Fate of Carbofuran in a Model Ecosystem

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Ring-^{14}C- and carbonyl-^{14}C-labeled carbofuran (2,2-dimethyl-2,3-dihydrobenzofuranyl 7-*N*-methylcarbamate) were studied in a model ecosystem similar to that devised by Robert L. Metcalf and coworkers. Carbofuran was rapidly degraded in water. Carbofuranphenol, carbofuran, 3-ketocarbofuran, 3-hydroxycarbofuranphenol, *N*-hydroxymethylcarbofuran, and 3-hydroxycarbofuran and several other unknown spots were detected by thin-layer chromatography (tlc) and autoradiography in ether extracts from water. Radioactivity in a NaOH trap rose steadily, accompanied by rapid loss of radioactivity in the water treated with carbonyl-^{14}C-labeled carbofuran. No parent carbofuran was found in living organisms. However, large amounts of the parent carbofuran were found in dead crabs which died soon after application of the pesticides. Carbofuran was highly toxic to crabs, clams, *Daphnia*, and snails immediately after application to the model ecosystem, but all animals except one crab survived restocking 20 days later.

Carbofuran (2,2-dimethyl-2,3-dihydrobenzofuranyl 7-*N*-methylcarbamate) is a broad-spectrum insecticide used for the control of insect pests in corn and many other crops. This compound is highly toxic to mammals, having an LD_{50} of 11 mg/kg (Metcalf *et al.*, 1968). The metabolism of carbofuran is well documented in several species of animals and plants (Ashworth and Sheets, 1972; Cook *et al.*, 1969; Dorough, 1968a,b; Knaak *et al.*, 1970a,b; Metcalf *et al.*, 1968). Acetylcholinesterase inhibition by this compound and its analogs has been reported recently (Yu *et al.*, 1972). However, the environmental consequences of this compound are not well known. Recently, Metcalf *et al.* (1971) devised a simple model ecosystem which enables investigators to examine the fate of pesticides in a highly reproducible laboratory system. Booth *et al.* (1973) have recently used this system for determining the fate of herbicides. This paper reports the fate of carbofuran in this model ecosystem.

MATERIALS AND METHODS

Labeled Compounds. Ring-^{14}C- and carbonyl-^{14}C-labeled carbofuran were obtained from Niagara Chemical Division, FMC Corp., Middleport, N. Y. The specific activity was 4.5 mCi/mmol for both compounds.

Model Ecosystem. The components of the laboratory ecosystem were seedling sorghum plants (*Sorghum halopense*), saltmarsh caterpiller larvae (*Estigmene acrea*), algae (*Oedogonium cardiacum*), fresh water clams (*Corbicula manilensis*), fresh water crabs (*Uca minax*), frogs (species not identified), and a fresh water plant (*Elodea canadensis*). The clams, crabs, frogs, and *Elodea* were obtained from a local pet shop. All other organisms came from stock cultures in the laboratory. The overall procedures described by Metcalf *et al.* (1971) were followed with slight modification. Standard reference water was used in the system (Freeman, 1953). Five milligrams (50 μCi) of the labeled carbofuran in 0.5 ml of acetone was applied to leaves of 7-day-old sorghum plants in each tank. The pesticide was applied at a dosage equivalent to 1 lb of actual toxicant per acre. Experiments were carried out in two aquaria (tanks) for each compound and the aquaria were positioned in an environmental chamber which provided a temperature of 27 ± 1° and 2000 ft-candles of fluorescent light for 12 hr and dark for 12 hr. Saltmarsh caterpillars were killed after they ate sorghum leaves containing carbofuran and it was necessary to add more caterpillars for the first 5 days until all the leaves were consumed. Most of the organisms were killed shortly after introduction of carbofuran to the tank but the tanks were restocked every 5–7 days. Aquatic organisms stocked 20 days after the application of carbofuran to the tank survived in the water. The experiment required 30 days for completion. Hence, most aquatic organisms were exposed for only 10 days because of the toxic nature of carbofuran.

Sample Preparation. The organisms were placed on paper towels to remove surface water and then fresh weight was recorded. They were then extracted three times with about 1 ml of acetone and centrifuged at 1000*g* for 15 min. The precipitates were resuspended in about 2 ml of acetone and centrifuged as before. The extraction efficiency ranged from 12 to 92% because of the differences in the organism's ability to metabolize the parent compound. The combined supernatant was concentrated to 1 ml under N_2 current. An aliquot of 0.2 ml was taken to measure the radioactivity. The solvent in the remaining solution was removed completely by N_2 current to dryness and then a few drops of acetone were added to redissolve the residue for chromatography on a tlc plate. The insoluble residue after acetone extraction and the pellets after centrifugation were combined and placed in an empty scintillation vial and held at room temperature overnight to evaporate the acetone completely. From 0.5 to 1.0 ml of tissue solubilizer (Protosol, from New England Nuclear) and about 3 drops of water were added to the vial which was then tightly covered with a cap and heated at 55° for 20 hr. After cooling, about 0.3 ml of 20% benzoyl peroxide in toluene was added to decrease quenching and the vial was heated at 55° for 1 hr. Ten milliliters of Aquasol (New England Nuclear) was then added and the contents of the vial were ready for counting. One liter of water was extracted with about 200 ml of diethyl ether three times. The combined ether layer was dried over anhydrous Na_2SO_4 and concentrated to a small volume to measure the radioactivity and for chromatography on a tlc plate. The water layer was monitored for radioactivity (1 ml taken for counting), hydrolyzed with 0.025 *N* HCl at 70° for 20 hr, and then extracted with ether as before.

Thin-Layer Chromatography (Tlc). Microfiber absorbent sheets impregnated with silica gel (ITLC type, from Gelman Instrument Co., Ann Arbor, Mich.) in a solvent system consisting of 15% (v/v) acetone in *n*-hexane were used to separate the metabolites. Standard metabolites were cochromatographed with solvent extracts from the

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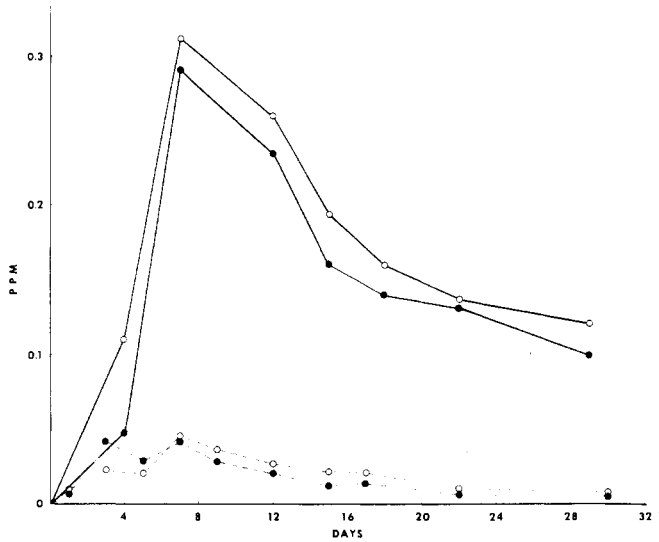

Figure 1. Radioactivity in tank water from model ecosystem containing organisms and ring-^{14}C- or carbonyl-^{14}C-labeled carbofuran: (●-●) ring ^{14}C, tank I; (O-O) ring ^{14}C, tank II; (●--●) carbonyl ^{14}C, tank I; (O--O) carbonyl ^{14}C, tank II.

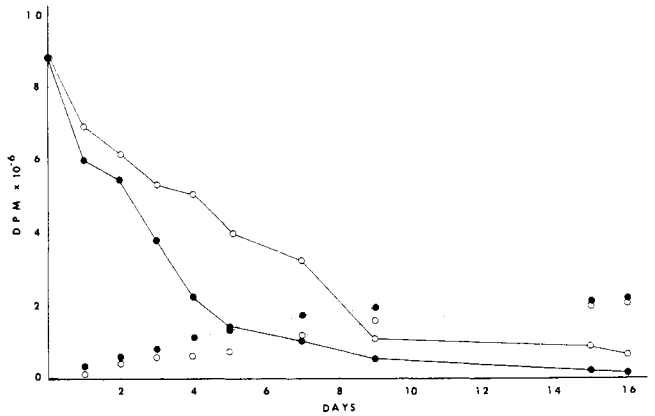

Figure 2. Results of sodium hydroxide trapping experiment: in H_2O (●-●) expt I; (O-O) expt II; in NaOH trap (●--●) expt I; (O--O) expt II.

water and organisms to identify the radioactive metabolites. After spraying with 15% methanolic KOH and with 0.01% methanolic *p*-nitrobenzenediazonium fluoroborate, spots with pink or yellow color were marked with a pencil and the strips were covered with no-screen X-ray film (Eastman Kodak Co.) in darkness for 4 weeks to locate the radioactive spots.

CO_2 Trapping Experiment. A 1000-ml erlenmeyer flask containing 400 ml of water (pH 7.5) from a tank used for rearing *Daphnia* was stocked with 100 *Daphnia*, 5 snails, and 4 μCi of [carbonyl-^{14}C]carbofuran. This flask was connected to a small air pump supplying about 25 ml of air/min. The air through the flask was then passed into a 500-ml erlenmeyer flask containing 200 ml of 0.1 *N* NaOH to trap CO_2 in the air. Radioactivity was monitored by taking 0.5 ml of the NaOH solution in the trap and changing at 1- or 2-day intervals.

Scintillation Fluid. Aquasol was used as the scintillation fluid to count the radioactivity of solubilized tissue. Water samples, tlc plates, and solvent extracts were counted using a fluid containing 120 g of naphthalene, 7 g of 2,5-diphenyloxazole, 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 1000 ml of 1,4-dioxane. An external method of standardization was used to correct quenching (Packard Tri-Carb liquid scintillation spectrometer Model 3320).

RESULTS AND DISCUSSION

Radioactivity in the water was monitored through the 30-day experimental period (Figure 1). Radioactivity reached a peak at the seventh day in both [ring-^{14}C]- and [carbonyl-^{14}C]carbofuran. However, radioactivity in the carbonyl-^{14}C-labeled tanks was much less than that of ring-labeled carbofuran. This indicated that carbofuran is hydrolyzed rapidly to carbofuranphenol and *N*-methylcarbamic acid. *N*-Methylcarbamic acid is further degraded to CO_2 and other metabolites.

In order to verify this conclusion, we placed [carbonyl-^{14}C]carbofuran in a closed aquatic system and monitored the radioactivity in water and in a CO_2 trap which contained NaOH. The results are presented in Figure 2. As the data show, radioactivity in water decreased rapidly, while radioactivity in the CO_2 trap increased steadily. Radioactivity in the CO_2 trap was only about 25% of the total ^{14}C put into the system. The radioactivity remaining in the water was less than 10% of the original radioactivity introduced into the water. The discrepancy between the radioactivity remaining in the water and that trapped in the NaOH trap probably can be explained by the inefficiency of the CO_2 trap. More NaOH traps should have been placed in the series in order to increase the efficiency of trapping. Nevertheless, we conclude that carbofuran in water is rapidly degraded and the carbonyl portion of the molecule is converted to CO_2. The exact nature of the conversion was not pursued in this study.

The concentrations of metabolites in the solvent extract and in the residue fraction from the organisms and water are presented in Tables I and II. In unhydrolyzed water, only small amounts (about 3 and 10%, respectively) of the radioactive materials can be extracted by diethyl ether

Table I. Concentration of Metabolites in Solvent Extract and in Residue Fraction for [ring-^{14}C]Carbofuran

	Concentration, equivalent ppm					
	Solvent extract[a,b]		Residue		Total	
Sample	Tank I	Tank II	Tank I	Tank II	Tank I	Tank II
Unhydr. H_2O	0.0027 (2.3)	0.0047 (3.9)	0.108	0.113	0.1107	0.1177
Hydr. H_2O	0.0295 (31.7)	0.0336 (33.6)	0.0635	0.0668	0.093	0.1004
Algae	0.860 (18)	0.770 (12.5)	3.915	5.380	4.775	6.150
Clam		1.087 (74.7)		0.368		1.455
Daphnia	1.083 (17.5)	1.095 (20.2)	5.050	4.330	6.133	5.425
Elodea	1.530 (33.6)	3.862 (56.5)	3.020	2.965	4.550	6.827
Fish	0.038 (13.5)	0.107 (15.5)	0.243	0.582	0.281	0.689
Frog	0.502 (32.5)		1.034		1.536	
Mosquito	1.007 (17.2)	1.135 (19)	4.850	4.820	5.857	5.955
Snail	1.390 (18.4)	1.900 (23)	6.180	6.360	7.570	8.260

[a] Diethyl ether was used to extract water and acetone was used to extract the organisms. [b] Values in parentheses are per cents and represent the amount of ^{14}C in terms of parts per million that could be extracted using the solvents shown.

Table II. Concentration of Metabolites in Solvent Extract and in Residue Fraction for [carbonyl-^{14}C]Carbofuran

	Concentration, equivalent ppm					
	Solvent extract[a,b]		Residue		Total	
Sample	Tank I	Tank II	Tank I	Tank II	Tank I	Tank II
Unhydr. H_2O	0.00032 (8.1)	0.00051 (11.7)	0.00363	0.00386	0.00395	0.00437
Hydr. H_2O	0.00141 (36.9)	0.00214 (43.6)	0.00241	0.00277	0.00382	0.00491
Algae	1.020 (23.7)	0.905 (25)	3.280	2.720	4.300	3.625
Clam	0.0286 (17.5)	0.0127 (16.4)	0.135	0.0646	0.1636	0.0773
Crab	0.152 (31.3)	0.390 (69.1)	0.334	0.174	0.486	0.564
Crab (2nd day)	2.800 (61)	0.499 (92)	1.800	0.0436	4.600	0.5406
Daphnia	0.157 (15.1)	0.225 (14.8)	0.883	1.300	1.040	1.525
Elodea	1.072 (16.5)	1.218 (22.2)	5.410	4.260	6.482	5.478
Fish	0.0423 (16.5)	0.0743 (17.8)	0.214	0.342	0.2563	0.4163
Mosquito	0.252 (24.8)	0.618 (28.8)	0.866	1.530	1.018	2.148
Snail	1.190 (21)	0.755 (12.8)	7.870	5.165	9.060	5.920

[a] Diethyl ether was used to extract water and acetone was used to extract organisms. [b] Values in parentheses are per cents and represent the amount of ^{14}C in terms of parts per million that could be extracted using the solvents shown.

Table III. Per Cent Distribution of Radioactive Metabolites in Solvent Extract from [ring-^{14}C]Carbofuran after Tlc Analysis

	I[a] (0.98)[c]	A[b] (0.83)	B (0.76)	C (0.70)	D (0.60)	E (0.53)	F (0.46)	II (0.36)	III (0.28)	IV (0.13)	V (0.06)	VI (0)
Unhydr. H_2O												
Tank I	10.2	23.2	3.6	3.8	3.7	4.3	7.7	7.0	8.1	5.1	8.5	14.9
Tank II	5.5	24.3	1.8	3.6	1.1	2.7	3.9	18.8	18.2	2.9	4.9	12.4
Hydr. H_2O												
Tank I	2.2	10.1	1.4	0.7	1.1	0.5	1.1	6.1	3.2	9.7	9.8	54.2
Tank II	2.6	8.2	1.0	1.9	0.7	1.0	1.5	9.8	5.7	6.9	9.7	51.2
Clam												
Tank II	0	1.2	0	0	0	0	0	0	17.6	0	0	81.2
Fish												
Tank I	16.8	8.0	0	0	0	0	13.85	2.18	0	0	0	59.2
Tank II	80.5	0	0	0	0	0	0	0	0	0	0	19.5
Frog												
Tank I	39.2	0	0	0	0	0	0	0	0	0	0	60.8
Mosquito												
Tank I	39.2	0	0	0	0	0	0	0	0	0	0	40.8
Tank II	38.9	0	0	0	0	0	0	0	0	0	0	61.1
Snail												
Tank I	19.3	27.1	0	0	0	0	0	0	0	0	0	53.6
Tank II	45.6	0	0	0	0	0	0	0	0	0	0	54.4

[a] Roman numerals indicate unknown spots. [b] Letters indicate known spots (cochromatography) in which A = carbofuranphenol, B = a mixture of carbofuran and 3-ketocarbofuranphenol, C = 3-ketocarbofuran, D = 3-hydroxycarbofuranphenol, E = *N*-hydroxymethylcarbofuran, and F = 3-hydroxycarbofuran. [c] Parentheses indicate R_f values.

from [ring-^{14}C]- and [carbonyl-^{14}C]carbofuran. After acid hydrolysis, however, about 35% of the radioactivity could be extracted by ether. About 20% of the radioactivity could be extracted by acetone from the organisms. Solvent extracts from water and organisms which had high radioactivity were analyzed by tlc and autoradiography.

Tables III and IV show the R_f values and per cent distribution for known and unknown spots in [ring-^{14}C]- and [carbonyl-^{14}C]carbofuran. There are more spots than were reported previously (Ashworth and Sheets, 1972; Dorough, 1968a,b; Knaak *et al.*, 1970a,b; Metcalf *et al.*, 1968). However, we have only a limited number of standard metabolites and many of the spots cannot be identified at the present time.

There was some carbofuranphenol in the ether extract from water (about 10 and 24%, respectively, for unhydrolyzed and hydrolyzed water) in [ring-^{14}C]carbofuran tanks (Table III). Surprisingly, there was also some parent compound in the ether extract from water. Spot B in Table III consisted of parent carbofuran and 3-ketocarbofuranphenol which cannot be separated in this tlc system. Spot B in Table III constituted about 1–4% of the total radioactivity for the ether extract from water in ring-labeled carbofuran tanks. Spot B in Table IV is exclusively parent carbofuran, since the carbonyl-^{14}C-labeled moiety is not present in 3-ketocarbofuranphenol and therefore 3-ketocarbofuranphenol would not show any radioactivity. In addition, it is possible that other potential ring metabolites that had lost the carbonyl moiety might be formed but would not be detected since the label would be lost. However, in comparison with the ring-labeled part of the experiment, it is not likely that many of these would be formed since these compounds would be detected with the ring label. The parent carbofuran in carbonyl-labeled tanks constituted about 85% of the radioactivity in the ether extract from the unhydrolyzed water and about 10% in the ether extract from hydrolyzed water (Table IV). The other four metabolites (3-ketocarbofuran, 3-hydroxycarbofuranphenol, *N*-hydroxymethylcarbofuran, and 3-hydroxycarbofuran) reported by previous workers (Metcalf *et al.*, 1968) were also found in the ether extract from the water. Spots E in Tables III and IV are tentatively referred to as *N*-hydroxymethylcarbofuran when compared with the report of Metcalf *et al.* (1968). However, we did not have this standard reference compound for cochromatography.

A large amount of radioactivity in the acetone extract from organisms was found at the solvent front or in the

Table IV. Per Cent Distribution of Radioactive Metabolites in Solvent Extracts from [carbonyl-^{14}C]Carbofuran after Tlc Analysis

	I[a] (0.96)[c]	VII (0.86)	VIII (0.75 + 0.71)	B[b] (0.73)	C (0.63)	E (0.55)	IX (0.51)	F (0.48)	X (0.26–0)	VI (0)
Unhydr. H_2O										
Tank I	0.2	4.2	0	84.4	2.5	1.8	0.8	0.8	2.9	3.1
Tank II	1.8	2.7	0	89.7	1.8	1.8	0	0	0	2.1
Hydr. H_2O										
Tank I	1.0	8.0	0	10.6	21.8	0	0	0	0	58.6
Tank II	0	0.5	0	9.3	46.4	2.3	2.3	2.3	6.8	32.0
Crab										
Tank I	86.6	0	0	0	0	0	0	0	0	13.4
Tank II[d]	28.0	0.7	0	32.5	0	0	19.5	19.5	0	19.5
Crab (2nd day)[e]										
Tank I	0	0	0	41.6	0	0	8.4	8.4	8.4	41.6
Tank II	0	0	0	80.0	0	0	0	0	0	20.0
Elodea										
Tank I	3.5	15.0	5.2	0	2.2	1.1	2.2	2.2	37.2	32.0
Tank II	5.0	7.5	0.5	0	3.8	5.0	0	0	49.8	28.0
Mosquito										
Tank II	71.5	0	0	0	0	0	0	0	0	28.5
Snail										
Tank I	71.4	0	0	0	0	0	0	0	0	28.6
Tank II	59.0	0	0	0	0	0	0	0	0	41.0

[a] Roman numerals indicate unknown spots. [b] Letters indicate known spots (cochromatography) in which B = carbofuran, C = 3-ketocarbofuran, E = *N*-hydroxymethylcarbofuran, and F = 3-hydroxycarbofuran. [c] Parentheses indicate R_f values. [d] Crab was dying. [e] Crabs were dead second day after introduction of carbofuran to tanks.

lower portion of the tlc plate. Identity of these spots is not known. We found the parent carbofuran in two crabs which were dead the second day after applying [carbonyl-^{14}C]carbofuran to the tanks and in one of the crabs which was introduced to the tanks 20 days after applying [carbonyl-^{14}C]carbofuran. The latter crab was moribund at the end of the experiment on the 30th day. A second crab stocked in the same tank on the 20th day did not die and no parent carbofuran was found in this living crab at the end of the experiment on the 30th day. Apparently, the crabs did not metabolize carbofuran extensively, since 61–92% of the radioactivity was extracted by acetone from the whole body (Table II). In other organisms only about 20% of the radioactivity could be extracted (Tables I and II).

The insoluble residues after solvent extraction from the water and organisms were not analyzed further. Therefore, their chemical nature is not known. Presumably, they are conjugated with glucose (Ashworth and Sheets, 1972; Knaak *et al.*, 1970a,b; Metcalf *et al.*, 1968) or other large molecules, as they are very polar metabolites. Residues after acetone extraction from organisms constituted about 80% of the total radioactivity in the body (Tables I and II).

In summary, carbofuran was hydrolyzed rapidly in water. Hydroxylation of the benzofuranyl moiety constituted the major degradation pathway. No parent carbofuran was found in the living organisms. However, we did find carbofuran in dead crabs. Carbofuran was highly toxic to crabs, *Daphnia*, snails, and clams immediately after application to the system. However, most of the organisms survived restocking 20 days after introduction of the compound to the tank.

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