Metabolism of Carbofuran in Mugho Pine

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Radiolabeled carbofuran applied to mugho pine roots was translocated primarily to needles. Metabolism was slower than reported in other plants and animals. At least two and possibly three metabolic pathways were operative. 3-Hydroxycarbofuran and an unknown metabolite (unknown II), tentatively identified as *N*-hydroxymethylcarbofuran, were detected from metabolism of [*carbonyl*-^{14}C]carbofuran. Carbofuran phenol, 3-ketocarbofuran phenol, 3-hydroxycarbofuran, and two unknown metabolites were detected using [*ring*-^{14}C]carbofuran. Metabolites were mainly in the nonconjugated form in early samples but later most were in the aqueous phase as glycosides. Acid hydrolysis freed all metabolites and treatment with β-glucosidase or β-glucuronidase freed all except unknown II. Metabolism of unknown II to carbofuran phenol was indicated by corresponding increases in phenol as unknown II declined. Increases in 3-ketocarbofuran phenol were accompanied by decreased levels of 3-hydroxycarbofuran. Direct hydrolysis to carbofuran phenol or rapid hydrolysis of unknown II to carbofuran phenol was indicated.

Metabolism of systemic insecticides in trees has been studied by few workers. In view of the growing concern over environmental contamination, it has become important to know the pathways and rates of metabolic breakdown of insecticides. Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) granules applied to soil effectively controlled the European pine shoot moth *Rhyacionia buoliana* (Schiffermüller) (Lepidoptera: Olethreutidae) on various ornamental pines and Christmas trees in western Washington (Pree and Saunders, 1972). Bioassay of foliage indicated that carbofuran residues persisted for 2 years in the field (Pree and Saunders, 1973). These studies were designed to determine the rate and pathways of carbofuran metabolism in mugho pines, *Pinus mugo* Turra.

The metabolism of carbofuran has been investigated in agricultural crops, insects, and mammals (Bowman and Beroza, 1967; Leuck *et al.*, 1968; Dorough, 1968a,b; Ivie and Dorough, 1968; Metcalf *et al.*, 1968; Green and Dorough, 1970; Hicks *et al.*, 1970; Knaak *et al.*, 1970a,b). These papers revealed that potential metabolic pathways in each plant and or animal were by hydrolysis, oxidation, and conjugation. Most authors found similar metabolites but the relative importance of each varied. Hicks *et al.* (1970) commented on this variation and suggested that examination of each species exposed might be necessary.

METHODS

General. Roots of 30–45 cm tall, 3- to 4-year-old mugho pines were soaked in water and gently washed to remove soil. Each tree was placed in a beaker covered with aluminum foil to protect the roots from light. Glass marbles provided a solid matrix for the root system. A 2 × 2 ft polyethylene covered chamber enclosed the foliar part of the tree. Air was evacuated from the chamber through a glass tube placed in the side and passed through 4 *N* NaOH twice to collect CO_2. Air supply to the tree did not have CO_2 removed; therefore CO_2 trapped contained atmospheric CO_2 as well as CO_2 from the tree.

Radioactive solutions were prepared by placing a measured amount of ^{14}C-labeled carbofuran in ethyl acetate in a beaker, evaporating the solvent, and dissolving the carbofuran deposit in deionized water. Samples of ^{14}C-labeled (sp act. 2.7 Ci/mol) and ring-^{14}C-labeled carbofuran (position 7a) (sp act. 2.2 Ci/mol), and carbofuran and metabolite standards were supplied by Niagara Chemical Division, FMC Corporation, Middleport, N. Y. Roots of the tree treated with carbonyl-^{14}C-labeled carbofuran (20 μCi, or 1.485×10^7 cpm) remained in the radioactive solution throughout the test (24 days). In two tests with ring-^{14}C-labeled carbofuran (60 μCi or 31.05×10^6 and 31.5×10^6 cpm, respectively), the radioactive solution was replaced after 35 days with a dilute nutrient solution adapted from Walker *et al.* (1955). The nutrient solution contained 0.005 *M* $NH_4H_2PO_4$, 0.003 *M* KNO_3, 0.002 *M* $Ca(NO_3)_2$, and 0.001 *M* $MgSO_4$. In the latter two experiments, the roots were aerated continuously by bubbling air through the solution. Levels of test solution were maintained by adding water or nutrient solution as required.

Needle samples (2–2.5 g) were collected at various dates during the experiments. At completion, the tree was dismantled into roots, trunk, current growth wood, needles, and buds. Trunk tissues included all woody parts except shoots of the current year's growth.

Extraction and Cleanup Procedures. Methods used were similar to those of Cook *et al.* (1969) and Cassil *et al.* (1969). Tissue samples were ground for 5 min in a blender with 50 ml of 0.5 *N* HCl, diluted to 250 ml with additional 0.5 *N* HCl, refluxed for 1 hr, and filtered hot through a Whatman No. 1 filter paper. The boiling flask and filter were washed with an additional 100 ml of hot 0.5 *N* HCl. The filtrate was cooled to room temperature and the aqueous phase extracted three times with 100-ml aliquots of CH_2Cl_2 (reagent grade). The CH_2Cl_2 extracts were dried over anhydrous Na_2SO_4, filtered, and reduced to *ca.* 5 ml over low heat and a stream of dry air and 100 ml of CH_3CN (reagent grade) were added. The CH_3CN was extracted twice with 25-ml aliquots of C_6H_{14} saturated with CH_3CN. The C_6H_{14} fraction was discarded and the CH_3CN fraction concentrated to *ca.* 5 ml, diluted to 100 ml with tap water (pH 6.5), and extracted twice with 100-ml aliquots of CH_2Cl_2. Water samples were checked for radioactive content. Methylene chloride extracts were dried over anhydrous Na_2SO_4, filtered, and concentrated for chromatography or further cleanup.

Samples from the tree treated with carbonyl-labeled carbofuran were concentrated to *ca.* 130 ml and poured through a 20 × 250 mm i.d. glass column that contained 17 g of silica gel (Florisil 60–100 mesh) topped with a glass wool plug and packed in C_6H_{14}. The flask was washed with an additional 15 ml of CH_2Cl_2 that was added to the column. When most of the CH_2Cl_2 fraction had penetrated into the silica gel, 100 ml of ethyl acetate–hexane (70:30) was added. Collection was continuous until no solvent remained on the column. The column was not used

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Table I. R_f Values of Carbofuran and Derivatives on Eastman Prepared Silica Gel Plates

Chemical name	Designation in text	R_f EH[a] (3:1)	R_f EB[b] (3:1)	R_f HAT[c] (3:1:1)
2,3-Dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate	Carbofuran	0.50	0.48	0.51
2,3-Dihydro-2,2-dimethyl-3-hydroxy-7-benzofuranyl *N*-methylcarbamate	3-Hydroxycarbofuran	0.28	0.35	0.26
2,3-Dihydro-2,2-dimethyl-3-keto-7-benzofuranyl *N*-methylcarbamate	3-Ketocarbofuran	0.48	0.46	0.42
2,3-Dihydro-2,2-dimethyl-7-hydroxybenzofuran	Carbofuran phenol	0.69	0.71	0.54
2,3-Dihydro-2,2-dimethyl-3,7-dihydroxybenzofuran	3-Hydroxycarbofuran phenol	0.46	0.44	0.29
2,3-Dihydro-2,2-dimethyl-3-keto-7-hydroxybenzofuran	3-Ketocarbofuran phenol	0.55	0.53	0.51

[a] Ether–hexane. [b] Ether–benzene. [c] Hexane–acetone–toluene.

with ring-labeled carbofuran samples because some of the metabolites, *e.g.*, carbofuran phenol, remained on the column.

The combined methylene chloride–ethyl acetate–hexane fractions were concentrated to *ca.* 1 ml over low heat and a stream of dry air. The remaining solvent was evaporated by heating gently under vacuum. Immediately, 0.5 ml of ethyl acetate was added and the sample was ready for thin-layer chromatography. Preliminary tests showed that 90–96% of the ^{14}C-labeled carbofuran and 87–93% of carbofuran phenol introduced into untreated samples were recovered.

Carbofuran and free metabolites were separated from conjugated metabolites by grinding samples in 50 ml of acetone, filtering, and concentrating to *ca.* 5 ml over low heat and a stream of dry air, diluting to 100 ml with tap water, and extracting with CH_2Cl_2. Extraction was facilitated by refrigeration of the aqueous phase (at 5°) to avoid semistable emulsions that formed at room temperature. The CH_2Cl_2 fraction was prepared for chromatography as previously described. Conjugated metabolites were extracted (into CH_2Cl_2) after acid hydrolysis or enzyme action.

Enzyme Studies. To test the concept that carbofuran and metabolites in the aqueous phase (after CH_2Cl_2 extraction) were conjugated with sugars, aliquots of the aqueous phase were subjected to enzymes known to free conjugated carbamate metabolites (Kuhr and Casida, 1967) and other enzymes which appeared potentially applicable. β-Glucuronidase (containing some sulfatase activity), amyloglucosidase, cellulase, sulfatase (containing some β-glucuronidase activity) (Sigma Chemical Co., St. Louis, Mo.), or β-glucosidase (Worthington Biochemical Corporation, Freehold, N. J.) were added (1 mg/ml) to aliquots of the aqueous phase buffered at pH 4.5 with a Na_3PO_4–citric acid buffer system. Chitinase (Worthington Biochemical) was buffered at pH 6.5. Samples were shaken for 24 hr at 22° and extracted with CH_2Cl_2, and the aqueous phase was acidified to pH 1.2 with 0.5 *N* HCl, refluxed, cooled, and extracted again with CH_2Cl_2. Methylene chloride fractions were prepared for chromatography. Semistable emulsions that formed in samples containing β-glucosidase or β-glucuronidase were broken by centrifuging (8000*g*) for 10 min.

Thin-Layer Chromatography. Extracts were cochromatographed with known standards of carbofuran and metabolites on Eastman silica gel plates containing no fluorescent indicator. Chromatograms were developed in ether–hexane (3:1) and visualized by spraying with *p*-nitrobenzenediazonium fluoroborate to produce a reddish color with carbofuran metabolites having free hydroxyl groups. Metabolites without free hydroxyl groups were visualized by treatment with ethanolic NaOH (1 *N*) to free hydroxyl groups prior to treatment with fluoroborate solution.

R_f values for carbofuran and metabolites, their chemical nomenclature, and the designated name used in the text are presented in Table I. Ether–hexane was the standard system but auxiliary systems were included to confirm or further identify metabolites that were poorly separated by ether–hexane.

Twenty microliters of plant extract and 10 μl of metabolite solution made up as 4 mg/ml were applied to each spot for chromatography. Fresh developing solution was used each day. X-Ray film (Kodak Blue Brand) was exposed to the chromatograms in total darkness at 0° for varying time intervals, depending on the amount of radioactivity.

Isotope Counting. One-milliliter aliquots of the CH_2Cl_2 fraction were pipetted into planchets and counted with a Nuclear-Chicago Model D-47 gas flow detector connected to a Model C-110 B automatic sample changer and Model 181 B scaler. Volatilization of radioactive compounds from CH_2Cl_2 was reduced by adding 0.15 ml of a 5% shellac (4 lb cut orange shellac) in $CHCl_3$ to each planchet (Getzin and Rosefield, 1966). Samples were counted 1–2 hr after plating. Aqueous samples were plated onto ringed planchets and counted after air drying for 24 hr.

Carbon dioxide traps were replaced weekly. Carbonate in the NaOH was precipitated as $BaCO_3$, washed with acetone, dried, and assayed for radioactivity using a correction for infinite thickness. Radioactive spots on chromatograms were excised and placed in planchets for counting. Corrections for solvent effects in counting were related to counts per minute in reagent grade CH_2Cl_2.

Radioactivity in residues (solid material) after refluxing was estimated by total combustion using a derivation of the method described by Wang and Willis (1965). Ten per cent of the residue (by weight) was placed in a 250-ml boiling flask and 100 ml of a solution of 166 ml of H_2SO_4, 83 ml of H_3PO_4, and 12.5 g of CrO_3 was added. The sample was heated for 3 hr (below boiling) until digested. Carbon dioxide was trapped in 4 *N* NaOH and assayed as described. Air was passed through the boiling flask to ensure gas movement to the trapping solution.

RESULTS

Metabolism of [*carbonyl*-^{14}C]Carbofuran. Radioactivity was detected in needles within 3 days and increased *ca.* 10,000 cpm/g per week during the experiment. Roots con-

Table II. Metabolism of [*carbonyl*-^{14}C]Carbofuran in Mugho Pine

Sample	% of original ^{14}C as[a]			
	Carbofuran	3-Hydroxy-carbofuran	Unknown II[b]	Total
Water solution, 24 days	54.87	0.56	0.33	55.76
Roots, 24 days	7.83	0.64	0.49	8.96
Needles				
3 days[c]	0.16	0	0	
7 days[c]	1.43	0	0.07	
14 days[c]	2.93[b]	0.12	0.11	
24 days[c]	3.01	0.40	0.90	4.31
Trunk, 24 days	1.83	0.05	0.20	2.08
Current growth wood, 24 days	0.25	0.02	0.07	0.34
$^{14}CO_2$, 0–24 days				3.78
% recovery				75.19

[a] Acid-hydrolyzed samples. [b] R_f 0.44 in ether–hexane (3:1). [c] Based on total weight of needles at 24 days.

Table III. Metabolism of [*ring*-^{14}C]Carbofuran in Mugho Pines

Sample	% of original ^{14}C as[a]								
	Carbofuran	3-OH	7-OH	3-C=O 7-OH	U-I[c]	U-II[d]	U-III[e]	Residue	Total
Water solution[b]									
35 days	36.26	0.37	1.11	2.39	0.25	0.87	Tr	0	41.25
70 days	12.96	0.10	2.03	1.84	0.06	0.36	14.95	0	32.30
Roots, 70 days	0.40	0.03	0.20	0.27	0.55	0.05	0.26	0.99	2.75
Trunk, 70 days	0.65	0.10	0.49	0.18	0.02	0.22	0.32	0.21	2.20
Current growth wood, 70 days	0.16	0.02	0.11	0.05	0.01	0.05	0.11	0.06	0.57
Needles									
14 days[f]	2.69	0.14	0.29	0.15	0.10	0.12	0.25	Tr	
35 days[f]	4.06	0.60	1.16	0.61	0.18	0.35	1.68	0.02	
70 days[f]	3.12	1.59	2.60	1.94	0.56	0.95	4.21	0.34	15.31
Buds	0.01	Tr	0.01	Tr	Tr	Tr	0.01	Tr	0.03
CO_2, 0–70 days									0.07
Total									94.48

[a] Acid-hydrolyzed samples. [b] Plants were removed from treatment solution after 35 days and placed in fresh nutrient solution for 35 days. [c] R_f 0.00 in ether–hexane (3:1). [d] R_f 0.44 in ether–hexane (3:1). [e] Water soluble, not extracted with CH_2Cl_2 after acid hydrolysis. [f] Based on total weight of needles at 70 days.

tained almost 9% of the original input whereas 4.31% was found in needles. Lesser amounts were present in trunk or current growth wood tissue. After acid hydrolysis *ca.* 95% of the radioactivity partitioned into the CH_2Cl_2 phase. Over 50% of the initial input remained in the water solution. Seventy-five per cent of the input was recovered. Carbon dioxide (as $BaCO_3$) accounted for 3.78% of the original input (Table II). Carbon dioxide production began within 12 hr and increased to approximately 200,000 cpm per week after the first 7 days of the experiment. Some radioactivity possibly was lost as CO_2 from the aqueous solution or from inefficiencies in CO_2 trapping.

Chromatography of needle extracts (Table II) showed only carbofuran after 3 days but the sample was small (2.0 g) and minute quantities of metabolites may have been undetectable. Carbofuran metabolites accounted for *ca.* 0.07 and 0.23% of the radioactivity in the organosoluble fraction after 7 and 14 days, respectively. In the 24-day needle samples, 70% of the radioactive compounds in the organic fraction (3.01% of input) was carbofuran. An unidentified metabolite (unknown II) chromatographed at R_f 0.44 in ether–hexane and was the major organosoluble metabolite. Unknown II and 3-hydroxycarbofuran were the only organosoluble metabolites detected with [*carbonyl*-^{14}C]carbofuran. Unknown II was detected 7 days earlier than 3-hydroxycarbofuran.

Organosoluble metabolites occurred in all tree parts but accumulation was greatest in the needles (Table II). Unknown II was the major metabolite in all tissues except roots. Over 98% of the labeled carbofuran remaining in the aqueous solution was not metabolized.

Metabolism of [*ring*-^{14}C]Carbofuran in Mugho Pine. Carbofuran in needles accounted for *ca.* 2.69% of the total radioactivity after 14 days, 4.06% after 35 days, and 3.12% after 70 days (Table III). Carbofuran phenol was the main organosoluble metabolite in each sample. Lesser amounts of 3-hydroxycarbofuran, 3-ketocarbofuran phenol, unknown I (R_f 0.00), and unknown II were present in all samples. Radioactivity in the aqueous phase (unknown III) not extracted in CH_2Cl_2 was 4.21% of the total input in the 70-day sample.

Percentages of 3-hydroxycarbofuran, carbofuran phenol, and 3-ketocarbofuran phenol approximately quadrupled between 14 and 35 days, but increased less between 35 and 70 days.

Radioactivity was concentrated in leaf tissues (Table III). Bud tissues held less radioactivity than others. Trunk and roots contained approximately the same amount of radioactivity. Roots contained more inextractable radioactivity than other tissues and held only small amounts of carbofuran. Radioactivity that remained in water solution after 35 days was mostly carbofuran. Carbofuran represented 40% of the total radioactivity in the nutrient solution after 70 days (Table III). Carbofuran phenol and 3-ketocarbofuran phenol were the most important metabolites in solution but were a small portion of the total.

Carbofuran phenol was the most important organosoluble metabolite, but all metabolites found were present in all tissues. Needles held the highest per cent carbofuran. Unknown III (water soluble not extracted in CH_2Cl_2) was most prevalent in needle tissues and in solutions. Unknown I was most concentrated in root and needle tissues.

Forty-one per cent of the radioactive input remained in

Table IV. Distribution of Conjugated and Free Metabolites of Ring-^{14}C-Labeled Carbofuran in Mugho Pine Needles

Compound	% of original ^{14}C as					
	14 days		35 days		70 days	
	Organo-soluble[a]	Water soluble[b]	Organo-soluble[a]	Water soluble[b]	Organo-soluble[a]	Water soluble[b]
Carbofuran	2.93	0.11	4.01	0.28	3.36	0.48
3-Hydroxycarbofuran	0.08	0.19	0.14	0.40	0.31	0.72
Carbofuran phenol	0.01	0.09	0.03	0.93	0.04	1.78
3-Ketocarbofuran phenol	0.14	0.08	0.26	0.33	0.41	0.75
Unknown I[c]	Tr	0.12	Tr	0.29	0.07	1.40
Unknown II[d]	0.10	0.11	0.25	0.19	0.34	0.41

[a] Soluble in CH_2Cl_2 before acid hydrolysis. [b] CH_2Cl_2 soluble after acid hydrolysis. [c] R_f 0.00 in ether–hexane (3:1). [d] R_f 0.44 in ether–hexane (3:1).

the treatment solution after 35 days at which time the roots were placed in fresh nutrient solution. After an additional 35 days 32.3% of the radioactivity was present in the nutrient solution (Table III). This radioactivity was sorbed by roots and was released into the nutrient solution during the second half (35–70 days) of the experiment. Carbon dioxide accounted for 0.07% of the radioactivity. Recovery was 94.48% of the total.

Separation of carbofuran and free metabolites from conjugates (Table IV) showed that, though all were present in free forms, most of each metabolite was held as a conjugate. Treatment with β-glucosidase or β-glucuronidase freed all except unknown I and unknown II which were freed by acid hydrolysis. Carbofuran phenol existed almost entirely in the conjugated form. Metabolites were not present as salts since acidification (to pH 1.2) did not increase organosoluble radioactivity. Chromatography of extracts in other solvent systems revealed no other metabolites.

Unknown II was found with [*ring*-^{14}C]carbofuran and with [*carbonyl*-^{14}C]carbofuran. Samples of unknown II from each experiment were purified by thin-layer chromatography (in ether–hexane) and the spots eluted in ethyl acetate. Cochromatography of the two extracts resulted in a single spot of R_f 0.44 in ether–hexane. Alkaline hydrolysis of unknown II (using ring-^{14}C-labeled material) yielded carbofuran phenol. Similar treatment of unknown I yielded water soluble metabolites. Alkaline hydrolysis of unknown II using carbonyl-labeled material resulted in loss of the radioactivity into the aqueous phase.

In a second experiment using similar amounts of [*ring*-^{14}C]carbofuran, unknown II was the major metabolite in needles (Table V). 3-Hydroxycarbofuran, carbofuran phenol, 3-ketocarbofuran phenol, and unknown I were the other organosoluble metabolites. There was more 3-hydroxycarbofuran than carbofuran phenol in the 14- and 35-day samples but both were present in approximately equal amounts after 49 days. The data suggest that unknown II and 3-hydroxycarbofuran were the first metabolites produced. 3-Ketocarbofuran phenol levels remained almost constant after 14 and 35 days but increased greatly between 35 and 49 days. Carbofuran phenol increased greatly between 35 and 49 days whereas the percentage of unknown II remained almost constant. This suggested a possible conversion of unknown II to carbofuran phenol.

Enzyme Studies. Treatment of the aqueous phase (before acid hydrolysis) with β-glucosidase, β-glucuronidase, or sulfatase freed quantities of all metabolites except unknown II, indicating that these were held as glycosides. All other enzymes were ineffective. Acid hydrolysis of enzyme-treated samples yielded small additional quantities of each metabolite except unknown II. Sulfatase added to β-glucosidase or β-glucuronidase released small additional amounts of each metabolite including unknown II.

Analysis of aqueous and organic phases revealed *ca.* 15% loss of radioactivity. The experiment was repeated in a sealed container with a CO_2 trap but no radioactive CO_2 was detected. This loss was not observed when boiled enzyme (β-glucosidase) was added to the control. When samples were centrifuged, much of the enzyme was deposited at the H_2O–CH_2Cl_2 interface.

Table V. Metabolism of [*ring*-^{14}C]Carbofuran in Mugho Pine Needles

Compound	% of original ^{14}C as[a]		
	14 days	35 days	49 days
Carbofuran	3.29	5.29	9.58
3-Hydroxycarbofuran	0.16	1.79	2.32
Carbofuran phenol	0.04	0.18	2.45
3-Ketocarbofuran phenol	0.10	0.29	1.52
Unknown I[b]	0.06	0.85	0.89
Unknown II[c]	0.33	4.66	5.74

[a] Acid-hydrolyzed samples extracted with CH_2Cl_2. [b] R_f 0.00 in ether–hexane (3:1). [c] R_f 0.44 in ether–hexane (3:1).

DISCUSSION

Most radioactivity accumulated in needles. Lesser quantities were found in roots, trunk, and current growth wood. Buds contained the smallest amounts. This agrees with the translocation pattern in field samples of needles and buds (Pree and Saunders, 1973). Ashworth (1970) found a similar pattern in tobacco leaves *vs.* tops and suggested that this was due to greater transpiration from large leaves than from the terminal bud.

Metabolism of carbofuran in mugho pines was slower than reported in other plants and animals. This correlates with carbofuran persistence for 2 years in mugho pine foliage in the field (Pree and Saunders, 1973). In bean plants, 68% of injected carbofuran was metabolized after 3 days (Dorough, 1968a). Knaak *et al.* (1970a) found 95% of carbofuran applied to alfalfa roots in sand was metabolized after 30 days. The half-life in tobacco plants was about 4 days (Ashworth, 1970). Metcalf *et al.* (1968) found that over 50% of carbofuran was hydrolyzed within 24 hr in white mice and Dorough (1968b) found that 45% was hydrolyzed within 32 hr in rats. Carbofuran fed orally to laying hens was 54% hydrolyzed after 6 hr (Hicks *et al.*, 1970). A dairy cow fed ^{14}C-labeled carbofuran excreted over 90% of the radioactivity within 24 hr (Ivie and Dorough, 1968). A cow fed carbofuran-treated alfalfa residues excreted 77 and 38%, respectively, of ring and carbonyl labels in urine and 22 and 18%, respectively, in feces within 4 days (Knaak *et al.*, 1970b). The half-life of carbofuran in mugho pine was not accurately estimable from these studies because of sorption by roots. This phenomenon may have significance in the control of some root-feeding species since Saunders (1970) found that drenches applied to container-grown spruce controlled the black vine weevil, *Brachyrhinus sulcatus* (F.).

Hydrolysis, oxidation, and hydroxylation reactions are involved in metabolism of carbofuran in mugho pines. At least two and possibly three metabolic pathways were operative. 3-Hydroxycarbofuran and unknown II were produced from carbonyl-labeled carbofuran. Unknown II was the major metabolite in all tissues except roots where about equal amounts of 3-hydroxycarbofuran occurred. Carbon dioxide production indicated that hydrolytic reactions also occurred. Carbofuran phenol, 3-ketocarbofuran phenol, 3-hydroxycarbofuran, and unknown II were recovered from [*ring*-^{14}C]carbofuran treated trees. Unknown I was found only in trees treated with ring-labeled carbofuran. Similar metabolites were produced in both experiments with ring-labeled carbofuran but their relative abundance was dissimilar. In one test unknown II was the major organosoluble metabolite in all plant parts except roots where 3-ketocarbofuran phenol occurred in slightly larger amounts. In the other test carbofuran phenol was the major metabolite with 3-hydroxycarbofuran, 3-ketocarbofuran phenol, unknown I, and unknown II present in lesser amounts.

Carbofuran metabolism in solution around roots was minimal until mineral nutrients were added. These nutrients may have increased metabolism by promoting bacterial growth. All metabolites found in plant tissues were present in solution.

Metabolites found by other workers have generally included those found in this study but the major metabolite has often varied. Carbofuran phenol was a major metabolite in plants, insects, and animals (Metcalf *et al.*, 1968). Dorough (1968a) found that 3-hydroxycarbofuran was the most abundant metabolite in bean plants. 3-Hydroxycarbofuran, 3-hydroxycarbofuran phenol, and carbofuran phenol were most common in tobacco plants (Ashworth, 1970). 3-Ketocarbofuran phenol and 3-hydroxycarbofuran phenol were most abundant in alfalfa (Knaak *et al.*, 1970a).

Unknown II was the major metabolite in the experiment with [*carbonyl*-^{14}C]carbofuran and in one experiment with [*ring*-^{14}C]carbofuran and was present to a lesser extent in a third. Cochromatography of purified samples of carbonyl-labeled and ring-labeled unknowns produced a single radioactive spot, indicating that this was the same compound and that the carbamate moiety or a portion thereof remained on the molecule. Hydrolysis (in alkali) of ring-labeled unknown II to carbofuran phenol showed that alterations to the initial molecule occurred on the carbamate moiety. This suggested that this metabolite was *N*-hydroxymethylcarbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-hydroxymethylcarbamate). The R_f of unknown II in ether–hexane placed it between 3-hydroxycarbofuran and 3-ketocarbofuran, a position similar to that found for *N*-hydroxymethylcarbofuran by Metcalf *et al.* (1968). Dorough (1968a) found traces of 3-hydroxy-*N*-hydroxymethylcarbofuran and 3-keto-*N*-hydroxymethylcarbofuran in bean plants. Many of these workers had no authentic standard and based their observations on indirect methods. Metcalf *et al.* (1968) synthesized an authentic standard, but indicated that because of its hydrolytic instability only small quantities were likely to occur in either plants or animals. *N*-Hydroxymethyl metabolites are often major breakdown products of other methyl carbamate insecticides (Kuhr, 1968, 1970; Casida and Lykken, 1969). Oonnithan and Casida (1966, 1968) identified *N*-hydroxymethyl metabolites from carbaryl, propoxur, aminocarb, and several other methyl carbamates added to rat liver microsomes. Methods for synthesis of *N*-hydroxymethyl derivatives were described by Balba *et al.* (1968), Balba and Casida (1968), Durden *et al.* (1970), and Fahmy and Fukuto (1972). More work on synthesis and systematic identification of *N*-hydroxymethyl standards is necessary before positive identification of unknown II as *N*-hydroxymethylcarbofuran can be made. Another possible metabolite which might have a similar R_f is *N*-demethylcarbofuran (2,3-dihydrol-2,2-dimethyl-7-benzofuranyl carbamate). *N*-Demethylpropoxur has been reported by Shrivastava *et al.* (1970) from mosquitos.

Metabolites were mainly in the free form in early samples. In later samples (35, 49, and 70 days) most were retained in the aqueous phase until freed by enzyme action or acid hydrolysis. Although all metabolites occurred in the free form in these later samples, only 3-hydroxycarbofuran and 3-ketocarbofuran phenol were present in appreciable quantities. Hydrolysis with β-glucosidase or β-glucuronidase produced organosoluble metabolites, indicating that conjugation was mainly as glycosides. Unknown II, however, was not freed by enzyme action and could be detected only after acid hydrolysis. Acid hydrolysis of enzyme-treated samples failed to release this metabolite. The loss in radioactivity observed in enzyme treated samples may have been due to increased absorption. However, loss occurred only with active enzyme and not where boiled enzyme was added.

Levels of conjugated metabolites freed by hydrolysis did not greatly increase between 35 and 70 days. Radioactivity in the aqueous phase increased greatly but most was not extractable after enzyme action or acid hydrolysis. This suggested that quantities of extractable aglycones had equilibrated.

Carbon dioxide production from [*ring*-^{14}C]carbofuran was slight, and most of the radioactivity taken up remained in the plant. Some of the ^{14}C may have been incorporated into plant constituents during biosynthesis. Some glycosides may not have been destroyed by hydrolytic processes.

Several pathways in metabolism of carbofuran are evident from these studies. The relative importance of each pathway varied in different trees. Results obtained in these experiments suggested that some of the initial metabolic products were in turn converted to others. The position of the ^{14}C label on carbonyl-labeled carbofuran allowed detection of only two metabolites, but radioactivity in CO_2 produced indicated hydrolysis to at least one phenol form.

Unknown II, tentatively identified as *N*-hydroxymethylcarbofuran, was the major metabolite produced in one experiment with ring-labeled carbofuran. Metabolism of unknown II to carbofuran phenol is suggested by an increase in carbofuran phenol as the unknown II content levelled off. Direct hydrolysis of carbofuran to carbofuran phenol was most important in another experiment. Rapid hydrolysis of unknown II to carbofuran also may have occurred. Production of 3-ketocarbofuran phenol was probably by hydrolysis of 3-ketocarbofuran, a highly unstable derivative (Metcalf *et al.*, 1968) of 3-hydroxycarbofuran. Although 3-ketocarbofuran was not isolated in these studies, the occurrence of this pathway is evidenced by increases in 3-ketocarbofuran phenol as the 3-hydroxycarbofuran content levelled off (Table V). The transient occurrence of this metabolite has been postulated by Knaak *et al.* (1970b).

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Carbofuran. Comparative Toxicity and Metabolism in the Worms *Lumbricus terrestris* L. and *Eisenia foetida* S.

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Eisenia foetida, a manure worm, showed characteristic symptoms of carbofuran poisoning (coiling and random muscle contractions). There appeared to be, however, a wide difference between this species and *Lumbricus terrestris*, the common dew worm, in susceptibility to this carbamate insecticide. Compared to the toxicity toward the dew worm this compound was one-sixth as toxic to *Eisenia* when injected and approximately half as toxic when applied in the soil. The insecticide appeared to repel *Eisenia* but not *Lumbricus*. Both worms took up carbofuran in quantities proportional to their size; however, *Eisenia* excreted 95% of this material in 48 hr compared to only 10% excreted by *Lumbricus*. Approximately half of the excreted material in the case of *Eisenia* was unchanged insecticide. Of the insecticide broken down by the worms in a 48-hr period, *Eisenia* retained only 5% as metabolites whereas *Lumbricus* retained 87%. This comparative study emphasized the importance of selecting truly representative species for the evaluation of new insecticides.

Stenersen *et al.* (1973) have recently reported on the severe toxicity of the carbamate insecticide carbofuran (Furadan) to the earthworm *Lumbricus terrestris*. However, apparently conflicting reports on the effects of this relatively new carbamate on earthworms have been released by the manufacturer (FMC Corporation, Niagara Chemical Division) in a technical review paper in June 1972. This discrepancy was not real, however, since FMC Corporation used as their experimental animal a species of worm that is not strictly an earthworm. This species, *Eisenia foetida*, inhabits animal dung and manure and feeds by ingesting organic debris without ever surfacing (Satchell, 1967). It can tolerate colder temperatures than *Lumbricus* and can mate the year round. Although this manure worm has the highest nitrogen excretion rate of any worm species (0.4 mg/g of worm per day), it is of little direct significance to field fertility since it lives only in excreta.

The wide difference in susceptibility of these two species presented an excellent opportunity for a comparative study in an effort to better understand the reasons for the high toxicity of carbofuran toward the agriculturally important species *Lumbricus*.

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METHODS

Five hundred *Eisenia foetida* were purchased from Brazos Worm Farms (Waco, Tex. 76705) and 500 were supplied by the FMC Corporation (Niagara Chemical Divison, Middleport, N. Y.). These worms, referred to by the dealer as "red-gold hybrids," were kept under conditions identical with those for *Lumbricus*. The latter were purchased and maintained as described by Stenersen *et al.* (1973). All worms used were adults and weighed either 3-5 g (*Lumbricus*) or 0.5-0.7 g (*Eisenia*).

Toxicants were obtained, prepared, and administered as outlined by Stenersen *et al.* (1973).

All worms to be injected were prechilled on ice for 15 min. The maximum injection for *Lumbricus* was 5 μl and the maximum for *Eisenia* was 1 μl.

Selection of carbofuran-treated *vs.* untreated soils by the two species was tested by presenting both soil types in the same container. Round plastic buckets (20-cm diameter) were used. Carbofuran (recrystallized) was incorporated into the treated soils at 1.5-2.0 ppm. The soil types