Fate of Benomyl in Animals

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A rat and a dog eliminated >99% of single, oral doses of [2-^{14}C]benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] *via* the urine and feces within 72 hr. The major metabolite was methyl 5-hydroxy-2-benzimidazolecarbamate (5-HBC), which is present in the urine as glucuronide and/or sulfate conjugates. Based on residue analyses, dairy cows and chickens display the same route of metabolism and elimination. Other possible degradation products in animal systems include methyl 2-benzimidazolecarbamate (MBC) and methyl 4-hydroxy-2-benzimidazolecarbamate (4-HBC). Residue data from all studies, including 2-year chronic feeding tests with dogs and rats, demonstrate that benomyl and its metabolites do not accumulate in animal tissues. Milk from dairy cows and eggs from chickens contain detectable residues (0.1 ppm or less) only at high dietary levels of benomyl (25–50 ppm). Synthesis methods for all three breakdown products and for [2-^{14}C]benomyl are described.

Benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, is the active ingredient in DuPont Benlate benomyl fungicide. The compound was introduced in 1968 as a fungicide and mite ovicide candidate (Delp and Klopping, 1968), and a substantial amount of information is available in the literature on its behavior and fate in plant systems. The most recent articles include reports by Upham and Delp (1973), Siegel (1973), Baude *et al.* (1973), and Hammerschlag and Sisler (1973).

In animals, methyl 5-hydroxy-2-benzimidazolecarbamate (5-HBC) has been found as a metabolite in the urine of rats fed a diet containing 2500 ppm of benomyl (Gardiner *et al.*, 1968). Enzyme hydrolysis was used to liberate the metabolite from its glucuronide and/or sulfate conjugates. Recently, a method for the high-speed liquid chromatographic analysis of benomyl and its metabolites in cow milk, urine, feces, and tissues has been reported (Kirkland, 1973).

This paper presents the results of comprehensive studies on the fate of benomyl in animal systems, which include rats, dogs, dairy cows, and chickens. The data from the rat and dog studies are based in part on the use of [2-^{14}C]benomyl. Other data were obtained by the residue method of Kirkland (1973). Synthesis methods for [2-^{14}C]benomyl and for benomyl breakdown products are given.

EXPERIMENTAL SECTION

Rat Study with [2-^{14}C]Benomyl. A male ChR-CD rat (172 g) was given a diet of ground Purina Laboratory Chow containing 1% corn oil and 2500 ppm of nonradiolabeled benomyl. Twelve days later, the animal (264 g) was given by intragastric intubation an acetone–peanut oil mixture (2 ml; 1:9, v/v) containing [2-^{14}C]benomyl (7.7 mg, 3.24 μCi/mg, >99% radiochemical purity). The rat was immediately placed in a glass metabolism unit (Stanford Glassblowing Labs., Inc.) through which dry, CO_2-free air was drawn (500 ml/min). The exit gases were passed through a sodium hydroxide trap (200 ml, 4 *N*), then through a cupric oxide oxidation tube maintained at 700° to convert any ^{14}C-labeled organic volatiles to $^{14}CO_2$, and finally through a second NaOH trap (150 ml, 4 *N*). Urine and feces were collected at 24-hr intervals. Gas trap samples were taken at the end of approximately 6 and 24 hr for each 24-hr period. The rat (268 g) was sacrificed after 72 hr.

Aliquots of urine and gas trap contents were analyzed for ^{14}C by liquid scintillation techniques on a Nuclear-Chicago liquid scintillation system 6801. Counting efficiency was determined by internal standardization with ^{14}C-labeled toluene. Small organs were analyzed *in toto* for ^{14}C by wet combustion (Smith *et al.*, 1964). Larger organs were homogenized with water in a glass and Teflon tissue homogenizer and aliquots analyzed by wet combustion. The skin and fur were removed from the carcass and analyzed separately. The carcass itself was ground in a meat grinder prior to wet combustion. The results of the analyses for ^{14}C distribution are shown in Table I.

The urine from the first 24-hr interval contained the bulk of ^{14}C activity eliminated from the rat. A fresh urine aliquot (5 μl) was spotted on a 250-μ silica gel GF tlc plate and developed for 15 cm in ethyl acetate–methanol–glacial acetic acid (100:100:4, v/v/v) followed by development for 15 cm in methanol–water (2:1, v/v) [tlc procedure I]. The plate was placed next to Ansco Non-Screen X-Ray film for 9 days. Figure 1A shows the radioautogram and demonstrates that one major (at 11 cm) and two minor (at 6 and 2 cm) ^{14}C-containing materials were in the fresh urine.

Fresh urine (5 ml) was adjusted to pH 5 by addition of 6 *N* hydrochloric acid and hydrolyzed enzymatically at 35° for 24 hr following the addition of a β-glucuronidase–arylsulfatase solution (Boehringer and Mannheim Corp., New York, N. Y., 0.5 ml). An aliquot (5 μl) of this hydrolyzed urine sample was spotted on a tlc plate and developed as before. Figure 1B shows the radioautogram. After enzyme hydrolysis, there was only one major metabolite which was different in R_f value from all of the three previously detected compounds. These data suggest that the fresh urine contained glucuronide and/or sulfate conjugates of the true metabolite of the 2-^{14}C-labeled starting compound administered to the rat.

Figures 1C and 1D show radioautograms of these same samples, respectively, where the tlc plates were developed for 15 cm in ethyl acetate–dioxane–methanol–ammonium hydroxide (160:20:5:0.5, v/v/v/v) [tlc procedure II]. In these examples, there is a much clearer separation between the conjugates at the origin of the plate and the liberated metabolite at 5 cm. These figures show that very little if any benomyl or methyl 2-benzimidazolecarbamate (MBC) was present in the urine (<5%).

In Figure 1B, the silica gel from the tlc plate was removed in increments and counted by liquid scintillation. Eighty per cent of the total detected activity was in the metabolite spot at 12 cm. The remainder of the activity was spread fairly equally up the plate as shown in Figure 1B with only 6% remaining at the origin. This streaking phenomenon and the amount of material left at the origin of the plate were believed to be caused by the nature of the urine sample applied to the plate, which after enzymatic hydrolysis was very dark and viscous. The single

Biochemicals Department and Haskell Laboratory for Toxicology and Industrial Medicine, E. I. du Pont de Nemours & Co., Inc., Wilmington, Delaware 19898.

Table I. Distribution of Radioactivity after Treatment of Rats with [2-^{14}C]Benomyl or [2-^{14}C]MBC

	% recovered radioact.					
	[2-^{14}C]Benomyl[a]			[2-^{14}C]MBC[b]		
Sample analyzed	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
External fractions						
Urine	78.9	85.3	85.8	83.5	87.6	88.0
Feces	8.7	12.2	13.1	9.6	11.0	11.3
$^{14}CO_2$	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Acid wash of cage			0.4			0.3
Fur			0.2			0.1
Body fractions						
Blood sample			<0.01			<0.01
Brain			<0.01			0.01
Fat sample			<0.01			<0.01
G.I. tract and contents			0.2			0.3
Heart			<0.01			<0.01
Kidneys			<0.01			<0.01
Liver			0.2			0.1
Lungs			<0.01			<0.01
Muscle sample			<0.01			<0.01
Spleen			<0.01			<0.01
Testes			<0.01			<0.01
Carcass (less fur)			0.02			0.02
	87.6	97.5	100	93.1	98.6	100

[a] Overall recovery was 91.8% of calculated ^{14}C dose. [b] Overall recovery was 100.4% of calculated ^{14}C dose.

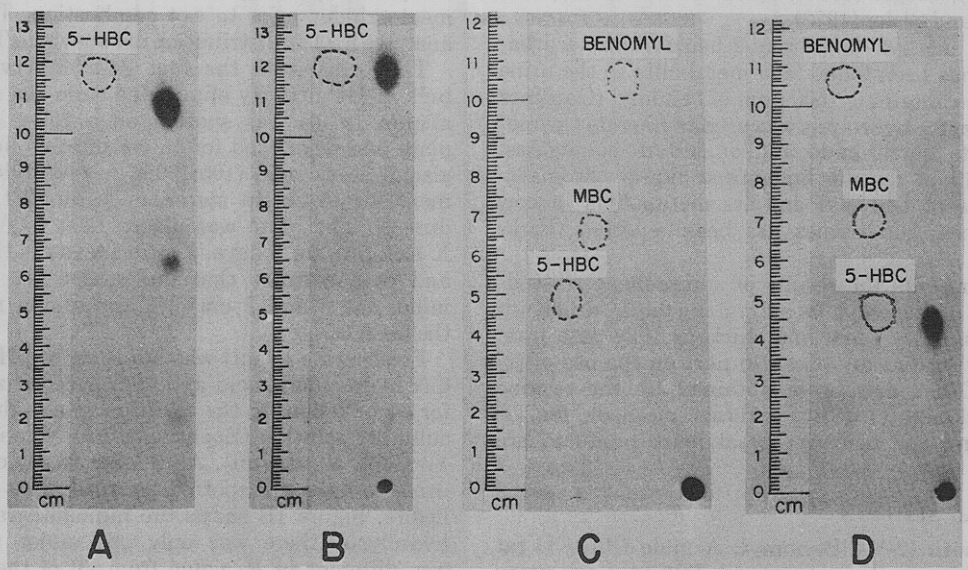

Figure 1. Tlc radioautograms of urine from a rat dosed with [2-^{14}C]benomyl: (A) fresh urine, tlc procedure I; (B) enzyme-hydrolyzed urine, tlc procedure I; (C) fresh urine, tlc procedure II; (D) enzyme-hydrolyzed urine, tlc procedure II.

major metabolite had the same R_f value as 5-HBC, a previously identified metabolite (Gardiner *et al.*, 1968).

To confirm the identity of the metabolite, the remaining enzymatically hydrolyzed urine sample was diluted with water (250 ml) and extracted continuously with diethyl ether for 48 hr. Seventy-three per cent of the activity was extracted. The extract was evaporated to dryness and the residue dissolved in methanol (5 ml) for tlc cleanup. An aliquot (200 μl) was streaked on a tlc plate which had been previously rinsed with methanol and heated to 250° to remove organic matter. The plate was developed as indicated for Figure 1B. The 5-HBC area was eluted from the plate with distilled-in-glass acetone (98% of the ^{14}C activity in the extract was in this area) and was subjected to mass spectroscopic analysis.

Figure 2 shows the mass spectra of both the isolated metabolite and an authentic sample of 5-HBC prepared by procedures given earlier (Gardiner *et al.*, 1968). The low molecular weight region of the spectrum of the isolated metabolite was complicated by extraneous material associated with the tlc procedure, but the presence of a molecular ion at m/e 207 and principal fragments at m/e 175, 162, and 148 confirms the identification of the metabolite as 5-HBC.

Rat Study with [2-^{14}C]MBC. In an experiment similar to the above, a male ChR-CD rat (178 g) was given a diet of ground Purina Laboratory Chow containing 1% corn oil and 2500 ppm of nonradiolabeled MBC. Fourteen days later, the animal (298 g) was given by intragastric intubation an acetone–peanut oil mixture (2 ml; 1:9, v/v) containing [2-^{14}C]MBC (5.28 mg, 4.74 μCi/mg, >99% radiochemical purity). The rat was immediately placed in the glass metabolism unit and the experiment proceeded as before. The rat (310 g) was sacrificed after 72 hr.

The results of the analyses for ^{14}C distribution are also shown in Table I. The urine from the first 24-hr interval again contained the bulk of ^{14}C activity eliminated from the rat. This urine was analyzed to produce almost identi-

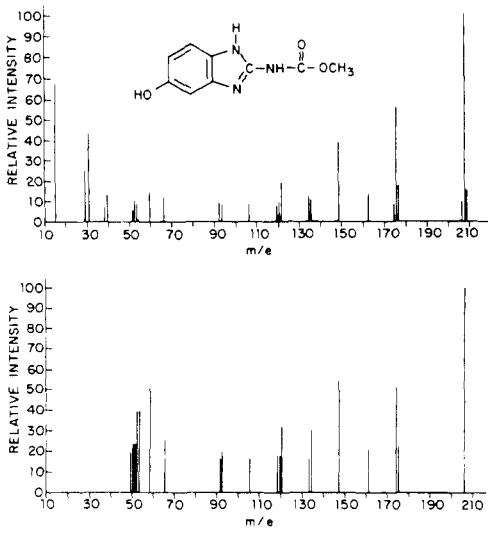

Figure 2. Mass spectra: (upper) synthetic 5-HBC; (lower) isolated rat urine metabolite.

cal results with the [2-^{14}C]benomyl study. One major and two minor ^{14}C-labeled materials were in the fresh urine. After enzyme hydrolysis and direct tlc, at least 85% of the total activity was in the single metabolite spot corresponding to 5-HBC (*vs.* 80% in the [2-^{14}C]benomyl test). No residual [2-^{14}C]MBC was detected in the urine (<5%). To confirm the identity of 5-HBC, the hydrolyzed urine was extracted continuously with diethyl ether for 48 hr as before. Eighty-eight per cent of the ^{14}C activity was extracted from the urine (*vs.* 73% in the [2-^{14}C]benomyl test). After tlc prior to mass spectral analysis, the 5-HBC area contained 96.6% of the ^{14}C activity in the extract. The mass spectrum confirmed the identity of the MBC metabolite as 5-HBC.

Dog Study with [2-^{14}C]Benomyl. A male beagle dog was fed a diet of Purina Dog Chow containing 2500 ppm of unlabeled benomyl. After 7 weeks, he was given a gelatin capsule containing [2-^{14}C]benomyl (30.8 mg, 3.24 μCi/mg, >99% radiochemical purity), and was maintained on the benomyl-containing diet for 3 days while daily urine and feces samples were collected and pooled. The dog was sacrificed 72 hr after treatment and all tissues were frozen and retained for ^{14}C analyses.

Samples of urine, feces, and tissues were analyzed for ^{14}C content by procedures similar to those described for the rat studies. Results are given in Table II. About 99% of the detected ^{14}C was eliminated from the animal *via* the feces (83%) and urine (16%) in 72 hr.

The 0–48-hr feces sample was air dried and ground in a mortar and pestle. Aliquots were hydrolyzed and extracted using the method of Kirkland (1973). About 77% of the total ^{14}C was present in the final extract and was examined by tlc. Approximately 82% of this ^{14}C corresponded in R_f value to MBC, while another 12% corresponded to 5-HBC. The major product was removed from the tlc plates and analyzed by mass spectrometry, which confirmed the presence of MBC in the extract. This analytical procedure would not distinguish between benomyl and MBC in the original feces.

Dairy Cow Feeding Study with Benomyl. Eight Guernsey dairy cows were confined to barn stanchions at a test farm near Newark, Del. Each cow received a daily ration of dried alfalfa hay (20 lb) plus a grain concentrate (10 lb) during a 1-week pretest period. During the next 32 days, two cows each were fed levels of 0, 2, 10, and 50 ppm of benomyl (from Benlate benomyl fungicide, 50% WP), based on the total weight (30 lb) of the daily ration for each cow. The benomyl was admixed with the grain concentrate portion of the ration. Benomyl ingestion levels were about 0.06, 0.3, and 1.4 mg/kg per day, respectively. Total amounts of benomyl ingested at the three feeding levels during 32 days were 0.88, 4.36, and 21.8 g, respectively. There were no apparent adverse effects on the animals or on milk production. One cow at each level was sacrificed at the end of the 32-day feeding test and the other after a 1-week withdrawal period during which untreated ration was fed. Samples of milk, urine, feces, and tissues were frozen and analyzed for benomyl and/or metabolite residues by the method of Kirkland (1973).

Table II. Distribution of Radioactivity after Treatment of a Dog with [2-^{14}C]Benomyl

Sample analyzed	% recovered radioact.[a] 24 hr	48 hr	72 hr
External fractions			
Urine	11.9	16.1	16.2
Feces	*b*	82.5	83.4
Body fractions			
Adrenals			<0.01
Blood sample			<0.01
Bone marrow sample			<0.01
Brain			<0.01
Fat sample			<0.01
G.I. tract and contents			0.13
Heart			<0.01
Kidneys			<0.01
Liver			0.31
Lungs			<0.01
Muscle sample			<0.01
Pancreas			<0.01
Prostate			<0.01
Spleen			<0.01
Stomach and contents			<0.01
Testes			<0.01
Thyroid			<0.01
	11.9	98.6	100

[a] Overall recovery was 93.3% of calculated ^{14}C dose. [b] No feces during first 24 hr.

The development of the final Kirkland (1973) residue method took place during the course of this study. As certain samples from the cows were being analyzed by liquid chromatography (lc) for the major animal metabolite (5-HBC), a second but similar metabolite was detected. Consequently, it was postulated that the unknown compound might be an isomer of 5-HBC. The material occurred in sufficient amounts in urine from cows on the 50-ppm feeding level to permit identification by lc separation and mass spectrometry.

Figure 3 shows a liquid chromatogram of a cow urine extract in which a trapping procedure was carried out on the unknown material and on the peak corresponding to 5-HBC. Aliquots of the urine extract (5 × 100 μl) were injected sequentially and the two peaks separately isolated and combined. The isolated fractions (in 0.1 *N* acetic acid–0.1 *N* sodium acetate) were extracted into distilled-in-glass ethyl acetate (2 × 5 ml), which was filtered through a bed of anhydrous sodium sulfate and evaporated to dryness in a nitrogen stream. The residues were subjected to mass and infrared spectroscopic analysis. The peak corresponding to 5-HBC in Figure 3 produced the expected mass spectral pattern of 5-HBC. The unknown peak produced a very similar mass pattern, highly suggestive of methyl 4-hydroxy-2-benzimidazolecarbamate (4-HBC). The identity of this unknown was confirmed by the synthesis of an authentic sample of this material and direct mass spectrographic comparison. Figure 4 shows the mass spectral patterns of the unknown peak and the authentic sample of 4-HBC.

The final residue method of Kirkland distinguishes be-

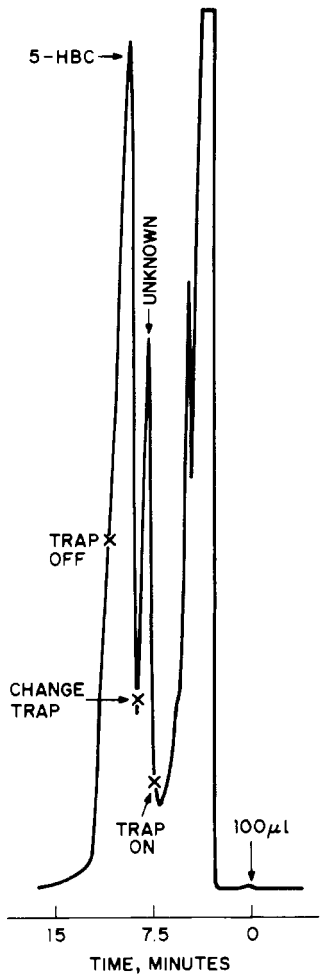

Figure 3. Lc separation of cow urine extract.

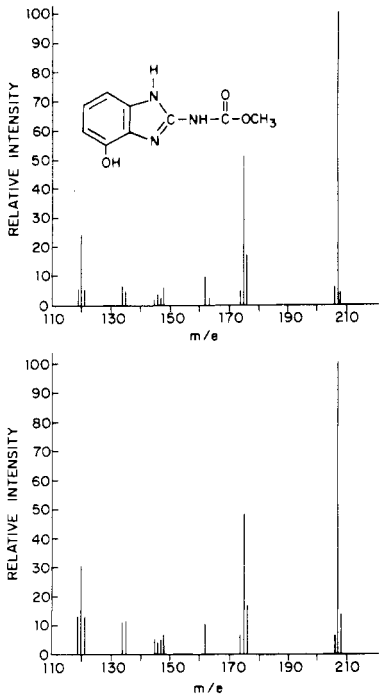

Figure 4. Mass spectra: (upper) synthetic 4-HBC; (lower) isolated cow urine metabolite.

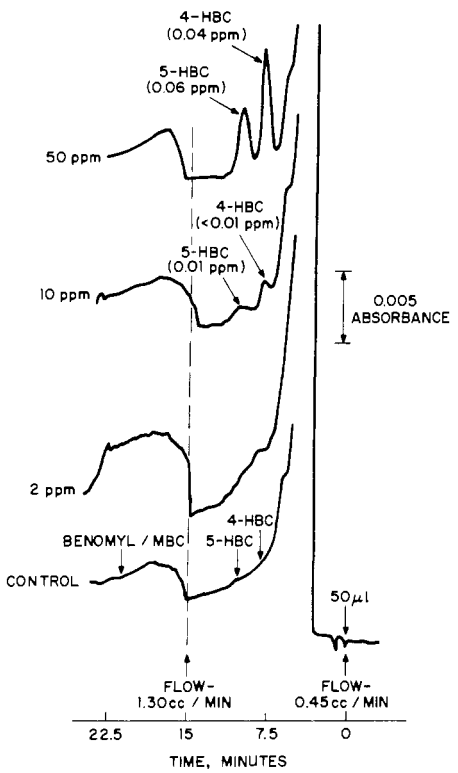

Figure 5. Lc separations on extracts of milk from benomyl-treated cows (4 days on treatment).

tween 5-HBC, 4-HBC, and benomyl–MBC, but measures the benomyl and/or MBC residue as a composite. The results of milk analyses are given in Table III. Milk samples from each cow were analyzed separately, but a.m. and p.m. milkings were composited on an equal basis prior to analysis. Additional data on separated milk indicated about a 2:1 distribution of residue between the aqueous and fat fractions, respectively. Typical lc scans on whole milk samples are shown in Figure 5. The cow tissues (liver, kidney, subcutaneous fat, lean muscle) contained no detectable residues (<0.1 ppm of benomyl-MBC; <0.05 ppm of 5-HBC or 4-HBC). Cow urine and feces residue data are given in Table IV. All positive results are corrected for average recovery of added material, based on data from control samples fortified with benomyl and/or MBC, with 5-HBC and with 4-HBC. These recovery data were reported earlier (Kirkland, 1973).

Chicken Feeding Study with Benomyl. Twenty-four White Leghorn laying hens, 3–5 lb each, were fed a diet of Purina Layena for approximately 3 weeks. At the end of this period, they were divided into three equal average weight and equal average egg-producing groups of eight hens each to receive the following diets: group I, Purina Layena (control); group II, Purina Layena + 5 ppm of benomyl (10 ppm of Benlate benomyl fungicide, 50% WP); group III, Purina Layena + 25 ppm of benomyl (50 ppm of Benlate benomyl fungicide, 50% WP). The hens were placed on their respective diets for 4 weeks. During this period, composite samples of eggs were taken on the last 2 days of each week (contents were separated from the shells). At the end of 4 weeks, composite samples of feces were taken and one-half of the animals in each of the three groups was sacrificed to obtain liver, fat, and muscle (breast). The four hens remaining in each group were placed on a control diet for 1 week prior to sacrifice. The presence of benomyl in the diet for 28 days did not adversely affect feed consumption, body weight gain, or egg production.

Samples of eggs, tissues, and feces were analyzed for benomyl and/or metabolite residues by the method of Kirkland (1973). Results are shown in Table V. Certain minor modifications to the method of Kirkland were necessary in the lc conditions to maximize the separation of

Table III. Milk Residue Data (ppm[a]); Benomyl Dairy Cow Study

Days on diet	10 ppm[b]				50 ppm[b]			
	110[c]		123[c]		128[c]		149[c]	
	5-HBC	4-HBC	5-HBC	4-HBC	5-HBC	4-HBC	5-HBC	4-HBC
0[d]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
1	0.01	<0.01			0.05	0.02		
2			0.01	<0.01			0.04	0.02
4	0.01	<0.01			0.06	0.04		
5			0.01	<0.01			0.05	0.02
7	0.01	<0.01			0.05	0.02		
8			0.01	<0.01			0.06	0.02
10	0.01	<0.01			0.06	0.04		
11			0.01	<0.01			0.04	0.02
13	0.01	<0.01			0.05	0.04		
14			0.01	<0.01			0.05	0.02
16	0.01	<0.01			0.05			
17			0.01	<0.01			0.05	0.02
19	0.01				0.05	0.02		
20			0.01	<0.01			0.04	0.01
22	0.01	<0.01			0.06	0.04		
23			0.01	<0.01			0.05	0.02
25	0.01	<0.01			0.05	0.02		
26			0.01				0.06	0.02
28	0.01	<0.01			0.06	0.04		
29			0.01	<0.01			0.04	0.01
31	0.01				0.05			
Benomyl withdrawn								
1			<0.01	<0.01			0.02	0.01
2			<0.01	<0.01			<0.01	<0.01
3			<0.01	<0.01			<0.01	<0.01
4			<0.01	<0.01			<0.01	<0.01
5			<0.01	<0.01			<0.01	<0.01

[a] Benomyl and/or MBC residue not detected in any milk sample (limit of detection, 0.02 ppm). 5-HBC and 4-HBC not detected in milk from control cows or cows on the 2-ppm feeding level (limits of detection, 0.01 ppm). [b] Benomyl in diet. [c] Cow number. [d] Pretreatment period.

Table IV. Urine and Feces Residue Data (ppm[a]); Benomyl Dairy Cow Study

Days on Diet	Sample	2 ppm[b]				10 ppm[b]				50 ppm[b]			
		69[c]		159[c]		110[c]		123[c]		128[c]		149[c]	
		5-HBC	4-HBC	5-HBC	4-HBC	5-HBC	4-HBC	5-HBC	4-HBC	5-HBC	4-HBC	5-HBC	4-HBC
0[d]	Urine	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
	Feces	<0.05		<0.05		<0.05		<0.05		<0.05		<0.05	
8	Urine	0.5		0.5		4.0	0.4	1.2		19	1.9	13	1.9
	Feces			<0.05				0.15				1.0	
15	Urine	<0.1	<0.1	0.6		0.5	0.1	0.7		4.2	0.4	1.6	
	Feces	<0.05				0.09				1.3			
22	Urine	<0.1		<0.1		0.6	<0.1	1.1	0.1	0.3		18	0.1
	Feces			<0.05				0.30				1.1	
29	Urine	0.5		<0.1		0.5		5.5		1.6	0.1	2.3	
	Feces	<0.05				0.15				1.1			
Benomyl withdrawn													
2	Urine			<0.1				<0.1				<0.1	
	Feces			<0.05				<0.05				<0.05	

[a] Benomyl-MBC residue not detected in any sample (detection limit, 0.1 ppm). No 5-HBC or 4-HBC detected in urine or feces from control cows; no 4-HBC detected in feces from treated cows (detection limits, 0.1 ppm in urine, 0.05 ppm in feces). [b] Benomyl in diet. [c] Cow number. [d] Pretreatment period.

naturally occurring interfering materials from peaks of interest, particularly in the tissue samples. The lc carrier for this work was an equal volume mixture of 0.2 *N* sodium acetate and 0.2 *N* acetic acid. Inlet pressure was held constant at 225 psi. Under these modifications, the 4-HBC peak elutes in about 10 min, 5-HBC at about 12 min, and benomyl-MBC at about 35 min. Recoveries averaged 81% for benomyl-MBC and 67% for both 5-HBC and 4-HBC. Positive residue results in Table V have been corrected for the appropriate average recovery factor.

Analyses of Tissues from 2-Year Rat and Dog Feeding Studies. Tissue samples were obtained from rats and dogs at sacrifice after 2-year dietary administration of 2500 ppm of benomyl. Samples were composited by sex prior to analysis by the method of Kirkland (1973). Residue data are shown in Table VI.

Synthesis of [2-^{14}C]Benomyl. [2-^{14}C]Benomyl was prepared from [^{14}C]thiocarbonyl-labeled thiourea by the steps illustrated in Figure 6. The [^{14}C]thiourea (30 mg, 0.39 mmol, 20 mCi, New England Nuclear Corporation) was received as a solution in ethanol (6.6 ml). This solution was added to thiourea (1490 mg, 19.6 mmol) in a

Table V. Residue Data; Benomyl Chicken and Egg Study

Sample description	5-HBC residues found, ppm[b]		
	Control[a]	5[a]	25[a]
Eggs			
7 days	<0.02	<0.02	0.03
14 days	<0.02	<0.02	0.06
28 days	<0.02	<0.02	0.03 (<0.02)[d]
Breast, 28 days	<0.02	<0.02	<0.02
Fat, 28 days	<0.02	<0.02	<0.02
Liver, 28 days	<0.1[c]	<0.1	<0.1 (<0.1)
Feces, 28 days	0.16[e]	1.6	7.5 (0.60)

[a] Benomyl in diet, ppm. [b] Benomyl–MBC and 4-HBC not detected in any sample with 0.02-ppm detection limits (0.1-ppm detection limits in feces). [c] Sensitivity of method limited to 0.1 ppm by natural interferences. [d] Numbers in parentheses are after a 1-week withdrawal period. [e] Natural interference.

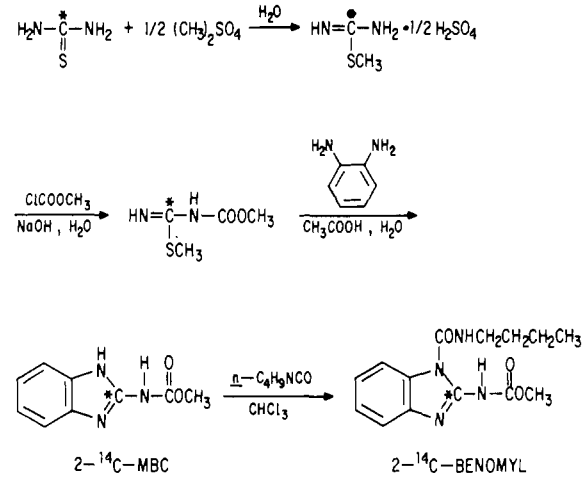

Figure 6. Synthesis of [2-^{14}C]benomyl.

50-ml wide mouth reaction flask and the ampoule was rinsed with ethanol (7 ml). A four-necked head fitted with a mechanical stirrer, a thermometer, a reflux condenser, and a dropping funnel was attached to the flask, and the mixture was heated with stirring, adding ethanol through the condenser until the solid had completely dissolved. The warm solution was slowly evaporated with stirring by a nitrogen stream introduced *via* a glass tube inserted through the condenser.

Water (5 ml) and dimethyl sulfate (1.9 ml) were added to the residue through the condenser head, and the mixture was stirred until the initial heat evolution had subsided (3 min). The clear solution was then heated on a steam bath for 1 hr with stirring. The solution was cooled to about 20° and surrounded by a water bath (18–22°). Freshly distilled methyl chloroformate (3.1 ml, 39 mmol, bp 70°) was added all at once, followed by dropwise addition of 10% aqueous sodium hydroxide (25 ml) over a period of 30 min. Stirring was continued until no further precipitation occurred (25 min). Glacial acetic acid (2.3 ml) was then added dropwise with stirring to minimize foaming. *o*-Phenylenediamine (2.2 g, 20 mmol) was added all at once from a long stem funnel placed in one of the necks of the four-necked head, and the mixture was heated slowly with stirring on a steam bath. There was a copious evolution of methyl mercaptan between 35 and 65°. The mixture was kept at 80° for 30 min, cooled to room temperature, and filtered on a (medium) fritted glass filter. The product was washed with water and acetone (three times each), and dried by suction to give MBC (3.50 g, 18.3 mmol, 92% yield).

The [2-^{14}C]MBC from above was suspended in chloroform (36 ml, dried over $CaCl_2$) in a 50-ml round-bottomed flask fitted with a $CaCl_2$ drying tube and a reflux condenser. Redistilled *n*-butyl isocyanate (2.12 g, 21.4 mmol) was added and the mixture stirred at room temperature for 18 hr. The now clear solution was filtered ("Darco") and placed in a 150-ml suction erlenmeyer provided with a one-hole rubber stopper containing a piece of glass tubing. The erlenmeyer was placed in a water bath at 25°, and a nitrogen stream was introduced through the glass tubing until almost all of the chloroform had evaporated (the exit nitrogen was bubbled through a xylene trap to scrub ^{14}C-labeled material). The oily residue was triturated with hexane (15 ml, dried over $CaCl_2$), and the precipitate filtered, washed with hexane, and dried, giving [2-^{14}C]benomyl as a white fluffy solid (4.97 g, 17.1 mmol, 93% yield, sp act. 0.91 mCi/mmol, 3.11 μCi/mg). Overall, the yield from [^{14}C]thiourea to [2-^{14}C]benomyl was 86%, based on the weight of the diluted thiourea starting mate-

Table VI. Residue Data from Benomyl Chronic Feeding Studies; Rat and Dog Tissues

Tissue	Dietary level, ppm	Residue,[a] ppm					
		Male			Female		
		Benomyl–MBC	5-HBC	4-HBC	Benomyl–MBC	5-HBC	4-HBC
Rat							
Muscle	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	<0.05	0.52	<0.1	0.06	0.51	<0.1
Fat	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	<0.05	0.08	<0.1	<0.05	<0.05	<0.1
Liver	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	0.61	2.5	<0.1	0.20	1.7	<0.1
Kidney	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	1.4	22	0.45	0.20	2.8	<0.1
Dog							
Muscle	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
Fat	0	<0.05	1.2[b]	<0.1	<0.05	<0.05	<0.1
	2500	0.15	0.14	0.1	<0.05	<0.05	<0.1
Liver	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
Kidney	0	<0.05	<0.05	<0.1	<0.05	<0.05	<0.1
	2500	<0.05	<0.05	<0.1	<0.05	0.12	<0.1

[a] Benomyl–MBC residue corrected for average recovery of 79%; 5-HBC corrected for 65% recovery; 4-HBC corrected for 55% recovery. [b] Apparent contamination.

rial. Based on total ^{14}C, the yield was also 86%. (*Note:* similar yields have been obtained in additional preparations of [2-^{14}C]benomyl.)

The radiochemical purity was determined by tlc–radioautographic techniques. Aliquots of the [2-^{14}C]benomyl were dissolved in chloroform, immediately applied to cellulose tlc plates, and immediately developed in chloroform to separate benomyl from possible impurities, particularly the [2-^{14}C]MBC intermediate. Since benomyl degrades slowly to MBC while in dilute solution, rapid analysis is necessary. The radiochemical purity was >99%.

Synthesis of 5-HBC and 4-HBC. 3,4-Dinitrophenol (mp 134.5–135.5°) and 2,3-dinitrophenol (mp 145–146°) were prepared by the nitration of *m*-nitrophenol (Eastman) using the procedure of Holleman and Wilhemy (1902) as modified by Gardiner *et al.* (1968).

These materials were reduced to the corresponding diaminophenols using the "Brown Square" External Hydrogenator (Brown and Brown, 1962; the apparatus is manufactured by Delmar Scientific Laboratories, Inc., Maxwood, Ill.). In this glass apparatus, hydrogenation is carried out at atmospheric pressure using hydrogen generated from the hydrolysis of sodium borohydride with aqueous acetic acid, whereby a simple, pressure-activated device controls the rate of hydrogen evolution. Using this apparatus, the yields of diaminophenols were much improved as compared with the Parr shaker method described previously by Gardiner *et al.* (1968).

Typically, 3,4-dinitrophenol (1.84 g, 0.010 mol) was dissolved in absolute ethanol (20 ml) and 5% palladium on carbon (0.184 g) was added. About 20 ml of the 1 *M* aqueous $NaBH_4$ was consumed during hydrogenation. Water (20 ml) was added to dissolve the precipitate, and the solution was filtered hot to remove the catalyst, which was subsequently washed with 50% aqueous ethanol. The combined filtrate and washings were vacuum concentrated to a brown solid (3,4-diaminophenol). This material was immediately treated at steam bath temperatures for 1 hr with methoxycarbonyl-*S*-methylisothiourea (3.0 g, 0.025 mol) dissolved in water (50 ml) to give solid 5-HBC (1.8 g, 0.0072 mol, 72% yield). After one recrystallization of the 5-HBC from *N,N*-dimethylformamide–water (1:1, v/v), the pure product (1.5 g) was obtained. *Anal.* Calcd for $C_9H_9N_3O_3$ (mol wt 207.2): C, 52.17; H, 4.38; N, 20.28. Found: C, 52.02; H, 4.45; N, 20.15.

4-HBC was prepared in the same manner. The intermediate 2,3-diaminophenol is soluble in ethanol so that the addition of water after the hydrogenation step was unnecessary. The residue was treated with methoxycarbonyl-*S*-methylisothiourea as described above to give solid 4-HBC (1.5 g, 0.0072 mol, 72% yield). *Anal.* Calcd for $C_9H_9N_3O_3$ (mol wt 207.2): C, 52.17; H, 4.38; N, 20.28. Found: C, 52.13; H, 4.32; N, 20.11.

RESULTS AND DISCUSSION

[2-^{14}C]Benomyl was administered to a rat by intragastric intubation. After 24 hr, 78.9% of the radioactivity was detected in the excreted urine and 8.7% was found in the feces (Table I). Almost all of the radioactivity was eliminated from the rat within 72 hr. A total of 91.8% of the calculated ^{14}C dose was accounted for in this study, of which 99.5% was found in the urine and feces by the end of the 72-hr period.

The major metabolite in the rat urine was identified as methyl 5-hydroxy-2-benzimidazolecarbamate (5-HBC) by the tlc R_f value and by mass spectroscopic analysis (Figures 1 and 2). Very little if any parent compound (<5%) was found in the urine. The major metabolite was eliminated as three conjugated materials which could be enzymatically hydrolyzed with β-glucuronidase–arylsulfatase. These results are in agreement with published studies by nonradioactive methods (Gardiner *et al.*, 1968) and show

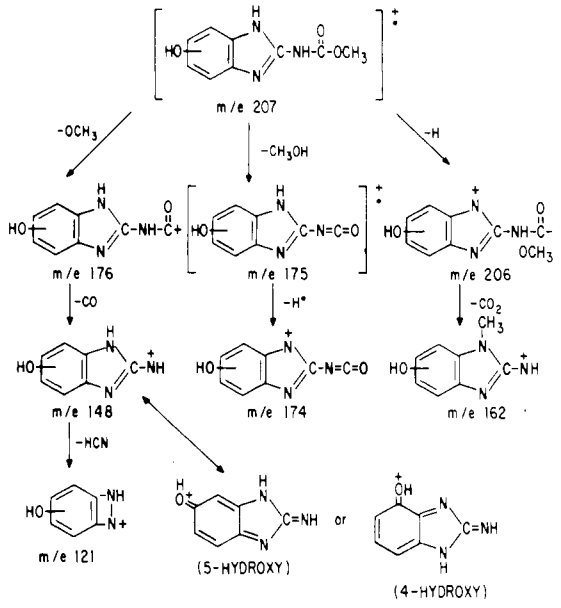

Figure 7. Mass fragments for 5-HBC and 4-HBC.

clearly that in the rat system there are no other major metabolites that were previously undetected when nonradiolabeled benomyl was employed.

It has been suggested (Gardiner *et al.*, 1968) that the route of metabolism from benomyl to 5-HBC could involve either a stepwise mechanism based on hydrolysis to methyl 2-benzimidazolecarbamate (MBC) followed by hydroxylation to 5-HBC or a mechanism whereby hydroxylation takes place prior to removal of the 1-butylcarbamoyl group. While the second possibility has not been ruled out, the separate study with [2-^{14}C]MBC showed that this compound is metabolized and eliminated from a rat in a manner identical with benomyl (Table I). MBC is an expected degradation product of benomyl and has been identified previously in aqueous solutions and within plants (Clemons and Sisler, 1969; Fuchs *et al.*, 1970; Peterson and Edgington, 1969; Sims *et al.*, 1969).

[2-^{14}C]Benomyl was dosed into a dog by gelatin capsule. Again, the bulk of ^{14}C activity (>99%) was eliminated from the animal within 72 hr (Table II). In this case, the major route of excretion was *via* the feces where benomyl and/or MBC and 5-HBC were detected.

In the benomyl dairy cow feeding study, a minor metabolite, 4-HBC, was identified (Figures 3 and 4). The mass patterns for 4-HBC and 5-HBC (Figures 2 and 4) are very similar and arise from fragments produced from the parent compounds as shown in the scheme in Figure 7. The identity of these fragments has been confirmed by high-resolution mass spectroscopy using MBC as the model compound. The 148 fragment is considerably stronger in the spectrum of 5-HBC since it can be stabilized by a *p*-quinone resonance form; the *o*-quinone resonance form in the spectrum of 4-HBC is less stable.

Except for the rat and dog study with ^{14}C-labeled materials, all samples in this report were analyzed by the residue method of Kirkland (1973). Results of special tests to verify that this method is suitable for the determination of metabolite conjugates are shown in Figure 8 and Tables VII and VIII. Figure 8 illustrates the effect of pH on the liberation of 5-HBC from its conjugate by heating at 90–95° for 1 hr. These data were obtained by adding 15 *N* phosphoric acid to samples of rat urine containing the 5-HBC conjugate. The pH of the solutions was measured after phosphoric acid addition and prior to hydrolysis. Hydrolysis of the fortified conjugate is appreciable only below pH 1 and sharply increases between pH 0 and 1. Although the data suggest a continued increase in yield as

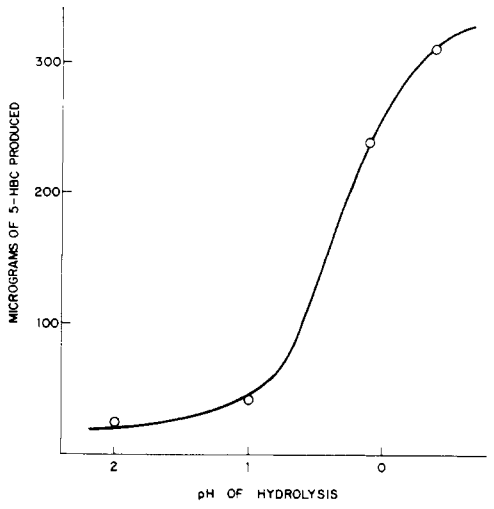

Figure 8. Hydrolysis of 5-HBC conjugates.

the pH is further decreased, at very low pH the level of background phenolic interferences is also increased (Suemitsu and Fujita, 1968), causing only an apparent increase in yield. Hydrolysis is complete at about pH 0, which is the pH specified by conditions in the final residue method.

Enzymatic hydrolysis was used to confirm that the metabolite conjugates are quantitatively hydrolyzed by the acidic hydrolysis procedure employed. Various amounts of β-glucuronidase-arylsulfatase enzyme solution were added to samples of rat urine containing the conjugates. The samples were adjusted to pH 4.8–5 with phosphoric acid and incubated at 37° overnight. They were then subjected to extraction and lc analysis by the method of Kirkland (1973). Table VII shows the data obtained on samples of urine from rats and dogs treated with high levels of benomyl and analyzed by both the acidic and enzymatic hydrolysis procedures under conditions of maximum hydrolysis for each. It can be noted that the two methods produce substantially the same results for free 5-HBC, giving added assurance that the acid hydrolysis is suitable for the determination of the conjugates.

Finally, the accuracy of the entire hydrolysis–isolation procedure of Kirkland (1973) was demonstrated for several types of matrices. This was accomplished by adding known amounts of the conjugates from rat urine (previously analyzed by both acid and enzymatic hydrolyses) to various samples, hydrolyzing in acid, and subjecting the hydrolysates to the analytical procedure. The results of these tests are given in Table VIII. Analysis at the 0.017-ppm conjugate level in raw milk was demonstrated. Because of the additional handling necessary in preparing and analyzing tissues, somewhat more variability in analyses of these samples was experienced. Recovery from all matrices averaged 87%.

Using the Kirkland method, samples from the dairy cow feeding study with benomyl were analyzed for residues. No residue of benomyl–MBC was found in milk (<0.02 ppm, Table III) or meat (<0.1 ppm) at dietary levels up to 50 ppm. With respect to 5-HBC and 4-HBC, no residue was found in milk (<0.01 ppm) or meat (<0.05 ppm) at the 2-ppm dietary level. At the 10-ppm dietary level, a residue of 5-HBC was just detectable in milk at 0.01-ppm sensitivity; no residue was found in meat (<0.05 ppm). At the 50-ppm dietary level, the total residue of 5-HBC and 4-HBC was about 0.1 ppm in milk; no metabolite residue was found in meat (<0.05 ppm). Constant levels of the metabolites were present in milk within 24 hr after benomyl feeding was begun. The residues were absent in milk (<0.01 ppm) by 48 hr after the feeding was halted.

Table VII. Lc Analysis of Urine from Treated Animals

Urine sample	Benomyl–MBC, ppm[a]		5-HBC, ppm[b]	
	Enzymatic hydrolysis	Acid hydrolysis	Enzymatic hydrolysis	Acid hydrolysis
Rats[c]	4.1, 12	57	1750, 1760	1510
Dog[c]	0.28	7.4	12	52

[a] Corrected for 85% recovery, calculated as MBC. [b] Corrected for 81% recovery. [c] Animals on diet containing 2500 ppm of benomyl.

Table VIII. Analysis of "Conjugate"-Fortified Samples

Sample	Fortification level, ppm	5-HBC Added,[a] μg	5-HBC Found, μg	% rec.
Cow urine	6.8	170	180	105
Raw milk	0.017	0.34	0.40	114
Raw milk	0.07	1.7	1.5	86
Raw milk	0.07	1.7	1.2	71
Raw milk	6.8	170	169	99
Raw milk	68	1700	1500	88
Beef muscle	0.34	8.5	4.4	52
Beef liver	0.34	8.5	7.4	87
Beef kidney	0.34	8.5	7.3	86
			Av	87%

[a] Added as "conjugate" from rat urine.

In the chicken feeding study, no residues (<0.02 ppm) of benomyl or its degradation products were present in breast or fat samples taken from sacrificed hens after 28 days on either the low-level (5 ppm) or high-level (25 ppm) benomyl diet as shown in Table V. No residues (<0.02 ppm) were detected in eggs from hens on the low-level test, while only 5-HBC (0.03–0.06 ppm) was found in the high-level egg samples. This small residue was not detectable in eggs 1 week after the hens were placed on an unfortified diet.

The data in Table VI on tissues from rats and dogs at the conclusion of 2-year feeding tests provide additional evidence that benomyl and/or its metabolites do not accumulate in animal tissues, especially in view of the total amounts of benomyl ingested over the 2-year periods.

In all animal systems studied, the basic route of benomyl metabolism involves hydroxylation to 5-HBC. Conjugates of this material are usually eliminated *via* the urine. The presence of 5-HBC has been demonstrated in excretion products from rats, dogs, dairy cows, and chickens. The 4-HBC isomer of the major metabolite has been detected in some systems, but 5-HBC was always the major hydroxylated metabolite. For example, in dairy cows at the 50-ppm feeding level, up to 19 ppm of 5-HBC was found in urine, whereas the concentration of 4-HBC did not exceed 1.9 ppm (Table IV). Chickens, which do not have a completely separate urinary tract, eliminated benomyl as 5-HBC in the "feces" (Table V).

CONCLUSIONS

Benomyl is normally metabolized in animals to methyl 5-hydroxy-2-benzimidazolecarbamate (5-HBC) which may then be conjugated and eliminated from the system *via* the urine or feces. Smaller amounts of an isomer, methyl 4-hydroxy-2-benzimidazolecarbamate (4-HBC), may also be formed. Based on studies with rats, dogs, dairy cows, and chickens, benomyl and its metabolites do not display propensity for accumulation in animal tissues.

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Factors Influencing the Metabolism of Mexacarbate by Microorganisms

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A screening of microorganisms (bacteria, mold, and fungi) from soil, water, and stock cultures was undertaken to determine the extent and mode of degradation of mexacarbate (4-dimethylamino-3,5-xylyl methylcarbamate) in the environment. All organisms showed an ability to degrade mexacarbate and two organisms, HF-3, a bacteria, and *Trichoderma viride*, a fungus, were selected for more extensive study. By mass culturing and isolating the metabolic products it was possible to identify 4-dimethylamino-3,5-xylenol (DMAX) and 4-methylamino-3,5-xylyl methylcarbamate (MAZ) as the major metabolites of *T. viride* and HF-3, respectively. By studying the effects of various cofactors, inhibitors, and carbon sources it was found that *T. viride* could be induced to form N-desmethylation products (MAZ) where in standard media only decarbamylation (DMAX) occurs. Using these same factors HF-3 would only form N-desmethylation products (MAZ). In both cases mexacarbate was found to be metabolized fastest in the absence of an added carbon source.

As the use of the chlorinated hydrocarbon insecticides decreases, the importance of carbamate and phosphate insecticides as possible alternatives will increase. One carbamate insecticide which has shown good effectiveness and may be utilized extensively for control of forest insect pests is mexacarbate (4-dimethylamino-3,5-xylyl methylcarbamate). Numerous workers have studied the chemical (photooxidation) and enzymatic (plants and animals) degradation of mexacarbate. In animals and plants the primary site of attack is at the carbamyl end of the molecule (Williams *et al.*, 1964; Krishna and Casida, 1966); however, Roberts *et al.* (1969) showed that oxidation at the *N*-dimethyl end of the molecule can occur in the insect cuticle. Deamination of the molecule to form the hydroquinone can occur and conjugates of 4-dimethylamino-3,5-xylenol and 2,6-dimethyhydroquinone have been identified. Photooxidation of mexacarbate attacks the molecule at the dimethylamino group (Abdel-Wahab and Casida, 1967). Four products are normally formed; the toxic methylamino and amino analogs and the less toxic methylformamido and formamido are formed.

Although enzymatic and chemical degradations of mexacarbate have been studied extensively, the microbial aspects of degradation have not. The object of the work reported in this paper was to demonstrate the ability of microorganisms to degrade mexacarbate and to clarify the general pathway of this metabolism.

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EXPERIMENTAL SECTION

Screening. A total of 35 aerobic organisms were isolated from water and soils, some of which were collected from mexacarbate-treated forests, and from housefly gut, as described by Matsumura and Boush (1967). Pure cultures of these organisms were grown on 10 ml of yeast–mannitol culture media for 24 hr before amending with 10 μl of a 10^{-3} *M* ethanol solution of ring-labeled [^{14}C]mexacarbate (4 mCi/mmol obtained from Dow Chemical Co. and purified by tlc). Cultures were incubated for 2 weeks. Each tube was extracted with chloroform (Matsumura and Boush, 1967) and the radioactivity was counted in the chloroform and water fractions in order to determine recoveries (80–100%). The chloroform extracts were evaporated to 0.1 ml and a portion of the residue was spotted on a silica gel G tlc plate. The mobile phase employed for separating the metabolites was a mixture of ethyl ether, hexane, and ethanol (77:20:3). Chromatograms were developed to 15 cm and subsequently autoradiographed using Kodak No-Screen Medical X-ray film.

Two distinct types of metabolic patterns (Figures 1A and 1B) were observed in the screening study. Two microorganisms were therefore selected for further study: a bacteria isolated from housefly gut (HF-3) for the A-type pathway and a fungus, *Trichoderma viride* (culture No. 12, Matsumura and Boush, 1967), for the B-type pathway.

Mass Culture. For culturing HF-3, a modified culture media containing inositol (carbon source) and NADH (0.1 mg/ml) was incubated for 24 hr and then amended with 15 mg of unlabeled mexacarbate in 10 ml of ethyl acetate. After 2 weeks, at 30°, the mixture was extracted with