification of specific amino acids by gas chromatography.

The rat blood was centrifuged to precipitate blood cells, which were washed with water and the washings added to the original serum (aqueous) phase. The aqueous phase was extracted with ethyl acetate, then heated to precipitate

Table V. Liver Microsomal Metabolism of [^{14}C]Oxamyl and DMCF Percentage Compositions of the Reaction Products

	Oxamyl	Oximino compd	DMCF	Mono-methyl oxamyl	Mono-methyl oximino	*N,N*-Dimethyl-oxamic acid
Oxamyl, control	85.7	12.9				
Oxamyl, 0.3 mg	37.6	15.5	31.6	3.8	1.1	8.8
Oxamyl, 1.0 mg	59.0	12.7	17.1	2.5	0.7	7.3
Oxamyl, 2.0 mg	60.0	10.4	19.6	2.1	0.5	6.8
DMCF, control			99.9			
DMCF, 0.3 mg			79.6			20.4
DMCF, 1.0 mg			87.9			12.1
DMCF, 5.0 mg			93.9			6.1

heat-coaguable protein. The original precipitated cells and the heat-precipitated protein were treated with Pronase and the resulting solutions characterized as described above.

Treatment of a Rat with DMCF. A male Charles River-CD rat, weighing 255 g, was maintained on a diet composed of ground Purina Laboratory Chow and 450 ppm unlabeled DMCF. Seven days later when the animal weighed 283 g, he was given by intragastric intubation 4 mL of an aqueous solution containing 1.1 mg (10.7 μCi) of ^{14}C-labeled DMCF and immediately placed in the metabolism chamber. Fractions were collected and, after the 72-h test period, this animal (297 g) was killed and dissected as previously described (Table II).

The rat urine samples were combined and freeze-dried. The resulting powdery residue was extracted in turn with ethyl acetate (3×) and methanol (3×). The insoluble residue after the extractions was resuspended in water. Overall recovery was 92%. Because of the low amount of radioactivity in the ethyl acetate extracts (0.3%) and aqueous suspension (5%), these fractions were not investigated further. A portion of the concentrated methanol extract of urine was evaporated to dryness, and reacted with BCl_3–$ClCH_2CH_2OH$ reagent. The reaction products were worked-up and analyzed by TLC as previously described.

Samples of freeze-dried liver, carcass, and hide were extracted with ethyl acetate to remove any organosoluble ^{14}C residues. The tissue after extraction was treated with Pronase and amino acids in the solubilized fraction were isolated by chromatography on 50–100 mesh Dowex 50 W-X8 (H^+ form) cation-exchange resin. Low specific activity of the amino acids precluded identification of specific amino acids by gas chromatography.

Rat blood was centrifuged to precipitate blood cells which were washed with water and the washings added to the original serum (aqueous) phase. The clear liquid was extracted with ethyl acetate to remove any organosoluble ^{14}C residues. After the extraction, the water-soluble radioactivity was characterized by gel filtration on Bio-Gel P-2. The original precipitated blood cells and a portion of the water-soluble serum radioactivity were each subjected to Pronase treatment, after which the soluble radioactivity was recharacterized on Bio-Gel P-2.

RESULTS AND DISCUSSION

Incubation with Liver Microsomes. The incubation of oxamyl with rat liver microsomes for 2 h resulted in a mixture of six compounds (Table V). In addition to intact oxamyl, major amounts of the corresponding oximino compound (I), DMCF (V), and *N,N*-dimethyloxamic acid (III) were found. Small amounts of the monomethyl oxamyl (VI) and oximino compound (II) were detected, indicating that N-demethylation was occurring at a slower rate. The control incubation (no microsomes) produced only the corresponding oximino compound (I) in approximately the same amount as the microsome incubations, suggesting that hydrolysis is not mediated by the liver enzyme system.

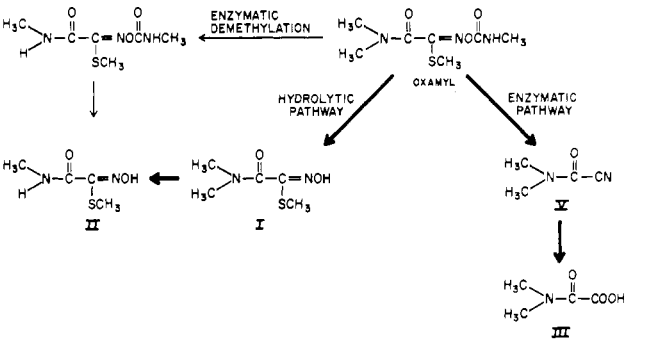


Figure 10. Rat liver microsomal metabolism of oxamyl.

Incubation of DMCF (V) with liver microsomes produced *N,N*-dimethyloxamic acid (III) as the only product (Table V) other than unreacted starting material. Liver microsomes were without effect on the oximino compound (I), and more than 99% of I was recovered intact. In the incubation of metabolite A with liver microsomes, >90% of the metabolite A was recovered intact in all treatments. A small amount of a very polar material with a longer retention time was detected by LC on Porasil (Figure 3). This is believed to be the glucuronide conjugate of the oximino compound, an oxidation product of metabolite A.

The identification of all metabolites from the incubations was confirmed by mass spectroscopic analysis after isolation by TLC and HPLC as appropriate. Only the minor metabolite from metabolite A was available in insufficient quantity for MS analysis.

Thus, oxamyl degraded in the presence of liver microsomes by two major and distinctly different pathways. The first route, which we have designated as the "hydrolytic pathway", since it does not appear to require the microsomal fraction, consists simply of hydrolysis of oxamyl to the corresponding oximino compound (I), which is itself resistant to further degradation by liver microsomes. The second, or "enzymatic pathway", involves degradation of oxamyl to DMCF (V) which is in turn degraded to *N,N*-dimethyloxamic acid (III). These two pathways are illustrated in Figure 10. Superimposed on both of these pathways is another slower enzymatic process which results in partial demethylation of the dimethyl carbamoyl group.

The results of these experiments were confirmed and extended by the in vivo studies with oxamyl, metabolite A, and DMCF in rats.

In Vivo Studies. Material Balance. Each rat displayed a remarkably similar pattern for the elimination of radioactivity regardless of which ^{14}C compound was administered (Table II). Most of the dose (68–72%) was eliminated within 72 h. Most of this material was recovered in the urine, with a lesser amount in the feces. Little, if any, radioactivity appeared in the expired air (<0.3%). Low levels of radioactivity were distributed

throughout the body at the conclusion of the experiment, and the pattern of distribution was the same among tissues and organs regardless of the compound administered.

Urinary Metabolites. TLC analysis of the fresh urine of rats treated with [^{14}C]oxamyl showed that no radioactivity moved off the origin, although nonradiolabeled reference samples of oxamyl and I mixed with the urine before streaking were located above the origin in their normal position. This TLC behavior also eliminates all of the following compounds as urinary elimination products, since these all have measurable R_f values (in parenthesis): II (0.66); *N,N*-dimethyl-1-cyanoformamide, DMCF (0.73); and *S*-oxide of oxamyl (0.15); the *S*-oxide of I (0.19); the *S,S*-dioxide of oxamyl (0.42); and the *S,S*-dioxide of I (0.53). This result was confirmed by ethyl acetate extraction of urine and feces. All of these compounds, if present in the free form in urine, will partition into ethyl acetate, but less than 1% of the radioactivity was ethyl acetate soluble and TLC of this extract showed only polar ^{14}C compounds which remained at the origin. Consequently, we conclude that radioactivity is eliminated only as very polar water soluble compounds.

Gel permeation chromatography of urine showed that the radioactivity was present as a very complex mixture of compounds which eluted from the columns earlier than I, indicating that the material was present as larger molecules, presumably conjugates. Anion-exchange chromatography indicated that these materials were acidic in nature, suggesting glucuronides and/or sulfates. Because of the apparent difficulty in purifying and identifying individual elimination products, degradation of these conjugates by acid hydrolysis was used to determine what aglycons related to oxamyl were present.

Approximately 79 and 72% of the original radioactivity in urine and feces, respectively, was accounted for as conjugates of two types of compounds related to oxamyl. Conjugates of I and II were cleaved by methanolic HCl back to I and II, which were determined by TLC and GC. This type conjugate was not cleaved by Lewis acid catalyst. On the other hand, Lewis acid (BCl_3–$ClCH_2CH_2OH$) cleaved conjugates of III and IV to produce the corresponding β-chloroethyl esters, which were determined by TLC. The results of these analyses are shown in Table III. These conjugates do not appear to be attacked by β-glucuronidase–aryl sulfatase enzyme treatment, just as the glucosides of compounds I and II formed in plants are not attacked by β-glucosidase.

Acid hydrolysis of these conjugates does not lead to truly quantitative results. The reagents do not completely cleave the conjugates, while in the same time interval some of the hydrolysis product is broken down even further. The figures given in Table III represent the aglycons actually recovered. It is felt that these figures represent minimum recoveries and that these types of conjugates are actually responsible for considerably more of the radioactive elimination products.

The urine of the rat treated with ^{14}C metabolite A was likewise shown to be free of any radioactive compounds except very polar water soluble ^{14}C conjugates. Indeed, the major component (45%) of the urinary radioactivity was positively identified as unchanged metabolite A itself (30% of the original dose). It was isolated by the established LC procedures for metabolite A, and the identity of the purified product was confirmed by GC–MS study. This is entirely consistent with the rather unreactive nature of metabolite A in the in vitro liver microsome test.

Two minor ^{14}C components of the urine which were associated with metabolite A in a fraction from gel filtration chromatography on Sephadex LH-20 were designated A and B (Figure 9). They accounted for 8 and 7%, respectively, of the original urinary radioactivity. When subjected to anhydrous methanolic HCl hydrolysis, 40% of the radioactivity in A was recovered as II and 4% as I. Less than 2% of the radioactivity in B appeared as I and II combined. When the remainder of the radioactivity from A and especially B (after methanolic HCl hydrolysis) was reacted with the Lewis acid catalyst BCl_3 in the presence of 2-chloroethanol, considerable quantities of III and to a lesser extent IV were liberated (as the 2-chloroethyl esters). The amounts of radioactivity were too low, however, for generation of good quantitative data.

When the rest of the radioactivity in urine (36%) (which was separated on the Sephadex LH-20 column) was subjected to methanolic HCl hydrolysis, about 11% of the urinary radioactivity was liberated as I and 4% as II. Characterization of the reaction products from BCl_3–2-chloroethanol treatment indicated the presence of small amounts of III and IV, but good quantitative data could not be obtained. In addition to the inherent difficulties with quantitation of these hydrolysis reaction products, this situation is further complicated by the facts that (1) the initial ^{14}C dose was rather low and (2) it was possible in this case to remove and identify almost half of the radioactivity in the urine as metabolite A itself. While the amounts of each aglycon produced can not be determined exactly, we have demonstrated that the same aglycons appear as conjugates in the urine of rats treated with metabolite A as appear in the urine of rats treated with oxamyl. A total of 64% of the urinary radioactivity has been accounted for; 45% as metabolite A and 19% as conjugates of I and II. Conjugates of III and IV are present and account for a substantial part of the remainder.

The urine of the rat treated with [^{14}C]DMCF was also found to be free of organosoluble ^{14}C metabolites. When the reaction with BCl_3–$ClCH_2CH_2OH$ was carried out on a portion of the methanol extract of urine, 15% of the total radioactivity was accounted for by conjugates of III and 7% as conjugates of IV. Furthermore, when a portion of the methanol extract of freeze-dried urine was fractionated on Dowex cation exchanger 27% of the radioactivity was retained and eluted with ammonium hydroxide like amino acids. The presence of a large fraction in which carbon-14 has been incorporated into normal natural products (amino acids) suggests that much of the remainder of the carbon-14 has become incorporated into other natural constituents also.

Tissue Residues. The tissues of the oxamyl-treated rats which contained the largest radioactive residues were examined for the presence of metabolites related to oxamyl. Ethyl acetate extraction of these tissues failed to remove any radioactivity (<1%), indicating that oxamyl, I, II, or V were not components of the tissue residues. Ethyl acetate extraction after acid hydrolysis again failed to remove radioactivity (<3%), indicating that these compounds were not present as conjugates either.

About 51 and 43% (Table IV) of the original radioactivity in rat skin/hair and rat blood, respectively, were found to be present as ^{14}C which had been reincorporated into natural amino acids (protein). The mass spectra of the standard *n*-butyl-TFA derivatized amino acids were in excellent agreement with those obtained from hydrolysis of rat protein.

Extracts of liver, carcass, and blood from the ^{14}C metabolite A treated rat showed no radioactivity in the form of I, II, or V (<1%). The protein-hydrolyzing enzyme Pronase liberated nearly all of the radioactivity remaining

in liver, carcass, and blood after extraction. When the Pronase-solubilized radioactivity was characterized by gel filtration chromatography on Bio-Gel, most of the radioactivity was recovered in the molecular weight fraction characteristic of amino acids. The amino acid fraction as isolated on a cation-exchange resin accounted for 64% of the Pronase-solubilized radioactivity in liver, 64% of that in the carcass, and 65% of that in the blood.

Ethyl acetate extracts of liver, carcass, hide, and blood from the [^{14}C]DMCF-treated rat showed very low amounts of organosoluble radioactivity. When the insoluble residues in liver were treated with Pronase, a protein-hydrolyzing enzyme, all the remaining radioactivity was solubilized. When this soluble radioactivity was characterized on Dowex cation exchanger, 45% was retained and eluted with 1 N ammonium hydroxide, behavior characteristic of amino acids. Similar results were obtained after Pronase treatment of carcass and hide where 89 and 60% of the ^{14}C residues were solubilized, respectively. Pronase appeared rather ineffective at digesting hide and hair protein. Of the solubilized radioactivity from carcass, 40% was retained on Dowex cation exchanger in the amino acids, while 23% of the solubilized radioactivity from hide appeared as amino acids.

When the blood serum (water soluble) radioactivity was characterized on Bio-Gel P-2 gel filtration medium, 46% of the radioactivity appeared in the high-molecular weight fraction (7.5–15 min) and 36% in the fraction characteristic of amino acids (22.5–32 min). When the high molecular weight fraction was treated with Pronase and recharacterized on Bio-Gel, 74% of the carbon-14 had been converted into the low molecular weight amino acid fraction. Pronase treatment of the precipitated blood solids resulted in solubilization of the small amount of radioactivity they contained. Although the remainder of the radioactivity in tissues has not been identified, the fact that it has not been found as known metabolites of oxamyl together with the finding of extensive labeling of normal amino acids suggests that many other natural products in tissues have also become radiolabeled as a result of complete breakdown of the oxamyl molecule.

CONCLUSIONS

The results of the in vivo studies may now be used to extend the findings in the in vitro liver microsome work. In the intact rat, the oximino compounds (I and II) produced initially by what we have called the hydrolytic pathway, are converted to conjugates and excreted in the urine. The remainder of the oxamyl, after undergoing conversion through DMCF to III and IV, is also converted to conjugates and excreted. The pattern of the in vitro metabolism is confirmed by the fact that the same conjugates appear in the urine regardless of which metabolite is administered to the rat. Of course, conjugates of I and II do not appear in the urine of the rat fed DMCF because the starting material is already less complex than the oximino compounds. No metabolites were observed which corresponded to the S-oxidation products of oxamyl or the oximino compound. Further metabolism of these animal metabolites has also been shown to result in incorporation of the radiolabel into normal tissue constituents, i.e., amino acids. This is consistent with the previously reported finding (Harvey et al., 1978) of incorporation of carbon-14 from oxamyl into normal natural products in plants.

ACKNOWLEDGMENT

We wish to thank Russell F. Bellina, Richard K. Brantley, and Julius Fuchs for synthesis of the reference compounds; Richard A. Jewell for synthesis of the ^{14}C metabolite A; Henry Sherman and A. M. Kaplan for assistance in the dosing of the animals and collection of samples; Richard A. Jackson and Robert W. Reiser for analytical assistance, especially the GC–MS studies.

LITERATURE CITED

Gehrke, C. W., Roach, D., Zumwalt, R. W., Stalling, D. L., Wall, L. L., Analytical and Biochemical Laboratory, Inc., Columbia, Mo., 1968.

Harvey, J., Jr., Han, J. C-Y., *J. Agric. Food Chem.* **26**, 536 (1978).

Harvey, J., Jr., Han, J. C-Y., Reiser, R. W., *J. Agric. Food Chem.* **26**, 529 (1978).

Kirkland, J. J., U.S. Patent 3488922 (Jan 13, 1970).

Lamkin, M. W., Gehrke, C. W., *Anal. Chem.* **37**, 383 (1965).

Nomoto, M., Narahashi, Y., Murakami, M., *J. Biochem.* **48**, 593 (1960).

Received for review August 19, 1977. Accepted March 13, 1978. Most of the present work was presented at the 174th National Meeting of the American Chemical Society, Division of Pesticide Chemistry, Chicago, Ill., Aug 30, 1977.