Degradation of Soil-Sorbed Carbofuran by an Enrichment Culture from Carbofuran-Retreated *Azolla* Plot

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The degradation of carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate), sorbed to three soils of varying physicochemical characteristics, by an enrichment culture from a carbofuran-retreated *Azolla* plot was studied. The concentration of carbofuran, sorbed to the alluvial soil, decreased to undetectable levels (less than 2 μg) within 3 days of inoculation with the enrichment culture; in contrast, 68–83% of the insecticide, sorbed to Pokkali and Kari soils, was recovered even 6 days after inoculation. Carbofuran was desorbed with great ease from alluvial soil but not from organic matter rich Pokkali and Kari soils. Evidently, biodegradation of soil-sorbed carbofuran was related to its desorption. Isotope studies revealed that degradation of carbofuran, sorbed to alluvial soil by the enrichment culture, led to a substantial accumulation of carbofuran phenol and evolution of CO_2, in stoichiometric amounts, from the side chain.

INTRODUCTION

Biodegradation is the major means of detoxification of the majority of soil-applied pesticides. Generally, conclusive evidence for a microbial role in the degradation of a soil-applied pesticide is provided by demonstrating its degradation in sterile vs nonsterile soils and in pure cultures of microorganisms isolated from soil. An important factor influencing the availability of a soil-applied organic compound in solution phase for microbial degradation is its sorption–desorption (Ensminger and Gieseking, 1942; Pinck and Allison, 1951; Estermann et al., 1959; Helling et al., 1971; Helweg, 1975; Ogram et al., 1985). A pesticide applied to a soil is partitioned between soil and solution phases (Rao and Davidson, 1980), and its relative distribution between these two phases is determined by physicochemical characteristics of both adsorbent and adsorbate. Sorption may increase the chemical degradation of sorbed pesticide as reported for the surface-catalyzed hydrolysis of atrazine (Russell et al., 1968; Armstrong and Chesters, 1968) and parathion (Saltzman et al., 1974) in soils. Also, sorption of a soil-applied pesticide and possibly its subsequent desorption would determine the amount of pesticide available for degradation by soil microorganisms. The present study is concerned with the degradation of soil-sorbed carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate) by an enrichment culture.

MATERIALS AND METHODS

Soils Used. Three soils of varying physicochemical characteristics (Table I) were used. The air-dried soils were sieved through a 2-mm mesh before use. These soils were never exposed to carbofuran before.

Carbofuran and Its Metabolites. Technical formulation of carbofuran (99.4% purity), analytical grade carbofuran phenol, 3-hydroxycarbofuran, and 3-ketocarbofuran were gifts from FMC Corp., Middleport, NY. Uniformly ring-^{14}C-labeled carbofuran (specific activity 39.4 mCi mmol^{-1}; 50% purity) and [*carbonyl*-^{14}C]carbofuran (specific activity 13.3 mCi mmol^{-1}; 98% purity) were also gifts from FMC. Before use, ^{14}C-labeled carbofuran was purified by thin-layer chromatography (TLC).

Sorption of Carbofuran in Soils. An aqueous solution of carbofuran was prepared by shaking water with carbofuran for 24 h and then sterilized by passing through a Millipore filter (0.45 μm). Twenty-gram portions of soils contained in presterilized 250-mL Erlenmeyer flasks were shaken with 25-mL aliquots of the aqueous solution of carbofuran (50 μg mL^{-1}) for 2 h on a wrist action shaker to allow sorption. After equilibrium was attained, the soil suspension was centrifuged at 22000*g* for 10 min and the supernatant was removed. This soil with sorbed carbofuran was washed twice with 25-mL portions of sterile distilled water to ensure that carbofuran remaining in the soil is in sorbed condition. The soil suspension was shaken for 2 h after each washing and centrifuged. After each of three centrifugations, the supernatant was analyzed for carbofuran, and the amount of carbofuran sorbed by the soil was determined by subtracting the cumulative amount of carbofuran in the supernatant from the total amount of carbofuran initially added to the soil.

Inoculation of Soil Containing Sorbed Carbofuran with Enrichment Culture. The carbofuran-degrading enrichment culture used in this study is the same as that used in an earlier study (Singh et al., 1990a) and was prepared by five additions of carbofuran to standing water of a carbofuran-retreated *Azolla* plot. *Azolla* is a fern harboring N_2-fixing blue-green alga (*Anabaena azollae*) and is used as a biofertilizer in rice (Singh, 1989). Carbofuran is regularly applied for controlling the pests of *Azolla*. Soil (20 g) with sorbed carbofuran [250 μg in Central Rice Research Institute (CRRI) soil and 350 μg in Pokkali and Kari soils] was inoculated with 0.4 mL of this enrichment culture. Uninoculated soil with sorbed carbofuran served as control. At 0, 3, and 6 days after incubation at room temperature (28 ± 2 °C), soil samples in duplicate flasks were extracted with chloroform–diethyl ether (1:1 v/v) and the residues were analyzed colorimetrically for carbofuran by TLC.

Isotope Studies. In a follow-up study, [U-*phenyl*-^{14}C]carbofuran and [*carbonyl*-^{14}C]carbofuran were used to determine the degradation products formed during degradation of soil-sorbed carbofuran by the enrichment culture. Labeled carbofuran was allowed to be sorbed by CRRI soil as described for nonlabeled carbofuran. After each of three centrifugations, radioactivity was determined in 0.5-mL aliquots of the supernatant. Soil containing sorbed carbofuran was inoculated with 0.4 mL of the enrichment culture. Uninoculated soil with sorbed carbofuran served as control. Both uninoculated and inoculated flasks in duplicate were closed with a rubber bung provided with an inlet and an outlet which were closed with a pinchcock. The assembly was then incubated at room temperature. At periodic intervals, the inlet was connected to an air generator through a trap containing 25 mL of 2 N KOH to absorb $^{14}CO_2$, if any, in the air, and $^{14}CO_2$ evolved from the reaction flasks was purged directly into 5 mL of $^{14}CO_2$ scintillation cocktail (repurged with nitrogen) containing pseudocumene (R. J. Harvey Instrument

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Table I. Physicochemical Characteristics of the Soils Used

location	soil	pH[a]	organic matter,[b] %	CEC,[c] mequiv/100 g of soil	clay,[d] %	silt,[d] %	sand,[d] %
CRRI, Cuttack, Orissa	alluvial	6.2	1.62	18.6	25.6	12.6	61.8
Alamakkara, Cochin, Kerala	acid sulfate Pokkali	3.6	7.1	31.2	45.6	7.8	45.6
Karimadi, Alleppey, Kerala	acid sulfate Kari	2.7	8.8	32.2	33.6	12.8	53.6

[a] Measured by taking 1:1.25 soil–water slurry. [b] Estimated by Walkey–Black method. [c] Estimated by ammonium acetate (pH 7.0) method. [d] Bouyoucos hydrometer method.

Table II. Degradation of Carbofuran Sorbed to CRRI, Pokkali, and Kari Soils by the Enrichment Culture from Carbofuran-Treated *Azolla* Plot

incubation, days	carbofuran recovered from 20 g of soil, %					
	CRRI		Pokkali		Kari	
	UI[a]	I[b]	UI	I	UI	I
0	94.8[c] (6.2)[e]	94.9 (6.2)	93.5 (3.6)	92.7 (3.6)	93.0 (2.7)	95.0 (2.7)
3	90.4 (6.4)	0 (6.4)	89.3 (5.0)	83.4 (5.0)	87.0 (3.6)	89.8 (3.6)
6	NE[d]	NE	85.6 (5.2)	68.5 (5.2)	82.5 (3.8)	83.0 (3.8)

[a] Soil containing sorbed carbofuran was not inoculated with enrichment culture. [b] Soil containing sorbed carbofuran was inoculated with enrichment culture. [c] Mean of duplicate estimations. [d] Not estimated. [e] Figures in parentheses indicate pH of soil–water slurry.

Corp.). The contents in each reaction flask were shaken with chloroform–diethyl ether (1:1 v/v) for extraction of residues and subsequent assay of the radioactivity by liquid scintillation after separation by TLC.

Effect of pH. The ability of the enrichment culture to degrade carbofuran was tested at different pH values in a soil-free mineral salts medium. Carbofuran (400 μg) in 1 mL of acetone was added aseptically to presterilized 100-mL Erlenmeyer flasks. After evaporation of acetone at room temperature, 20-mL aliquots of sterilized mineral salts medium [$MgSO_4 \cdot 7H_2O$, 0.2 g; K_2HPO_4, 0.1 g; $FeSO_4$ $7H_2O$, 0.001 g; $CaSO_4$, 0.04 g; water, 1 L of pH 4, 5, 6, 7, and 8 (pH was adjusted with 1 N NaOH or 1 N HCl)] was dispensed aseptically to each flask. Flasks were equilibrated for 4 h. The mineral salts medium containing 20 μg mL^{-1} carbofuran was inoculated with 0.2 mL of enrichment culture and incubated at 30 ± 1 °C in a BOD incubator. Uninoculated medium served as control. At periodic intervals, residues in duplicate flasks were extracted three times with 30-mL portions of chloroform–diethyl ether (1:1 v/v) and then analyzed colorimetrically after separation of residues by TLC.

Extraction and Residue Analysis. Residues from the soils were first extracted by shaking the soil sample with a 50-mL portion of chloroform–diethyl ether (1:1 v/v) on a wrist action shaker for 2 h. After centrifugation at 22000*g* for 10 min, the extract was partitioned in a separating funnel and organic solvent fraction was collected in a beaker. This extraction procedure was repeated with 40- and 30-mL portions of chloroform–diethyl ether. The chloroform–diethyl ether fractions from three extractions were pooled, evaporated to dryness or 0.5 mL, and then made up to 1 mL with methanol for analysis by TLC. In isotope experiments, the radioactivity remaining in the water phase after organic solvent extraction was determined by liquid scintillation. Residues finally eluted in methanol were separated by TLC (Ramanand et al., 1988a). The standards were located by spraying successively with 2 N NaOH in absolute methanol and a solution of *p*-nitrodiazonium fluoroborate (5 mg dissolved in 25 mL of methanol and 25 mL of diethyl ether) (Archer, 1976). Silica gel areas coinciding with the standards were scraped into tubes for colorimetric analysis of nonlabeled carbofuran or into 5 mL of scintillation cocktail to assay [^{14}C]carbofuran.

For colorimetric analysis of nonlabeled carbofuran, the residues in silica gel were treated with 1.25 mL of 0.3% sodium nitrite, 1.25 mL of 0.2% sulfanilic acid in 1 N HCl, and 2.5 mL of 4 N NaOH in a hot water bath (50–60 °C) for 20 min. Silica gel was removed by centrifugation (2800*g* for 30 min), the supernatant was made up to 10 mL, and the color was read at 490 nm in a Spectronic 20 spectrometer (Venkateswarlu et al., 1977). The carbofuran was quantified using a standard curve which was linear over the range 0–12 μg mL^{-1} carbofuran.

In isotope studies, the silica gel areas of the samples corresponding to authentic compounds (carbofuran, carbofuran phenol, 3-hydroxycarbofuran, and 3-ketocarbofuran) were scraped into 5 mL of scintillation cocktail (Optiphase Hisafe II liquid scintillation cocktail, FSA Laboratory Suppliers, Loughborough, Leies, England), and radioactivity was assayed in Rackbeta liquid scintillation spectrometer Model 1209 (LKB, Wallac, Finland) with built-in program for color and chemical quenching correction. Printing was done by a Facit B 1100 printer interfaced with liquid scintillation spectrometer. $^{14}CO_2$ absorbed in scintillation cocktail was assayed directly by liquid scintillation.

RESULTS AND DISCUSSION

The concentration of carbofuran sorbed to CRRI soil declined to undetectable levels (less than 2 μg) within 3 days of inoculation with carbofuran-degrading enrichment culture (Table II). However, carbofuran sorbed to Pokkali and Kari soils resisted degradation by the enrichment culture. Only 26% of carbofuran sorbed to Pokkali soil was degraded by the enrichment culture even after 6 days, while there was virtually no degradation of carbofuran sorbed to Kari soil. Decrease in the concentration of carbofuran in all three uninoculated soils was negligible even after 6 days of incubation. Evidently, soil-sorbed carbofuran was readily degraded by the enrichment culture only in CRRI soil.

A study using [*carbonyl*-^{14}C]carbofuran indicated that more than 90% of the radioactivity sorbed to CRRI soil was not detected in the soil 2 days after inoculation with the enrichment culture (Table III) as compared to a loss of only 6% from the uninoculated control. Interestingly, 60% of the *carbonyl*-^{14}C in soil-sorbed carbofuran was released as $^{14}CO_2$ from the inoculated soil in 4 days. $^{14}CO_2$ evolution from the uninoculated soil was negligible. However, during degradation of soil-sorbed [*ring*-^{14}C]carbofuran by the enrichment culture, $^{14}CO_2$ evolution was negligible (Table IV). Radioactivity remaining in the water phase after organic solvent extraction was also negligible. About 70% of the *ring*-^{14}C in carbofuran sorbed to CRRI soil was accounted for in the organic solvent fraction of the inoculated soil at 5 days. TLC analysis of this extract showed that 85% of the radioactivity in the organic solvent extract was accounted for as carbofuran phenol at 5 days after inoculation. Authentic carbofuran phenol and the metabolite formed from carbofuran exhibited the same absorption maximum of 285 nm and R_f value of 0.78. Other degradation products, 3-hydroxycarbofuran and 3-ketocarbofuran, were also detected but only in traces. Evidence suggested that in inoculated CRRI soil with sorbed carbofuran carbofuran phenol accumulated as the major

Table III. Distribution of Radioactivity during Degradation of [*carbonyl*-^{14}C]Carbofuran Sorbed to CRRI Soil by the Enrichment Culture from Carbofuran-Retreated *Azolla* Plot

incubation, days	treatment	radioactivity recovered from 20 g of soil, %			
		methanol fraction	carbofuran[b]	CO_2	total recovery
0	uninoculated	94.8 ± 4.2[c]	91.7 ± 3.0		94.8
	inoculated				
2	uninoculated	91.5 ± 3.9	85.9 ± 6.1	6.6 ± 0.1	98.1
	inoculated	14.6 ± 1.2	7.7 ± 0.1	57.7 ± 2.3	72.3
4	uninoculated	88.3 ± 6.2	80.5 ± 4.5	7.2 ± 1.0	95.5
	inoculated	4.8 ± 1.0	3.8 ± 0.3	60.2 ± 2.9	65.0

[a] Soil initially contained 1.3×10^5 dpm of sorbed carbofuran 20 g^{-1} of soil. [b] After separation of the residues, extracted in chloroform–diethyl ether and eluted in methanol, by thin-layer chromatography. [c] Mean of duplicate estimations ± mean deviation.

Table IV. Distribution of Radioactivity during Degradation of [U-*phenyl*-^{14}C]Carbofuran Sorbed to CRRI Soil by the Enrichment Culture from Carbofuran-Retreated *Azolla* Plot at Room Temperature (Involving Partial Evaporation of Chloroform–Diethyl Ether)

incubation, days	treatment	radioactivity[a] recovered from 20 g of soil, %							
		aqueous phase	organic extract	carbofuran[b]	carbofuran phenol[b]	3-keto-carbofuran[b]	3-hydroxy-carbofuran[b]	CO_2	total recovery
0	uninoculated								
	inoculated	2.9 ± 0.3[c]	93.2 ± 4.6	84.0 ± 3.9	0.2 ± 0.1				96.1
3	uninoculated	2.9 ± 0.1	84.7 ± 3.0	73.2 ± 3.6	0.4 ± 0.1	1.0 ± 0.1	0.8 ± 0.0	0.9 ± 0.1	88.5
	inoculated	2.3 ± 0.5	75.0 ± 4.1	7.5 ± 0.9	61.9 ± 3.6	0.7 ± 0.0	1.2 ± 0.0	1.2 ± 0.2	78.5
5	uninoculated	4.8 ± 0.6	71.4 ± 2.3	59.3 ± 0.6	0.2 ± 0.0	0.7 ± 0.2	0.8 ± 0.1	1.1 ± 0.1	77.3
	inoculated	4.2 ± 0.2	70.4 ± 3.1	6.6 ± 0.0	60.4 ± 2.5	0.5 ± 0.1	0.9 ± 0.2	1.3 ± 0.2	75.9

[a] Soil initially contained 1.01×10^5 dpm of sorbed carbofuran 20 g^{-1} of soil. [b] After TLC separation of the residues extracted in chloroform–diethyl ether. [c] Mean of duplicate estimations ± mean deviation.

metabolite and ring cleavage is negligible. According to an earlier paper (Ramanand et al., 1988b), carbofuran was readily mineralized to CO_2 by an enrichment culture from a flooded soil held at 35 °C and retreated with carbofuran.

Degradation of sorbed carbofuran only in CRRI soil by the enrichment culture with great ease merits further discussion. In the present study using a 1:1.25 soil-to-water ratio, about 50% of carbofuran was held by the soil after 2 h of equilibration. However, when this soil with sorbed carbofuran was washed twice with distilled water, 56% of sorbed carbofuran was released. Thus, in CRRI soil, sorbed carbofuran was not strongly held by the soil particles, and its subsequent release into solution would explain the rapid degradation of sorbed carbofuran in CRRI soil by the enrichment culture.

In contrast, degradation of carbofuran sorbed to Pokkali or Kari soils was distinctly slow. Slow degradation of carbofuran sorbed to Pokkali soil was not related to pH. This soil had pH values of 5.0 at 3 days and 5.2 at 6 days after inoculation with the enrichment culture. In a soil-free mineral salts medium of different pH inoculated with enrichment culture, at pH 5.0, the concentration of carbofuran decreased to 61% and 96% of the original level, respectively, after 2 and 4 days of incubation. At pH 4.0, the decrease in carbofuran levels was negligible during 6 days of incubation of the medium after inoculation. In similarly incubated uninoculated controls, there was no appreciable decrease in the concentration of carbofuran at pH 4, 5, or 6. Kari soil had a pH of 3.8, and this low pH probably prevented, at least in part, the microbial degradation of carbofuran sorbed to this soil. Inasmuch as organic matter is the most important single factor responsible for sorption of carbofuran, these two organic matter rich soils showed high sorption values of carbofuran (Singh et al., 1990b). The force responsible for adsorption of carbofuran on these two soils appears to be very strong as only 5.4% and 15.4% of sorbed carbofuran was desorbed from Kari and Pokkali soils even after three repeated extractions using a soil-to-water ratio of 1:10 (Singh et al., 1990b). It is likely that in organic matter rich soils such as Pokkali and Kari carbofuran forms a type of complex preventing release for degradation by solution-phase microbes.

Microbial degradation of a pesticide in soil is largely governed by its bioavailability in solution phase. Microbial degradation is arrested or retarded as soon as the pesticide is sorbed by soil colloidal particles. Weber and Coble (1968) found that $^{14}CO_2$ evolution from diquat due to degradation by soil microorganisms was reduced to approximately half or totally absent following its addition to montmorillonite clay in an amount equilvalent to adsorb half or all of the [^{14}C]diquat added to the medium, respectively. Ogram et al. (1985) demonstrated that sorbed 2,4-D was resistant to microbial degradation both by solution phase or by sorbed bacteria, while solution-phase herbicide was degraded by solution phase as well as sorbed bacteria.

In summary, carbofuran sorbed in low organic matter CRRI soil was readily degraded by the adapted carbofuran-degrading enrichment culture due to its easy desorption from the soil. In contrast, degradation of carbofuran by the enrichment culture in Pokkali and Kari soils with low pH and high organic matter content was slow or absent. This inability of the enrichment culture to degrade carbofuran sorbed to Pokkali and Kari soils is attributed mainly to its slow desorption.

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