Metabolism and Excretion of an Insecticide (6-Chloro-3,4-dimethylphenyl *N*-Methylcarbamate) in the Rat

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The metabolic fate of the carbamate insecticide Banol, following acute oral administration to the rat, was investigated. Recovery in urine, feces, tissues, and as expired $C^{14}O_2$ of the administered dose of ring-methyl-C^{14}, carbonyl-C^{14}, and *N*-methyl-C^{14}-Banol was 100, 99, and 80%, respectively. Urinary water-soluble metabolites include methylamine and three to five groups of acidic conjugated components. One of these groups, the uronic acids, comprising 76 to 81% of the acidic components, was resolved into four components. Indirect evidence has been presented for the conjugation of Banol and a uronic acid to form a carbamate *N*-glucuronide. Physical stress affected the rate of release of $C^{14}O_2$ following administration of *N*-methyl-C^{14}-Banol. Physical stress appears to have a selective effect on the hydrolytic pathway of carbamate degradation.

Metabolic studies with various C^{14}-labeled carbamates using plants, animals, and isolated enzyme systems have recently been reported (Abdel-Waheb *et al.*, 1966; Dorough and Casida, 1964; Friedman and Lemin, 1966; Knaak *et al.*, 1965; Krishna and Casida, 1966; Leeling and Casida, 1966; Williams *et al.*, 1964). A comprehensive report on the fate of carbamate pesticides in mammals (Krishna and Casida, 1966) indicated the ease with which these compounds are eliminated. From 12 to 65% of administered doses of eight *N*-methylcarbamates were excreted in the urine, presumably as the intact carbamate because of the presence of a radioactive carbonyl-carbon atom. As these authors state, the high proportion of the radiocarbon from carbamate-carbonyl-C^{14} compounds excreted in the urine indicates the importance of nonhydrolytic detoxication mechanisms from which relatively stable carbamate metabolites could result.

The mammalian detoxication mechanisms were examined with a representative *N*-methylcarbamate to ascertain the extent of biodegradation and to characterize the metabolic products. The carbamate insecticide Banol (6-chloro-3,4-dimethylphenyl *N*-methylcarbamate) is an effective insecticide for control of certain ectoparasites and cotton insect pests (Lemin *et al.*, 1965). This paper reports the metabolism and excretion of acutely administered Banol in rats under normal and stress conditions. Certain metabolic products in urine have been characterized.

METHODS AND MATERIALS

The compounds used in this study are listed in Table I with their paper and thin-layer chromatographic properties in two solvent systems. Three samples of radioactive Banol (Upjohn Co., Kalamazoo, Mich.) containing 4.76, 5.75, and 6.10 mc. per mmole of C^{14} in the ring methyl, carbonyl, and *N*-methyl positions, respectively, were employed as tracers. These isotopic isomers were 87, 99, and 98% pure with respect to migrational behavior in several chromatographic systems. Compounds 2 and 3 were obtained from Aldrich Chemical Co., Chicago, Ill. Compound 4 was synthesized by the reaction of 6-chloro-3,4-dimethylphenyl chloroformate with concentrated ammonium hydroxide. The nonradioactive Banol and compound 5 were supplied by the Upjohn Co., Kalamazoo, Mich. The Banol was recrystallized twice from benzene until a constant melting point of 162° C. was obtained. Infrared and mass spectral analyses were consistent with the proposed structure of the 6-chloro-3,4-dimethylphenyl ester of carbamic acid. β-Glucuronidase was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio.

The specific activities of the C^{14}-labeled materials were determined with a Packard Tri-Carb liquid scintillation spectrometer, Model 3365 (Packard Instrument Co., LaGrange, Ill.), operated at 4° C. Counting efficiency and quench corrections for all radioactive samples were

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Table I. Chromatographic Behavior of Banol Metabolites and Related Compounds

	R_f in Solvent System[a]			
	I		II	
Compound	Paper[b]	TLC	Paper[b]	TLC
1 6-Chloro-3,4-dimethylphenyl *N*-methylcarbamate (Banol)	1.00	0.90	0.95	0.39
2 6-Chloro-3,4-dimethylphenol	1.00	0.90	0.95	0.62
3 3,4-Dimethylphenol	1.00	0.90	0.95	0.48
4 6-Chloro-3,4-dimethylphenyl carbamate	...	...	...	0.23
5 Trimethyl-*s*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione	...	...	...	0.20
6 Metabolite I(a)	0.73	0.57	0.00	0.00
7 Metabolite I(b)	0.48	0.49	0.00	0.00
8 Metabolite I(c)	0.28	0.36	0.00	0.00
9 Metabolite I(d)	1.00	...	0.00	0.00
10 Metabolite IV	0.20	0.29	0.00	0.00
11 Methylamine HCl	0.20	0.29	0.00	0.00
12 Methyl hydroxylamine HCl	...	0.43	0.00	0.00

[a] Solvent system I = butanol–acetic acid–water (4:1:5). II = benzene–ethyl acetate (6 to 1).
[b] Paper = Whatman No. 3MM. TLC = 250 microns of silica gel G.

determined by the internal or external standard method. The liquid scintillation solutions utilized are listed in Table II.

Administration of Compound. Osborne-Mendel (FDA-strain) male white rats weighing approximately 250 grams each were used for all experiments. C^{14}-Banol was dissolved in dimethylformamide (DMF) and dispersed in corn oil to give a final vehicle ratio DMF–corn oil of 1 to 9. The rats were given an oral dose of 5 mg. of Banol per kg. of body weight, with no more than 4 ml. per kg. of solvent administered. Unlabeled Banol was dissolved in acetone, diluted with corn oil and mixed with commercial ground chow to a level of 600 p.p.m. in the diet. In one instance only, rats on a diet of 600 p.p.m. were treated orally with C^{14}-Banol (5 mg. per kg.) to obtain larger quantities of labeled metabolic products. In general, unlabeled metabolites were collected from rats maintained on the 600-p.p.m. diet.

Sample Collection. Urine and feces were collected in individual metal (Wahmann Manufacturing Co., Baltimore, Md.) or glass metabolism units where cross contamination of feces by urine was kept at a minimum. Urine and feces (including wall rinses) were collected every 24 hours. Aliquots were removed for radioactivity assay, and the samples were kept at 0° C. under a few drops of toluene.

Expired CO_2 was collected continuously for 8 to 72 hours after treatment, in one of two systems utilizing either a commercial glass metabolism unit or a special glass apparatus for monitoring the effects of physical stress on evolution of CO_2. The commercial glass metabolism unit (Stanford Glass Blowing Laboratories, Palo Alto, Calif.) was initially used both with and without prescrubbing of the air with Drierite and Ascarite. The prescrubbing was eliminated when no differences in $C^{14}O_2$ collection were noted. A flow of air at the rate of 1 liter per minute swept the expired air through two traps in series containing 150 and 100 ml. of 3*N* NaOH. No provision was made for trapping C^{14}-methylamine or other volatile basic products after preliminary tests showed no radioactivity was trapped in an acidic medium. Calibration tests using C^{14}-sodium bicarbonate, either administered to rats or reacted with acid in a beaker, were performed with all metabolism cages to determine recoveries of $C^{14}O_2$. The alkali solutions were changed every 24 hours and immediately assayed for C^{14} activity. For the study of stress conditions on expiration of $C^{14}O_2$, the rat was held in a cage 20 cm. long, made from 5-cm. (I.D.) glass tubing. The cage was designed to afford the rat a minimum space in which to move and yet allow air to pass across his body. With these conditions, the animal was under stress at all times, as shown by the continual strained movements in the cage and the large quantity of CO_2 trapped. The authors intended that the animals be subjected to stress for extended periods. However, because of death of the animals, the authors were able to continue for a 24-hour period in only one instance. Therefore, data on CO_2 evolution were taken at 1- or 2-hour intervals only for the first 8 hours following treatments. Simultaneous experiments were performed with animals kept under normal, nonstress conditions. After a short initial period of activity following treatment handling, the normal rats were quiet and excessive levels of expired CO_2 were not observed. Because urine and feces could not be separated from rats kept in special stress cages, they were not collected.

Table II. Scintillation Counting Solutions[a]

		% Solvents	
		PPO[b]	POPOP
I	Dioxane + 10% naphthalene, w./v.	0.7	0.03
II	Toluene	0.5	0.03
III	Dioxane + 10% naphthalene, w./v. 5% Cab-O-Sil,[c] w./v.	0.7	0.03
IV	Toluene + 4% Cab-O-Sil, w./v.	0.4	0.01
V	(*a*) Toluene + 27% phenylethylamine, v./v. 27% methanol, v./v.	0.5	0.01
	(*b*) Toluene	0.5	0.01
VI	Dioxane + 10% naphthalene, w./v. 4% NaOH (3*N*), w./v. 9% methanol, v./v. 5% Cab-O-Sil, w./v.	0.7	0.03

[a] 15-ml., total volume of scintillation solutions.
[b] PPO = 2,5-diphenyloxazole (Packard Instrument Co.). POPOP = 1,4-bis-2-(5-phenyloxazolyl)-benzene (Packard Instrument Co.). All values expressed as w./v.
[c] Trade-mark of Cabot Corp., Boston, Mass.

Radioanalysis. Expired $C^{14}O_2$, collected in 3*N* NaOH, was assayed by using scintillation solution VI (Table II). Aliquots of alkali were pipetted into counting vials and diluted to a total volume of 1 ml. with 3*N* NaOH. Methanol (2 ml.) was added to precipitate Na_2CO_3 and the mixture was suspended in the dioxane scintillation solution. The vial was mixed on a Vortex shaker for 30 seconds to get a uniform suspension and chilled at 4° C. for 8 to 24 hours before reproducible counts were obtained.

Urine was assayed for C^{14} content by direct counting in dioxane (solution I) or suspension in dioxane–Cab-O-Sil (solution III). Solution I gave somewhat higher counts, but the corrected results (DPM) were the same.

Tissue and Feces. Feces were assayed by direct counting of dried samples in solution III, extracting with acetone and drying, and then counting both powder and acetone extract in solutions IV and II, respectively, or using Schöniger oxygen combustion (Jeffay, 1962). The combustion technique gave the most consistent results.

All tissue residues were combusted in the Schöniger apparatus. Samples were weighed, and aliquots were removed and allowed to air-dry overnight. The dried aliquots were then wrapped in a 2 × 2 inch cellophane square with a 3-inch cellophane wick and inserted into the sample holder. The 5-liter round-bottomed flask, the glass stopper of which contained a platinum sample holder (A. H. Thomas & Co., Philadelphia, Pa.), was fitted with a safety balloon and flushed with oxygen; the cellophane wick was ignited and inserted into the flask. Samples of less than 300 mg. (wet weight) were burned completely within 30 seconds. Following combustion, the flask was chilled in an ice and water bath until the safety balloon collapsed. A serum septum was inserted over the balloon and 15 ml. of scintillation solution V(a) was injected. The flask was held at 0° C. for 15 minutes and was allowed to warm to room temperature, then 5 ml. of solution V(b) was injected to rinse the walls of the flask. After 10 minutes, the flask was opened and a 15-ml. aliquot was removed for counting. The sample was chilled for 8 to 24 hours before reproducible count rates were obtained. Calibration of the Schöniger method with C^{14}-benzoic acid

showed greater than 95% recovery of radioactivity when samples of less than 300 mg. (wet weight) of tissue were burned. Combustion was incomplete with tissue samples larger than 300 mg. or when the samples were not dried completely.

Separation. Ion exchange chromatography was used to separate metabolic components. Rexyn 201 (Cl) (Fisher Scientific Co., Fairlawn, N. J., 100- to 200-mesh) and DEAE cellulose (Schleicher and Schuell Co., No. 71, Type 20) were used to separate acidic components, and Rexyn 101 (Na) (100- to 200-mesh) was used to separate components. The quaternary amine resins were used in the chloride form and the sulfonated polystyrene polymers as the sodium salt. All exchangers were activated by equilibrating with the strongest eluting solution for several hours before use.

Chromatographic columns of Rexyn 201 and 101 were prepared by washing 80 grams of activated resin into a 3.5-cm. (I.D.) column with water. Forty grams of activated resin reacted for 10 to 15 minutes with urine and the unreacted urine was filtered off. The urine-treated resin was rinsed with 2 to 5 liters of water and washed onto the column. Gradient elution techniques using either HCl or NaCl were used to elute the resin-bound components. Gradients included 1-liter volumes of water and 0.1*N*, 0.2*N*, and 0.4*N* acid or salt. (In the initial experiments, columns were rinsed with ethanol and acetone after the acid or salt gradient to remove any bound materials. This procedure was eliminated because, except with the ring methyl-labeled Banol urine, no materials were bound to the column after the gradient elution.) Fractions of 4.4 ml. were collected, and every third fraction was analyzed for C^{14} activity by using 0.1-ml. volumes in 10 ml. of scintillation solution I. If the presence of a peak was doubtful, adjacent fractions were further analyzed for radioactivity.

Gradient elution techniques using DEAE cellulose were essentially the same as reported by Knaak *et al.* (1965). A 1 × 29 cm. column was packed under 10-p.s.i. air pressure with 10 grams of DEAE cellulose. The urine was added and the column was washed with water until no radioactivity eluted. An elution gradient of 400 ml. each of 0.01, 0.05, and 0.1*M* Tris-HCl buffer (pH 7.5) was then used. Fractions of 1.3 to 1.5 ml. were collected and analyzed for radioactivity. Individual radioactive fractions that produced peaks were evaporated to dryness under vacuum at 40° C. and taken up in minimum quantities of methanol or water.

Thin-layer and paper chromatography were performed using silica gel G (0.25 or 0.50 mm.) and Whatman No. 1 or No. 3 MM paper. The solvent systems included: butanol fraction of a butanol–water–acetic acid mixture (4:5:1, v./v./v.); benzene–ethyl acetate (6 to 1, v./v.). Radioactive spots were located by cutting the paper or thin-layer plates into 0.5 to 1-cm. strips, eluting the material in small quantities of water, and counting in scintillation solution I. Radioautography using high resolution x-ray film (Type K, Eastman Kodak Co., Rochester, N. Y.) was utilized only where activity was high enough to permit development in 2 to 3 days.

The amount of acidic (or basic) components in urine from rats treated with carbonyl- or *N*-methyl-labeled Banol was determined quantitatively by reaction of the urine with excess amounts of either acidic or basic exchange resin. After the resin was washed, the difference in C^{14} activity between the nonbound material and the original urine was calculated as an estimate of either acidic or basic components. The neutral content was obtained by difference.

Characterization. Metabolities were characterized by three methods: cochromatography with known compounds, specific tests for functional groups, and enzymatic, acid, and basic hydrolytic degradation. Reference materials used for chromatography, solvent systems, and adsorbents used are listed in Table I. Nonradioactive material was detected by thin-layer chromatography. When solvent system II was used for development, the plates were sprayed with a solution containing 500 mg. of $KMnO_4$, 15 ml. of H_2SO_4, and 24 ml. of water, and then heated at 100° C. for 2 to 3 minutes. When solvent system I was used for development, the plates were sprayed with 1% eosin in ethanol. Organic materials were detected on paper with ethanolic eosin by quenching of background fluorescence.

Specific tests used with thin-layer or paper chromatograms included: naphthoresorcinol for uronic acids, antiesterase for carbamate esters, and 1% ninhydrin in acetone for amines. Other reactions carried out on isolated metabolic components included acid and base hydrolysis, β-glucuronidase hydrolysis, and colorimetric determination of formaldehyde using a Hantzch reaction as modified by Nash (1953).

A modification of the procedure described by Hawk *et al.* (1949) was used to test for the presence of glucuronic acid conjugates on paper chromatograms. The paper was sprayed with 4% ethanolic naphthoresorcinol, dried, and sprayed with 1*N* HCl. The wet paper was put in an oven and heated at 100° C. for 1 minute. The presence of glucuronic acid was indicated by the appearance of green-yellow spots just before the paper charred.

Acid and base hydrolyses were carried out at 100° C. in the presence of 3*N* NaOH or 1*N* HCl. Incubation times varied from 0.25 to 1.0 hour. The reaction solutions were neutralized and extracted with methylene chloride to separate organic and aqueous materials. The components were subjected to thin-layer and paper chromatography for comparison with known materials.

Two β-glucuronidase hydrolysis experiments were carried out by the method described by Voigt (1963). The initial experiment utilized a carbonyl-labeled metabolite collected and pooled from several ion exchange columns, rechromatographed on paper, and removed from an R_f area of 0.60 to 0.85. One thousand Fishman units of enzyme were incubated with the sample at 37° C. (pH 4.6) for 24 hours. The reaction was stopped, and the solution was extracted with ethyl ether. Aqueous and organic phases were counted for radioactivity and chromatographed.

The second series of experiments used an *N*-methyl-labeled metabolite as a substrate. The metabolite was separated from urine by ion exchange chromatography, but was not purified further by separation on paper. Two levels of enzyme concentration (15,000 and 30,000 units) were used in these experiments at 37° C. (pH 4.6) for 24 hours. The reaction was stopped, and the solution was

extracted with methylene chloride, counted, and chromatographed. One experiment was modified to include 10 μmoles of semicarbazide HCl in the reaction mixture to trap any formaldehyde that might be formed during the hydrolysis reaction. After incubation, the reaction mixture was treated with 10% zinc chloride and saturated barium carbonate to remove the protein components. The precipitate was removed by centrifugation, and the clear product treated with the Nash reagent (1953). The solution was extracted with chloroform, and chromatographed on thin-layer chromatoplates with ethyl acetate as solvent. A radioactive yellow spot with an R_f of 0.27 indicated the presence of formaldehyde in the reaction if the conjugated component was formed through condensation of glucuronic acid and an *N*-methylol metabolite of Banol.

A preparation of carbamate-sensitive liver esterase was used to test for the presence of antiesterase carbamate esters. The enzyme source was prepared from a 10% (w./v.) aqueous suspension of a liver-acetone powder. The aqueous suspension was filtered and the filtrate sprayed directly on developed thin-layer or paper chromatograms. The chromatogram was equilibrated at room temperature for 30 minutes. The substrate, 1-naphthyl butyrate (21 mg. per ml. in water-ethanol, 3 to 1, plus 1 drop Triton X-100 per 5 ml.), was sprayed directly on the chromatogram and allowed to react for 5 minutes. The chromatogram was then oversprayed with a saturated ethanolic solution of *p*-nitrobenzenediazonium fluoroborate. Colorless areas indicated the presence of antiesterase compounds. The sensitivity of the method is approximately 0.1 μg. of Banol.

RESULTS

Distribution and Excretion of Banol. The distribution and excretion of C^{14} radioactivity following orally administered doses of *N*-methyl C^{14}-, carbonyl C^{14}-, or ring-methyl C^{14}-labeled Banol are given in Table III. Excretion of radioactivity in urine, feces, and as $C^{14}O_2$, using two to 10 rats per 48 hours' study, was 75, 97, and 96% (after correction for the 87% purity of the ring-labeled Banol) of the administered dose of *N*-methyl-, carbonyl- and ring methyl–labeled Banol, respectively. Residual radioactivity was detected in several tissues, and amounted to 1.7 and 5.2% of the administered dose of carbonyl- and *N*-methyl–labeled Banol. No assay was made of tissues from animals treated with ring methyl–labeled Banol.

Table III. Distribution of Carbon-14 48 Hours after Oral Administration of Banol C^{14} to Male Rats[a]

Tissue or Source	% Administered C^{14} Recovered		
	Ring methyl label	Carbonyl label	*N*-Methyl label
CO_2	0.2	52.4	37.6
Urine	103.6	34.8	33.6
Feces	6.1	9.9	3.7
Intestine		0.3	1.2
Blood		0.7	1.0
Liver		0.4	2.1
Kidney		0.1	0.3
Testis		Trace (<0.1)	0.1
Other[b]		0.2	0.5
Total	109.9	98.8	80.1

[a] Dose 5 mg. of Banol per kg. for all treatments administered as solution in DMF–corn oil (1 to 9, v./v.) at a rate of 4 ml. per kg. Radioactivity administered at levels of 1.83 μc. per rat (ring label), 1.74 μc. per rat (methyl label), or 1.54 μc. per rat (carbonyl label). Results are average values of 4 to 6 rats per treatment with 2 to 3 assays of each tissue.
[b] "Other" tissues include: spleen, heart, lung, tibial muscle, abdominal muscle, skin, diaphragm, fat, brain, stomach, adrenal, prostate, thymus, fat, and epididymus.

Effect of Stress on $C^{14}O_2$ Evolution. The effect of stress on evolution of $C^{14}O_2$ from carbonyl- and *N*-methyl–labeled Banol over an 8-hour test interval is given in Table IV. Within 48 hours, 52.4% of the administered dose of carbonyl-labeled Banol was expelled, whereas 37.6% of the administered dose of *N*-methyl–labeled Banol was expelled over this time interval. The $C^{14}O_2$ expelled over an 8-hour test interval was 49.8 and 27.1% of the administered dose for the carbonyl- and *N*-methyl–labeled Banol.

Urinary Metabolites of Banol. The acidic urinary metabolites of Banol (*N*-methyl, carbonyl, and ring methyl label) from male rats were chromatographed on quaternary amine ion exchange resin as indicated in Figure 1. The chromatogram indicates the peak shape, position, and relative C^{14} concentrations of each metabolite. All three isotopic positions showed similar results. Recoveries of radioactive materials from the ion exchange column were complete with the carbonyl- and *N*-methyl–labeled Banol. However, with the ring methyl–labeled Banol, some unknown material remained on the column after the normal gradient elution. Similar results were obtained with DEAE cellulose column chromatography. Basic metab-

Table IV. Rate of Expiration of $C^{14}O_2$ as Affected by Stress Conditions

Time, Hours	Total $C^{14}O_2$ Expired within 8 Hours, %[a]			
	Carbonyl Label		*N*-Methyl Label	
	Normal	Stress	Normal	Stress
1	6.9	30.3		
2	22.1	55.3	19.0	25.0
3	44.3	73.1		
4	62.1	81.7	56.0	51.0
5	72.8	85.0		
6	80.6	91.1	81.0	76.0
7	90.2	94.1		
8	100.0	100.0	100.0	100.0

[a] Within 8 hours, carbonyl- and *N*-methyl–labeled Banol were excreted as $C^{14}O_2$ at 95 and 72%, respectively, of the total $C^{14}O_2$ recovered over a 48-hour test interval.

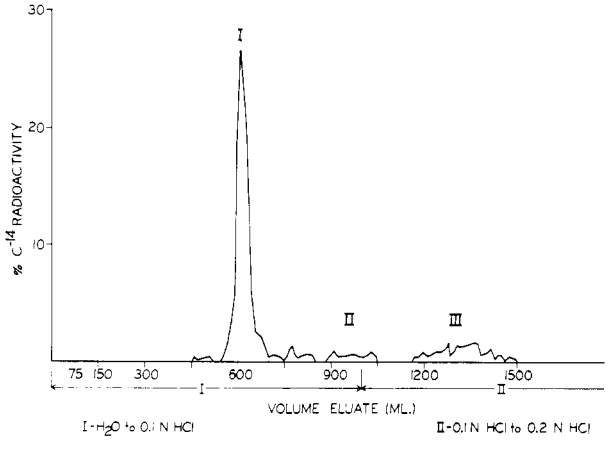

Figure 1. Ion exchange chromatograph of acidic urinary metabolites of Banol

olites, bound by sulfonated polyester resins, were not present when carbonyl- or ring methyl–labeled Banol was used. An estimate of the acidic, basic, and neutral components of *N*-methyl–labeled Banol in urine showed that from 49 to 51% of the components were acidic, 42 to 46% were basic, and 3 to 9% were not bound by either resin, and were believed to be neutral.

One basic metabolite from *N*-methyl–labeled Banol (designated metabolite IV) was eluted from ion exchange chromatographic columns. The peak was collected from several columns, pooled, and rechromatographed on paper and thin-layer chromatoplates. Its R_f value of 0.20 was identical with that of methylamine, it was positive to ninhydrin, and it cochromatographed as a single spot with a known sample of methylamine.

Three (and possibly five) definitive acidic metabolites of Banol were separated on ion exchange columns. These metabolites, designated metabolites I, II, and III, contained 76 to 81, 14 to 15, and 5 to 9%, respectively, of the total radioactivity of the acidic components. Glucuronic acid eluted from similar ion exchange columns simultaneously with metabolite I. Metabolite I, collected from several columns, was pooled and rechromatographed on paper with the results shown in Table V. Metabolite I from *N*-methyl and carbonyl-labeled Banol was resolved into three radioactive areas on Whatman No. 3 MM paper. With ring methyl–labeled Banol, four radioactive areas were resolved. These metabolites were designated as Ia, Ib, Ic, Id. Metabolites Ia, Ib, and Ic contained all three radioactive positions, whereas Id contained only the ring-methyl label.

A methylene chloride extract of urine, collected from rats treated with carbonyl-labeled Banol, was chromatographed on paper and thin-layer chromatoplates. The chromatograms showed traces of one radioactive area corresponding to Banol.

Enzyme Hydrolysis. Table VI shows the recovery of radioactivity from carbonyl- (Ia) and *N*-methyl-(I)–labeled metabolite after the reaction with β-glucuronidase. From 90 to 97% of the radioactivity was recovered in the aqueous phase in both the control and enzyme preparations. Chromatographic separation of the aqueous phase of the initial experiment, using carbonyl metabolite Ia, showed a significant difference from the control. The R_f area of the control peak ranged from 0.60 to 0.85; the enzyme reaction peak ranged from 0.05 to 0.30. These data indicated tentatively that some reaction had taken place with β-glucuronidase, causing formation of a more polar product. Chromatographic separation of the aqueous fraction of the second experiment using *N*-methyl-metabolite I showed no difference from the control. In contrast with the initial experiments, β-glucuronidase had essentially no effect on hydrolysis of metabolite I.

The modified β-glucuronidase experiment containing semicarbazide HCl showed a slight trace of C^{14}-formaldehyde only when whole urine was used. No trace of formaldehyde was noted when metabolite I was used as a substrate.

Table VI gives the results of the hydrolysis of *N*-methyl metabolite I by acid and base. Paper chromatography after acid hydrolysis showed no variations from the original extract. The basic reaction resulted in a loss of approximately 40% of the initial C^{14} radioactivity with the complete disappearance of peak I. The only product present had an R_f ranging from 0.0 to 0.35 and was believed to be methylamine. The results of acid hydrolysis were similar with both 1*N* and 2*N* HCl. Paper chromatographic separation of the aqueous fraction was similar to the controls. The organic fraction contained one component which had an R_f value on solvent system I of 0.55. The major component of the aqueous fraction was in the R_f range of 0.60 to 0.85. With solvent system II, no movement from the origin was detected with either extract.

Esterase Enzyme Inhibition. Metabolite I, separated by column and paper chromatography, gave a negative test for liver esterase inhibition. The limit of sensitivity for this test was about 0.1 μg. of Banol. No conclusions were reached about the relative inhibitory nature of the Banol metabolite because the concentration of metabolite was probably lower than the sensitivity of the method. In

Table V. Paper Chromatographic Separation of Peak I Showing Relative C^{14} Intensity of Each Area[a]

Distance, Cm.	Isotopic Position			
	Ring methyl	Carbonyl	*N*-Methyl	
9.5–10.0	+	−	−	
9.0– 9.5	+	−	−	I(d)
8.5– 9.0	−	+	−	
8.0– 8.5	+	++	+	
7.5– 8.0	++	+++	+	
7.0– 7.5	+++	++	++	I(a)
6.5– 7.0	++	++	++	
6.0– 6.5	++	+	+	
5.5– 6.0	−	−	−	
5.0– 5.5	+	+	+	
4.5– 5.0	+	+	+	I(b)
4.0– 4.5	+	+	+	
3.5– 4.0	−	−	−	
3.0– 3.5	−	−	−	
2.5– 3.0	−	+	+	
2.0– 2.5	+	+	+	I(c)
1.5– 2.0	−	−	+	IV
1.0– 1.5	−	−	+	
0.5– 1.0	−	−	−	
0.0– 0.5	−	−	−	

[a] Solvent system. Upper layer of a mixture of butanol–water–acetic acid (4:5:1, v./v.).

Table VI. Recovery of Radioactivity from Metabolite I Following Hydrolysis

	Radioactivity Recovered, %			
	Carbonyl Label[a]		*N*-Methyl Label[b]	
	Organic	Aqueous	Organic	Aqueous
Control	10	90	3	97
β-Glucuronidase	10	90	3	97
1*N* HCl	..	..	21	79
2*N* HCl	..	..	22	78
3*N* NaOH	..	..	3	97

[a] Carbonyl-labeled metabolite Ia, separated by ion exchange and paper chromatography, used as substrate.
[b] *N*-Methyl–labeled metabolite I, separated by ion exchange chromatography, used as substrate.

this test, a combination of radioactive and nonradioactive urine was used to collect metabolite I and no true estimation of concentration could be made.

DISCUSSION

Elimination of the carbamate insecticide Banol after subacute oral intoxication was essentially complete within 48 hours; only small quantities of fragments persisted in certain tissues. Studies on the in vivo effects of *N*-methylcarbamate esters on brain cholinesterase have demonstrated the rapid induction (within 5 minutes) and recovery (1 to 4 hours) of enzyme inhibition correlating with the intensity of cholinergic effects (Baron *et al.*, 1966). These and other studies suggest that the rapid in vivo biodegradability of carbamate esters with hydrolysis and the subsequent release of the carbonyl carbon as CO_2 are the major pathways for metabolism. Approximately 95% of the total $C^{14}O_2$ eliminated over a 48-hour interval was observed within 8 hours. Physical stress increased the rate of release of CO_2 during the first 2 to 3 hours. Immobilization has been shown to produce stress conditions because of neuromuscular exertion (Pearl *et al.*, 1966), and to result in a corresponding increase in the rate of activity of certain mammalian enzyme systems (Selye, 1946). The increased rate of CO_2 production during the first few hours suggests that stress directly affects those hydrolytic enzymes which degrade carbamates, but has no effect on the degradation of methylamine to CO_2. While the stress condition induced a higher rate of elimination of CO_2 within the first few hours after treatment, the total $C^{14}O_2$ evolved in 48 hours from both carbonyl- and *N*-methyl–labeled Banol was the same whether or not the animal was under stress. Thus, the pharmacodynamic effects of immobilization stress are believed to be oriented towards specific biological systems. The effect of stress on acute and subacute toxicity of carbamate esters should be studied further. The stress response may be a mechanism whereby the resulting increase in a nonspecific hydrolytic enzyme reaction gives the animal some increased protection against toxic contaminants.

Distribution and elimination of Banol in tissues, urine, and feces were essentially the same as reported by Krishna and Casida (1966). The presence of *N*-methyl- and carbonyl-C^{14} in certain tissues suggests the possibility of transcarbamylation after hydrolysis. The extent of this type of reaction appears to be slight, since less than 2% of the administered dose of the carbonyl-labeled material was in tissue residues. However, the effect of such transcarbamylation is unknown and its occurrence, even to a slight extent, may be important.

Partial characterization of urinary metabolites was attempted by several chromatographic techniques. Cochromatography with known compounds suggested that the basic metabolite present was methylamine and the neutral component consisted of unchanged Banol. Because limited numbers of standards were available for comparison of R_f values, possible ring or side chain oxidation products cannot be eliminated completely as potential neutral metabolites. Although the quantity of this metabolite(s) was on the order of <10% of the administered dose, the possibility of oxidation, especially with the production of unstable intermediate compounds, cannot be eliminated as a mechanism for detoxifying carbamates.

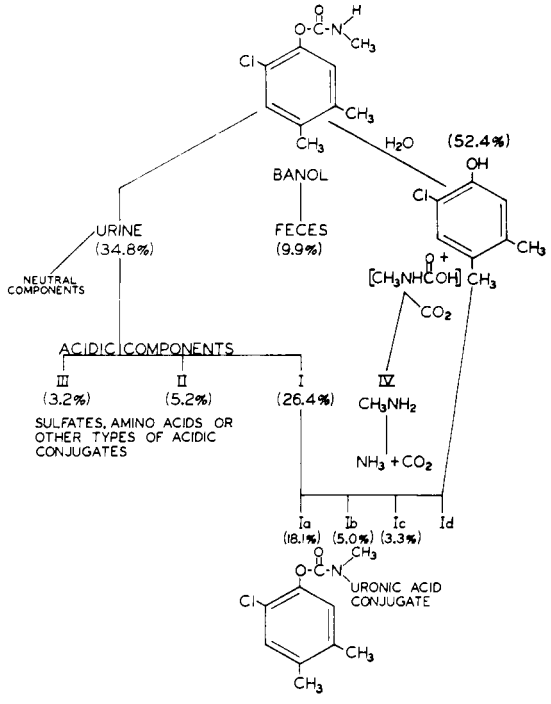

Figure 2. Proposed scheme for elimination of Banol by the rat

Figures in parentheses include per cent recovered of administered dose based upon data obtained by using the carbonyl-labeled Banol
Unknown metabolites II and III are acidic conjugates of Banol having an intact C—O—C(O)NHCH$_3$ skeleton
Unknown metabolites Ib and Ic are uronic acid conjugates of Banol having an intact C—O—C(O)NHCH$_3$ skeleton

Approximately 35% of the administered dose was present as acidic urinary metabolites and, with the exception of metabolite Id, contained the intact carbamate skeletal structure, C—O—C(O)NHCH$_3$. Because glucuronic acid migrated within the area of the major acidic radioactive metabolite (I), these compounds are believed to be uronic acid conjugates. The minor peaks probably represent other conjugating groups—e.g., sulfates, amino acids, etc. Four uronic acid conjugates were separated. The major component is believed to be an *N*-glucuronic acid derivative of Banol because of the lack of consistent response of glucuronidase, the absence of formation of free formaldehyde, complete alkaline degradation, and partial acid lability.

Results of the study using β-glucuronidase to hydrolyze an isolated carbonyl-labeled metabolite (Ia) were inconclusive. No organic-soluble material was obtained from the enzymatic reaction. However, a noticeable change in chromatographic migration was observed with the aqueous fraction. As these results were not duplicated in the experiments with the *N*-methyl–labeled metabolite, perhaps some error was made in the chromatographic procedure used in the first experiment. If a hydrolytic reaction takes place, a larger percentage of radioactive material would be expected in the organic phase, rather than only a change in chromatographic pattern of the aqueous material, as was observed. Therefore, it is suggested that no hydrolytic reaction took place with β-glucuronidase and metabolite Ia.

The *N*-glucuronic acid conjugates of carbamic acid esters and other nitrogen-containing compounds have recently been demonstrated as a major pathway for biological degradation (Bridges *et al.*, 1965; Tsukamoto *et al.*, 1963). These carbamates, carboxamides, and other compounds were not hydrolyzed by β-glucuronidase, were degraded with alkali, and were labile under strong acid conditions (Axelrod *et al.*, 1958; Bridges and Williams, 1962). The structure of an *N*-glucuronide of Banol is consistent with these conditions, and work is currently in progress to synthesize this compound for comparison with the isolated material.

The proposed scheme for elimination of Banol by the rat (Figure 2) suggests a direct conjugation of a uronic acid moiety to a carbamate as a principal pathway for elimination of the toxicant. As this metabolite from in vivo sources is not accumulated in a sufficient amount for more complete analysis, the authors cannot definitely confirm the proposed metabolic route.

Within 48 hours, 97% of the administered dose of carbonyl-Banol was eliminated from the body. The primary effect of Banol, as an inhibitor of a nervous system cholinesterase enzyme, takes place within minutes after intoxication. The toxic signs of poisoning that follow subacute doses are eliminated within several hours, presumably because of the ease of reversibility of enzyme inhibition and because of biological degradation. A limited number of studies of the metabolism in plants has been reported. The conversion of carbamates to water-soluble conjugates containing a C—O—C(O)N—CH_3 skeleton (Friedman and Lemin, 1966) and stored within the plant presents a possible source of significant error in the usual analytical determination. This is potentially misleading with regard to the nature of the residue present. As this type of metabolite can potentially be degraded to an intact carbamate after animal ingestion, more information is needed on the toxicology and analytical behavior of conjugated residues of carbamate esters.

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