of these compounds. If one arbitrarily assumes an average mol wt of 800 for the total liver lipids, one finds a specific activity of about 3.3 μCi/mol (9.2 dpm/mg) in line with the conclusions drawn above. The pathway from serine to carbohydrates as expected dilutes the specific activity considerably; glucose and ribose have 1.1 and 1.3 μCi/mol (13.8 and 19.2 dpm/mg, respectively), and an even lower specific activity of 0.6 μCi/mol (3.6 dpm/mg) is found in acetate-derived cholesterol.

The relative specific activities of the isolated compounds fall into an order which is compatible with the pathway postulated (Page et al., 1971, 1972a) leading from dichlorvos after cleavage of the P–O–vinyl bond and dechlorination via a hypothetical symmetrical two-carbon intermediate, to glycine and serine. At the glycine stage the carbon on the 1 position of the vinyl group has undergone a dilution of about 5×10^4 and proceeds from here with relatively little additional dilution through the metabolic pools of the other naturally occurring tissue constituents. There is no indication that a degradation pathway develops after prolonged exposure of animals to dichlorvos vapor which would differ measurably from that found to occur during short-term inhalation, gastric or intestinal infusion, and single and multidose oral ingestion of dichlorvos.

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LITERATURE CITED

Boyer, A. C., *J. Agric. Food Chem.* **15**, 282–286 (1967).
Burton, W. B., *J. Agric. Food Chem.* **19**, 869–871 (1971).
Curzon, G., Giltrow, J., *Nature* **173**, 314–315 (1954).
DeBersaques, J., *J. Chromatogr.* **31**, 222–223 (1967).
Hirs, C. H. W., Moore, S., Stein, W. H., *J. Am. Chem. Soc.* **76**, 6063–6065 (1954).
Hutson, D. H., Blair, D., Hoadley, E. C., Pickering, B. A., *Toxicol. Appl. Pharmacol.* **19**, 378–379 Abstr. (1971a).
Hutson, D. H., Hoadley, E. C., Pickering, B. A., *Xenobiotica* **1**, 593–611 (1971b).
Kirby, K. S., *Methods Enzymol.* **12B**, 87–92 (1968).
Loeffler, J. E., DeVries, D. M., Young, R., Page, A. C., *Toxicol. Appl. Pharmacol.* **19**, 378 Abstr. (1971).
Loeffler, J. E., Kirkland, V. L., DeVries, D. M., Collins, R. D., Young, R., Page, A. C., Biological Sciences Research Center, Shell Development Company, Modesto, Calif., unpublished work, 1972.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, Rose J., *J. Biol. Chem.* **193**, 265–275 (1951).
Page, A. C., DeVries, D. M., Young, R., Loeffler, J. E., *Toxicol. Appl. Pharmacol.* **19**, 378 Abstr. (1971).
Page, A. C., Loeffler, J. E., Hendrickson, H. R., Huston, C. K., DeVries, D. M., *Arch. Toxicol.* **30**, 19–27 (1972a).
Page, A. C., Tocco, D. J., Huston, C. K., Hendrickson, H. R., Loeffler, J. E., Biological Sciences Research Center, Shell Development Company, Modesto, Calif., unpublished work, 1972b.
Potter, J. C., Boyer, A. C., Marxmiller, R. L., Young, R., Loeffler, J. E., *J. Agric. Food Chem.* **21**, 734–738 (1973a).
Potter, J. C., Loeffler, J. E., Collins, R. D., Young, R., Page, A. C., *J. Agric. Food Chem.* **21**, 163–166 (1973b).
Roe, J. H., Daily, R. E., *Anal. Biochem.* **15**, 245–250 (1966).
Schneider, W. C., *Methods Enzymol.* **3**, 680–682 (1957).
Schultz, D. R., Marxmiller, R. L., Koos, B. A., *J. Agric. Food Chem.* **19**, 1238 (1971).
Stein, W. H., Moore, S., *Biochem. Prep.* **1**, 9–22 (1949).

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Fate of Croneton (2-Ethylthiomethylphenyl *N*-Methylcarbamate) in Rats

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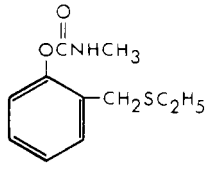
The fate of Croneton, 2-ethylthiomethylphenyl *N*-methylcarbamate, was determined in rats following both single oral or dietary exposure to the ^{14}C-carbonyl- and ^{14}C-ring-labeled insecticide. Greater than 95% of the [^{14}C]Croneton equivalents was excreted in the urine or as a combination of $^{14}CO_2$ (47%) and urinary products (41%) 72 h after a single oral dose. The feces contained 2–7% of the dose. A similar excretion pattern was observed during a 7-day feeding period. The principal urinary metabolites were Croneton sulfoxide (23–28% of the dose), phenol sulfoxide (20–23%), phenol sulfone (9–25%), and Croneton sulfone (3–11%) after a single oral dose and similar in the long term study. The carbamates were excreted primarily as free metabolites while the phenolic constituents were eliminated as acid labile conjugates. The 24-h acute oral LD_{50} values of Croneton, Croneton sulfoxide, and Croneton sulfone to mice were 71, 59, and 282 mg/kg, respectively.

Croneton, 2-ethylthiomethylphenyl *N*-methylcarbamate (Bay Hox 1901), is an experimental plant systemic insecticide with excellent aphicical properties. One of its principal projected uses is the control of these insects on vegetable, fruit, and cereal crops. Croneton is highly toxic to aphids, LD_{50} of 5 mg/kg, while the mammalian oral toxicity is rather low, with an LD_{50} of 411 mg/kg to rats (Bayer Information Bulletin E.1-715/29 359, 1974).



Croneton

The potential use of Croneton on food and feed crops

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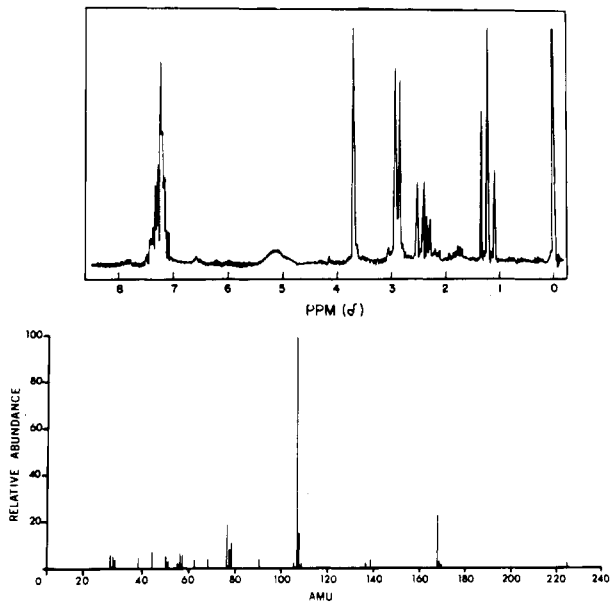

Figure 1. Nuclear magnetic resonance (upper) and mass spectra (lower) of Croneton.

necessitates studies of its fate in both plants and animals. This paper reports the results of investigations regarding the insecticide's metabolism in the rat. The fate of Croneton and its sulfoxide (SO) and sulfone (SO_2) analogues was determined in rats following a single oral exposure. Also, animals were exposed to the insecticide as a dietary supplement to determine the degree of residue accumulation which might occur following the more natural avenue of exposure.

MATERIALS AND METHODS

Chemicals. Croneton was radiolabeled with ^{14}C uniformly on the aromatic ring (7.05 mCi/mmol) and on the carbonyl moiety (5.88 mCi/mmol). The ^{14}C-ring label was supplied by The Chemagro Agricultural Division of Mobay Chemical Corp. and was greater than 99% radiochemically pure. The ^{14}C-carbonyl label was synthesized by the reaction of a 1.5 to 1 ratio of 2-ethylthiomethylphenol and [^{14}C]methyl isocyanate in dry benzene. Diacetoxydibutyltin was used as a catalyst.

For use as metabolite standards, 2-ethylsulfinylmethylphenyl *N*-methylcarbamate (sulfoxide), 2-ethylsulfonylmethylphenyl *N*-methylcarbamate (sulfone), 2-ethylsulfinylmethylphenol (phenol sulfoxide), and 2-ethylsulfonylmethylphenol (phenol sulfone) were synthesized by the method of Harvey et al. (1973), which utilized peracetic acid as the oxidizing agent. Structural confirmation was performed by nuclear magnetic resonance and mass spectrometry. For the NMR spectra, samples were dissolved in deuteriochloroform containing 1% tetramethylsilane and measured with a Model T-60 spectrometer (Varian Associates). Mass spectra were taken at 70 eV using a Finnegan Model 1015 C quadrupole mass spectrometer. Figure 1 illustrates spectra obtained from Croneton, while spectral data for the sulfoxide and sulfone are given below.

2-Ethylsulfinylmethyl *N*-Methylcarbamate. NMR spectra: δ 1.29 (t, 3 H, ethyl CH_3), 2.60 (q, 2 H, ethyl CH_2), 2.87 (d, 3 H, N-CH_3), 3.90 (d, 1 H, PhCH), 4.15 (d, 1 H, PhCH), 5.52 (b, 1 H, NH), and 7.05–7.55 (m, 4 H, Ph); mass spectra *m/e* 184 (1.9%), 107 (100%), 79 (18.1%), 78 (17.5%), 77 (30.6%), 58 (2.5%), 57 (20.6%), 51 (10.0%).

2-Ethylsulfonylmethyl *N*-Methylcarbamate. NMR spectra: δ 1.32 (t, 3 H, ethyl CH_3), 2.68–3.10 (m, 5 H, ethyl CH_2 and NCH_3), 4.28 (s, 2 H, $PhCH_2$), 5.27 (b, 1 H, NH), and 7.08–7.68 (m, 4 H, PH); mass spectra *m/e* 200 (5.5%), 107 (100%), 79 (10.0%), 78 (13.5%), 77 (16.5%), 58 (4.5%), 57 (9.0%), 51 (5.0%).

Croneton phenol, the phenol sulfoxide, and the phenol sulfone were also confirmed by NMR and MS. Because a molecular ion (*m/e* 225) was found for Croneton, the MS of Croneton phenol was easily distinguished. This was not the case for the oxidized material which could only be differentiated from respective phenols by the presence of *m/e* 58 and 57 ions arising from the carbamate moiety. More positive structural confirmation was obtained by NMR in these cases. In addition, the metabolite standards also were found to be identical with materials supplied by Chemagro.

Table I. Silica Gel Thin-Layer Chromatography of Croneton and Its Metabolites

Compound	R_f values in solvent systems[a] A	B	C
Croneton sulfoxide	0.15	0.29	0.15
Croneton sulfone	0.85	0.89	0.43
Phenol sulfoxide	0.39	0.63	0.38
Phenol sulfone	0.93	0.94	0.50
Croneton phenol	0.98	0.97	0.71
Croneton	0.97	0.94	0.64

[a] A, ethyl acetate; B, 8:2 acetonitrile-benzene, C, 6:4 hexane-acetone.

The purity of the synthesized compounds was determined by thin-layer chromatographic means using the solvent systems given in Table I and the two-dimensional system shown in Figure 2. In addition, purity was checked using high-pressure liquid chromatography under the following conditions: instrument, two M-6000 pumps plus a Model 660 solvent programer (Waters, Associates); detector, a Model 240 uv spectrophotometer detector set at 270 nm (Gilford Instrument Corporation); column, 0.25 in. × 30 cm μ Porasil; gradient elution, nonlinear 1% methanol in chloroform to 15% methanol in chloroform for 20 min at 1.0 ml/min. Under these conditions the six Croneton standards gave the elution patterns demonstrated in Figure 2.

Treatment. Albino rats (Cox-SD, Laboratory Supply Co.) weighing approximately 200 g each were used in all metabolism experiments. In the single oral dose studies, male and female animals were treated with ^{14}C-ring- and ^{14}C-carbonyl-labeled Croneton at a dosage level of 0.5 mg/kg. The specific activity of the compounds was adjusted to 2.0 mCi/mmol and delivered to the animals in 0.5 ml of water via a feeding needle. To determine the fate of the oxidation products of Croneton, female rats were similarly treated with the ^{14}C-ring- and ^{14}C-carbonyl-labeled sulfoxide and sulfone. Sufficient rats were treated so that all data collected represented the results obtained with a minimum of two animals.

[*Carbonyl*-^{14}C]- and [*ring*-^{14}C]Croneton were administered as a dietary supplement to female rats for up to 10 days. The animals were maintained on standard laboratory chow fortified with 6.6 ppm of the insecticide. At this concentration, animals consuming 15 g of feed per day received 0.5 mg/kg per day of the insecticide. Feeding of the Croneton-fortified diet was followed by a normal dietary regime for up to 1 week. Three rats were used for each predetermined sacrifice time. All animals were acclimated to their surroundings for 1 week prior to initiating treatment.

Sample Collection and Radioassay. Following treatment with either [*carbonyl*-^{14}C]- or [*ring*-^{14}C]-Croneton, the animals were placed in metabolism cages allowing for separation of urine and feces. Those treated

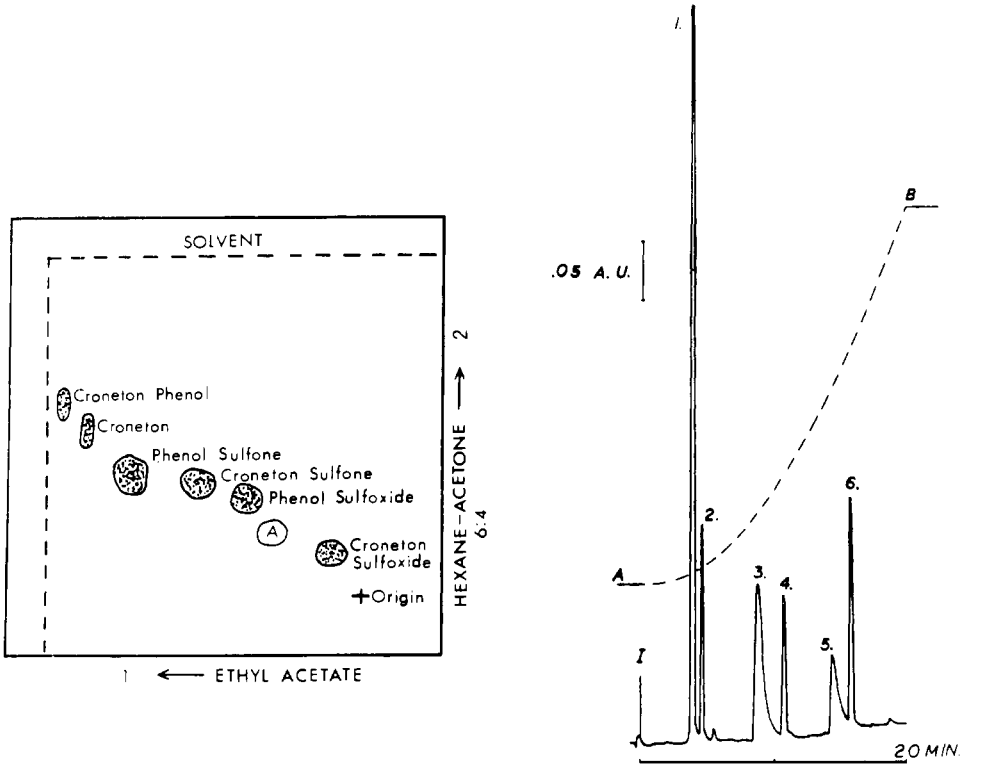

Figure 2. Thin-layer (left) and high-pressure liquid (right) chromatographic separations of Croneton and its six metabolites under conditions described in the text: (1) Croneton phenol; (2) Croneton; (3) phenol sulfone; (4) Croneton sulfone; (5) phenol sulfoxide; (6) Croneton sulfoxide; solvent gradient was 1% MeOH in $HCCl_3$ (A) to 15% MeOH in $HCCl_3$ (B).

with the ^{14}C-carbonyl-labeled material were placed in cages modified for collection of exhaled gases. Urine samples were radioassayed by direct liquid scintillation counting. Feces samples were collected at 24-h intervals over the 72-h experimental period and radioassayed by combustion of 0.5-g samples using a Packard Model 306 sample oxidizer. The $^{14}CO_2$ in the exhaled gases of rats was collected by drawing air from the cages through 300 ml of 1.0 N potassium hydroxide. Samples were then taken for radioassay at hourly intervals for 6 h posttreatment, then at 3-h intervals for the next 6 h and at 12-h intervals for the remainder of the 72-h experiment. Radioassay was accomplished by direct scintillation counting of 1.0 ml of the trap solution.

To obtain a measure of the rates of dissipation of [^{14}C]Croneton residues from selected tissues following single oral treatments, two animals were sacrificed after 8, 24, 48, and 72 h. Immediately after sacrifice, a blood sample was taken and the brain, fat, heart, kidney, liver, and a sample of skeletal muscle and skin were excised for radioassay. Radioassay of 0.2–0.5-g tissue samples, with the exception of fat where 50-mg samples were used, was accomplished in the same manner as the feces.

Following treatment of female rats with [*ring*-^{14}C]- and [*carbonyl*-^{14}C]Croneton sulfoxide, or [*ring*-^{14}C]Croneton sulfone, the animals were placed in metabolism cages as described above. Urine, feces, and exhaled gases were monitored for radiocarbon for 3 days after treatment. Tissue samples were not analyzed in this series of experiments.

Upon initiation of feeding [^{14}C]Croneton as a dietary supplement, separate collections of urine and feces were made on a daily basis, but no attempt was made to collect the exhaled gases. Sampling of excreta and tissues, and quantitation of the radiocarbon therein, were accomplished using the same techniques as employed in the single dose studies.

Samples collected during the course of the above experiments were stored at −20°C after preliminary radioassay. Periodic analysis of urine and liver over a 6-month period showed that the residues were stable at this temperature.

Analysis of Urinary Radiocarbon. Urine was lyophilized to dryness and the solid residue washed with methanol repeatedly until no radioactivity was detected in the wash. This process was quantitative in the recovery of the urinary radiocarbon while leaving much of the interfering urinary components behind. The methanol wash was then concentrated to a volume which allowed direct application to silica gel thin-layer plates. Several solvent systems were used to resolve the radioactive components of the urine (Table I). In all cases, cochromatography with standards was used to indicate the nature of the metabolites detected.

Analysis of the polar Croneton metabolites in the urine was carried out by reconstituting an appropriate amount of the methanolic fraction with distilled water. Extraction of this aqueous portion with chloroform removed the free carbamates and phenols and the aqueous layer was then adjusted to 0.5 N hydrochloric acid and incubated in a water bath at 37°C. The incubations were conducted in a sealed tube containing an equal volume of chloroform for a period of 6 days. Periodic shaking of the tubes extracted the hydrolyzed polar metabolites into the chloroform layer to prevent further decomposition. The chloroform extracts were combined and analyzed by TLC.

Toxicity Determinations. Oral LD_{50} values were determined for Croneton and its oxygen analogues in both rats and mice. In the case of rats, the techniques of Deichmann and LeBlanc (1943) were used to obtain an approximate LD_{50} only. The animals, 200-g female Cox (SD) albino rats (Laboratory Supply Company), were treated with one of a series of doses which increased by a factor of 1.5 on a milligrams per kilogram basis. The compounds were administered in aqueous solution via a feeding needle. For Croneton and its sulfone, 2%

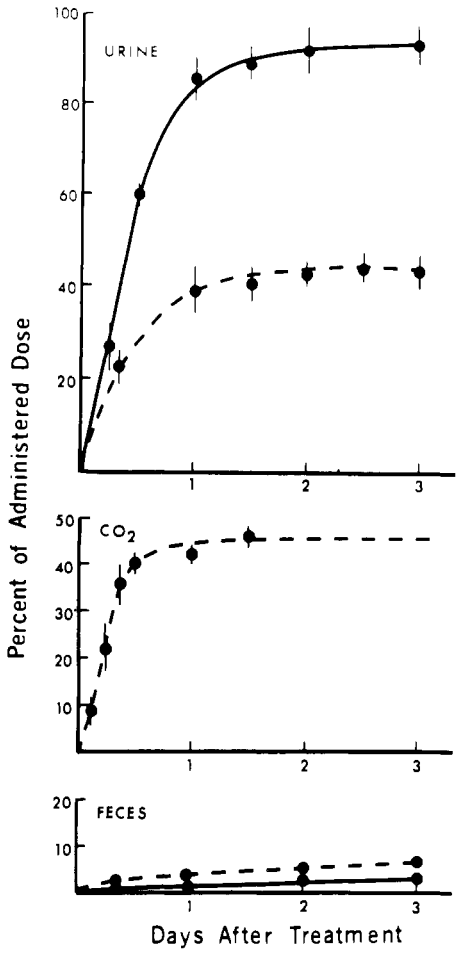

Figure 3. Cumulative elimination of [^{14}C]Croneton equivalents by rats following a single oral dose. Almost identical results were observed with Croneton sulfoxide and sulfone: (—) ^{14}C-ring label; (- -) ^{14}C-carbonyl label.

Tween-80 was used as the carrier. The approximate LD_{50} values were then calculated on a 24-h mortality basis. In the case of the mice, LD_{50} values were determined according to the techniques of Litchfield and Wilcoxon (1949), using log-dosage/probit analyses. Swiss female albino mice (Laboratory Supply Company) weighing 20 g were treated in groups of 5 with one of six dosage levels which varied over the range of the approximate LD_{50} calculated as above. Again, treatment was by feeding needle and in aqueous solution or Tween-80 as described above. Mortality values were calculated on a 24-h basis.

RESULTS AND DISCUSSION

Excretion. Administration of both ^{14}C-carbonyl- and ^{14}C-ring-labeled Croneton to male and female rats as a single oral dose resulted in rapid elimination of the radiocarbon. Figure 3 (dotted line) shows the pattern of elimination of ^{14}C-carbonyl-labeled Croneton, which was subsequently found to be virtually the same as the elimination pattern of [*carbonyl*-^{14}C]Croneton sulfoxide. An average of 47% of the dose was exhaled as $^{14}CO_2$ and this occurred predominantly within the first 8 h after treatment. An additional 41% of the dose was recovered in the urine over a 3-day period while only 7% of the dose was voided in the feces.

Approximately 96% of the [*ring*-^{14}C]Croneton dose was eliminated from the rats in the urine and about 2% in the feces (Figure 3, solid line). Almost identical patterns of excretion were exhibited by ^{14}C-ring-labeled Croneton sulfoxide and sulfone. Like the ^{14}C-carbonyl label, most of the excretion occurred during the first 24 h following

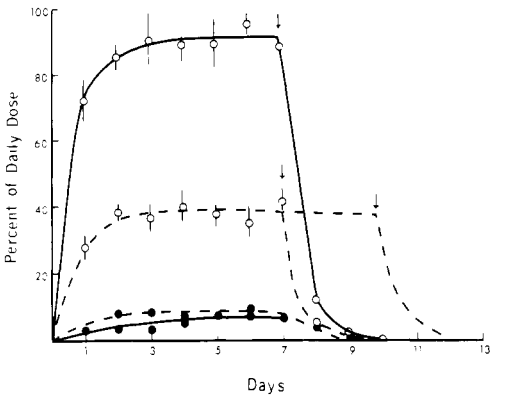

Figure 4. Elimination of [^{14}C]Croneton equivalents by rats fed 6.6 ppm of the insecticide in the diet and then returned to untreated feed. Arrows indicate time Croneton was removed from the diet: (—) [*ring*-^{14}C]-croneton; (- -) [*carbonyl*-^{14}C]croneton; (○) urine; (●) feces.

administration, and there was no difference in the rates or quantities of radiocarbon voided by males and females.

Excretion rates of radiocarbon from rats administered [*carbonyl*-^{14}C]- and [*ring*-^{14}C]Croneton as a dietary supplement (Figure 4) were very similar to those observed from the single oral treatments. Approximately 37% of the average daily dose of [*carbonyl*-^{14}C]Croneton was eliminated in the urine and 7% in the feces. After return to untreated feed, radiocarbon in the urine and feces declined to below 1% of the daily dose in 3 days. The rate of decline was the same for animals fed the treated diet for 7 and 10 days.

Elimination of [*ring*-^{14}C]Croneton equivalents in the urine averaged 89% of the average daily dose while only 5% was voided in the feces during the treatment period (Figure 4). Like the carbonyl-^{14}C continuous feeding study, a plateau was reached after 3 days on the treated diet. Excretion of radioactive residues declined to below 1% of the average daily dose 3 days after the [^{14}C]Croneton was removed from the diet. Cumulative excretion of the total radioactivity consumed during the 7-day feeding period was 86% at the time the animals were returned to an untreated diet, and 90% 7 days later.

Nature of Urinary Radiocarbon. Results of the analysis of urine from rats which received [^{14}C]Croneton as a single oral dose or in the diet are presented in Table II. These data represent the results obtained with the [*ring*-^{14}C]- and [*carbonyl*-^{14}C]Croneton. All metabolites identified as carbamates were detected in the urine of rats treated with both ring-^{14}C- and carbonyl-^{14}C-labeled Croneton. Those identified as phenolic hydrolysis products were detected only in the urine of rats treated with [*ring*-^{14}C]Croneton. These data, plus extensive TLC of the metabolites with authentic standards, provided the bases upon which identification of the metabolic products was made.

Qualitatively, the urinary metabolites were sufficiently similar following treatment of the rats with single and multiple doses of the carbamate to suggest that the metabolic pathway for Croneton in rats was not altered by continuous exposure of the animals to the insecticide in the diet. The parent carbamate was not voided in the urine, except possibly in trace amounts which appeared in extracts of acid-treated polar metabolites. While hydrolysis of the carbamate ester was evident, the parent phenol constituted only a small portion of the excreted radiocarbon, and this was almost entirely in the conjugated form.

Table II. Nature of Radiocarbon Eliminated in the Urine of Rats Treated with [^{14}C]Croneton

Metabolite	% of dose administered as: Single oral dose[a]	Dietary supplement[b]
Apolar	36.1	51.4
Croneton sulfoxide	23.0	28.2
Croneton sulfone	3.4	6.9
Parent phenol	0	0.4
Sulfoxide phenol	4.5	6.1
Sulfone phenol	2.9	4.5
Unknown A	2.3	5.3
Polar[c]	60.6	34.5
Croneton	1.0	0.9
Croneton sulfone	0	4.5
Parent phenol	0.9	7.7
Sulfoxide phenol	18.5	14.4
Sulfone phenol	22.0	3.6
Unknown B[d]	18.2	3.4
Total	96.7	85.9

[a] Combined urine voided over 3-day period following treatment. [b] Combined urine voided during 7-day feeding period. [c] Polar metabolites treated with acid to release indicated materials. [d] Radiocarbon not released by acid treatment.

The remaining metabolites which were identified resulted, at least in part, from sulfur oxidation (Table II). Croneton sulfoxide was the predominant carbamate metabolite in the urine while the sulfoxide and sulfone phenols were the predominant noncarbamate. Further oxidation of the sulfur resulted in the formation of the sulfone analogue which existed as the carbamate and phenol, but in lesser amounts than the sulfoxide derivatives. The majority of the phenolic metabolites were present as conjugates while the carbamate sulfoxide and sulfone were largely in the free form.

In the continuous feeding study, Croneton sulfone was recovered from the acid-treated polar metabolites in quantities equal to 4.5% of the total radiocarbon consumed by the animals. The majority of this metabolite was released immediately upon acidification. Its concentration was similar in urine of rats fed both radioactive Croneton feed preparations, and consistent results were obtained with replicate samples. It is, therefore, quite likely that Croneton sulfone was weakly conjugated in the rats and was excreted in this form. Croneton sulfoxide appeared in some extracts of the acid-treated polar metabolites, but the quantities were less than the sulfone and its presence was not consistent. It was concluded that the sulfoxide was not conjugated but was in the polar fraction in the free form as a result of incomplete extraction into the apolar phase.

The metabolite designated as unknown A (Table II) was tentatively identified as 2-ethylsulfonylmethylphenyl *N*-hydroxymethylcarbamate, or hydroxymethyl Croneton sulfone. Since the metabolite was produced in equal quantities with both the [*ring*-^{14}C]- and [*carbonyl*-^{14}C]Croneton, the ester linkage was assumed to be intact. This was further substantiated by treating the ring-^{14}C-labeled metabolites with 0.5 N sodium hydroxide to yield a product having TLC characteristics identical with sulfone phenol. Reaction of this product with methyl isocyanate gave a compound indistinguishable from Croneton sulfone. These data established that the ring moiety of unknown A was the sulfone phenol and that some modification of the carbamate group also had occurred. N-Methylhydroxylation was proposed since a positive color reaction was evident when the metabolite was treated with chromotropic acid. This test, which indicates the formation of formaldehyde from *N*-hydroxymethyl compounds, has been used to denote the presence or absence of *N*-hydroxymethyl metabolites of several carbamate insecticides (Dorough and Casida, 1964; Metcalf et al., 1968; Black et al., 1973).

Table III. Nature of Radiocarbon Eliminated in the Urine of Rats Given a Single Oral Dose of [^{14}C]Croneton Sulfoxide and Croneton Sulfone

Metabolites	% of dose[a] Croneton sulfoxide	Croneton sulfone
Apolar	35.1	23.3
Croneton sulfoxide	23.8	0
Croneton sulfone	4.9	7.8
Sulfone phenol	1.2	10.9
Unknown A	5.2	4.6
Polar	58.3	65.4
Total	93.4	88.7

[a] Combined urine voided over 3-day period following treatment.

Unknown A was consistently present in the free form (Table II) and was sometimes detected in very small amounts in extracts of the acid-treated polar materials. However, its low level and sporatic presence prevented confirmation of its identity as a conjugate.

Treatment of rats with [^{14}C]Croneton sulfoxide as a single oral dose resulted in the same metabolic pattern (Table III) as with Croneton, per se. The same free metabolites were detected in the urine, and the polar products were of a similar magnitude. There is little doubt that the parent carbamate is very rapidly converted to the sulfoxide form upon entering the animal body by ingestion. Data in Table II also confirm that the oxidation of the sulfoxide to the sulfone proceeds at a much slower rate than the initial oxidation. When the sulfone itself was given to the rats, the nature of the metabolites in the urine was as one might predict. All of the free metabolites were sulfone derivatives, and the amounts of polar metabolites were of the same general levels as observed with Croneton and Croneton sulfoxide treatments.

Tissue. Selected tissues were radioassayed for ^{14}C-labeled residues following single oral administration of [*carbonyl*-^{14}C]- and [*ring*-^{14}C]Croneton. The highest levels of residues were found in tissues of animals treated with the ^{14}C-carbonyl-labeled material (Figure 5). As compared to the ring-^{14}C-labeled residues, they were higher by a factor of 1.4 in the muscle, and by factors of 2.3, 5.8, and 9.1 for the kidney, blood, and liver, respectively, when averaged over the 72-h experiment. Obviously, these increased residues with the [*carbonyl*-^{14}C]Croneton treatment were derived only from the carbamate portion of the molecule. This would suggest that the radioactive carbon liberated upon hydrolysis was incorporated in natural tissue components.

Because of the very rapid metabolism and excretion of Croneton, and the low level of ring-^{14}C-labeled residues in rat tissue 8 h after treatment, an experiment was conducted to determine if peak residue levels occurred prior to the 8-h sampling period. Female rats were treated orally with 5×10^5 dpm (7.3 μg) of the ^{14}C-ring-labeled Croneton and then an animal was sacrificed at each 30-min interval for 8 h. Samples of the blood, liver, kidney, and muscle were collected and radioassayed. The point at which maximum [*ring*-^{14}C]Croneton equivalents occurred in the tissues ranged between 2 and 2.5 h after administration of the single oral dose, with the blood and muscle showing the earlier peak. These data clearly demonstrated the extreme rapidity with which Croneton and/or its

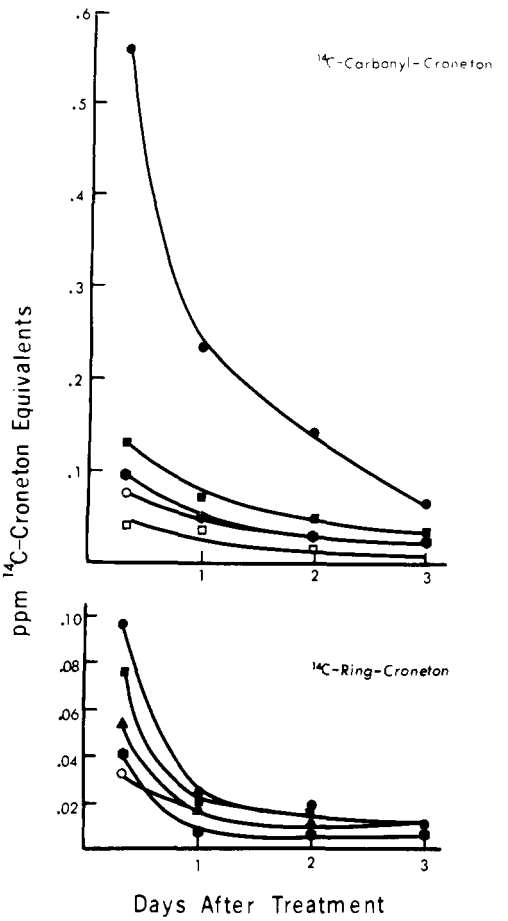

Figure 5. Dissipation of [^{14}C]Croneton equivalents from tissues of rats given a single oral dose of 0.5 mg/kg: (•) liver; (▪) kidney; (▲) muscle; (●) blood; (○) heart; (□) fat.

metabolites are absorbed from the gut, distributed throughout the body, and then eliminated. With such efficient metabolism and excretion, there appears to be little potential for residue accumulation.

Analysis of tissue samples collected from rats maintained on a diet containing 6.6 ppm of either [*carbonyl*-^{14}C]- or [*ring*-^{14}C]Croneton substantiated the results seen in the single oral dose studies (Figure 6). In those rats fed the ^{14}C-carbonyl-labeled material, the majority of the tissues increased in Croneton equivalents as the time of exposure increased. However, the rate of increase was very slow after the second day of exposure of the animals to the treated diet. At the end of 7 days the levels were 1.18, 0.29, 0.24, 0.10, 0.18, and 0.06 ppm for the liver, kidney, blood, muscle, brain, and fat, respectively. Those animals fed the ^{14}C-ring-labeled compound showed much lower ^{14}C-labeled residue levels in the same tissues and they increased very little after the second day of feeding.

When the animals were returned to an untreated diet, ^{14}C-ring-labeled residues in the tissues declined at a much faster rate than the ^{14}C-carbonyl-labeled residues. This pattern of dissipation would be expected if the latter residues resulted in part from the incorporation of the radioactive carbon into normal tissue constituents. [*carbonyl*-^{14}C]Croneton equivalents were between 0.04 and 0.11 ppm 7 days after removal from treatment, while the ^{14}C-ring-labeled residues had decreased below the 0.01-ppm level after only 2 days.

Because of the low levels of [*ring*-^{14}C]Croneton residues in the tissues, no attempt was made to characterize the radiocarbon therein. However, livers of rats fed [*carbonyl*-^{14}C]Croneton in the diet were evaluated as to

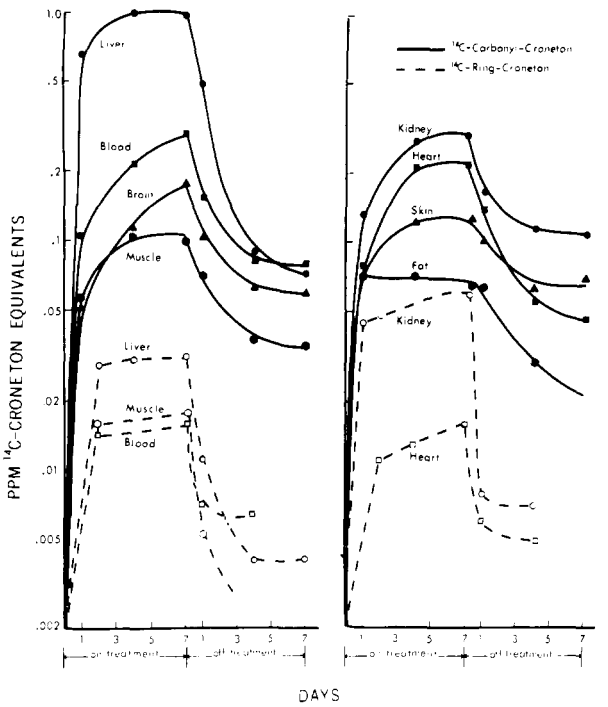

Figure 6. Accumulation and dissipation of [^{14}C]Croneton equivalents in tissues of rats exposed to 6.6 ppm in the diet and returned to untreated feed after 1 week.

extraction characteristics, but their levels were too low for evidence required for identification to be obtained. Homogenization of livers of rats fed 1, 2, 4, and 7 days with a 3:1 acetonitrile–water mixture removed approximately 30% of the ^{14}C-labeled residues in each of the samples. Treatment of the tissue solids with 1.0 N hydrochloric acid at 80°C for 1 h released an additional 44% of the residues; these could not be extracted from the aqueous phase with chloroform. The acetonitrile–water extract of the livers also was extracted with chloroform and, in this case, about two-thirds of the radiocarbon partitioned into the organosolvent layer. Treatment of the aqueous layer with 1.0 N hydrochloric acid for 1 h at 80 °C did not convert any of the radioactivity into chloroform-extractable materials.

Thin-layer chromatography of the chloroform extract of the liver homogenates was unsuccessful because of the small amount of radioactivity and the large quantities of oils and other interfering materials present. Treatment of the chloroform extractables with a coagulation solution (1.25 g of ammonium chloride and 35 ml of phosphoric acid diluted to 1 l.) resulted in all of the radioactivity being removed from the solution along with the precipitate. Thus, none of the ^{14}C-labeled residues in the liver of rats fed [*carbonyl*-^{14}C]Croneton exhibited extraction and/or partitioning characteristics typical of free or acid-released Croneton-type metabolites.

Toxicity. As an indicator of the biological significance of Croneton and its metabolites, the toxicities of the parent carbamate and its oxidized sulfur analogues were determined in both rats and mice (Table IV). The mice were much more sensitive to the carbamates than the rats. In mice, the toxicity of Croneton and its sulfoxide was not statistically different (71 and 59 mg/kg, $p < 0.05$) while Croneton sulfone was only about one-fourth as toxic. While less toxic to rats, the relative toxicities were approximately the same in rats as in mice.

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Table IV. Toxicities of Croneton and Its Oxidized Sulfur Analogues to Rats and Mice[a]

Compound	Approximate LD_{50}, mg/kg[b]		LD_{50}, mg/kg,[c]
	Rats	Mice	mice
Croneton	338	150	71
Croneton sulfoxide	150	67	59
Croneton sulfone	506	225	282

[a] Twenty-four h LD_{50} following single oral dose. [b] Determined by the approximation method of Deichmann and LeBlanc (1943). [c] Determined by log dosage/probit analyses of Litchfield and Wilcoxon (1949).

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LITERATURE CITED

Black, A. L., Chiu, Y. C., Fukuto, T. R., Miller, T. A., *Pestic. Biochem. Physiol.* **3**, 435 (1973).
Deichmann, W. B., LeBlanc, T. J., *J. Ind. Hyg. Toxicol.* **25**, 415 (1943).
Dorough, H. W., Casida, J. E., *J. Agric. Food Chem.* **12**, 294 (1964).
Harvey, J., Jelenik, A. G., Sherman, H., *J. Agric. Food Chem.* **21**, 679 (1973).
Litchfield, J. T., Jr., Wilcoxon, F., *J. Pharmacol. Exp. Ther.* **96**, 99 (1949).
Metcalf, R. L., Fukuto, T. R., Collins, C., Borch, K., El-Aziz, S. A., Munos, R., Cassil, C. C., *J. Agric. Food Chem.* **16**, 300 (1968).

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Metabolism of (+)-Limonene in Rats

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(+)-Limonene was administered intragastrically to rats and ten terpenoid metabolites were isolated from the urines. Of these metabolites, seven were identified by chromatographic and spectral analysis as *p*-mentha-2,8-dien-1-α-ol, *p*-mentha-2,8-dien-1-β-ol, *p*-mentha-1,8-dien-6-α-ol, *p*-mentha-1,8-dien-6-β-ol, *p*-mentha-2-ene-8,9-diol, *p*-mentha-1,8-dien-7-ol, and 4-isopropenyl-1-cyclohexene-1-carboxylic acid.

(+)-Limonene (*p*-mentha-1,8-diene) is a major component of the essential oils of many fruits and spices. (+)-Limonene and essential oils containing (+)-limonene are used extensively in the food and cosmetic industries. Until recently, however, little was known about the metabolism of this terpene in mammals.

In 1966, Wade et al. reported the isolation of uroterpenol (*p*-mentha-1-ene-8,9-diol) from human urine. Subsequent studies by Smith et al. (1969) showed that ingestion of limonene resulted in increased levels of uroterpenol in human urine.

Recently, two studies of the metabolism of (+)-limonene in rabbits and rats have been reported. Kodama et al. (1974) fed radioactive limonene to rabbits and found that 72% of the radioactivity appeared in the urine and 7% in the feces. Eight metabolites were indicated by thin-layer chromatography of the radioactive urine extract. Of the eight, Kodama isolated and identified six metabolites: *p*-mentha-1,8-dien-10-ol, uroterpenol, perillic acid (4-isopropenyl-1-cyclohexene-1-carboxylic acid), a dihydroxyperillic acid, *p*-mentha-1,8-dien-10-ol glucuronide, and uroterpenol glucuronide.

In the rat study Igimi et al. (1974) fed radioactive limonene and followed its absorption and excretion by analysis of urine, feces, bile, and expired air. The excretion of the radioactivity occurred predominantly in the urine (58% of the dose) with feces and bile making up 5.1 and 25%, respectively. Igimi et al. (1974) noted that thin-layer chromatography of the radioactive rat urine extracts indicated the presence of at least ten metabolites. By using comparative thin-layer chromatography these workers identified four metabolites: uroterpenol, perillic acid, 8,9-dihydroxyperillic acid, and uroterpenol glucuronide.

The purpose of this investigation was to isolate and identify the nonconjugated urinary metabolites of limonene in rats.

MATERIALS AND METHODS

(+)-Limonene obtained from J. T. Baker Chemical Co. was purified to greater than 99% by a combination of fractional distillation at reduced pressure (27 mmHg; 75°C) and by preparative gas–liquid chromatography (GLC). Uroterpenol was synthesized from (+)-limonene using the method described by Dean et al. (1967) and purified by silica gel column chromatography to greater than 99%, as judged by GLC. Perillyl alcohol was prepared from β-pinene using the method of Sato (1965) and purified by preparative GLC. Silica gel 60 (particle size less than 0.63 mm) was purchased from E.M. Reagents and used for column chromatography. Reagent grade solvents were used.

Animals and Dosing. Adult male Long-Evans rats (500–600 g) were used. Ten rats were each fed 0.25 ml of limonene per day for 10 days by pharynogogastric intubation. This dose of limonene corresponds to a dose of 40 mg/kg and the total amount of limonene fed to all the rats equaled 21 g. The animals were housed in individual stainless steel metabolism cages and they were allowed food (Purina Rat Chow) and water ad libitum.

Gas–Liquid Chromatography (GLC). A Varian Aerograph 200 gas chromatograph was used in conjunction with a Honeywell recorder and a Hewlett Packard 50B automatic attenuator. The chromatograph had a thermal conductivity detector. The purification of limonene was carried out on a $^3/_8$ in. × 20 ft preparative column containing 20% FFAP (Carbowax 20M plus 10% nitroterophthalic acid) on 40/60 mesh Chromosorb A (Varian Aerograph). The conditions used were as follows: injection

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