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Metabolism of Carbamate Insecticides by Resting Cells and Cell-Free Preparations of a Soil Bacterium, *Arthrobacter* sp.

K. Ramanand, M. Sharmila, Neera Singh, and N. Sethunathan

Laboratory of Soil Microbiology, Central Rice Research Institute, Cuttack-753006, India

Among the carbamate insecticides, carbofuran (2,3-dihydro-2, 2-dimethyl-7-benzofuranyl N-methylcarbamate) is one of the most widely used insecticides for broadspectrum control of common insect pests of economically important crops such as rice. We reported earlier (Ramanand et al. 1988a) that growing cells of a bacterium, Arthrobacter sp., isolated from a flooded soil held at 35°C and retreated with carbofuran, readily mineralized carbofuran to CO_2. In this report, we studied the degradation of carbofuran and related carbamate insecticides by the resting cells and cell-free preparations of this bacterium.

MATERIALS AND METHODS

The same carbofuran-degrading Arthrobacter sp., isolated earlier (Ramanand et al. 1988a) from a flooded soil held at 35°C and retreated with carbofuran was used in this study. Resting cells of Arthrobacter sp. were prepared as follows : A medium ($MgSO_4.7\ H_2O$, 0.2 g; K_2HPO_4, 0.1 g; $FeSO_4.7\ H_2O$, 0.001 g; $CaSO_4$, 0.040 g; $(NH_4)_2HPO_4$, 2.0 g; sucrose, 10.0 g; and distilled water, 1000 mL) supplemented with 0.1% yeast extract was dispensed in 100-ml portions in 250-mL Erlenmeyer flasks and autoclaved at 121°C for 15 min. The medium contained in each flask was supplemented with 10 ug of technical carbofuran in 1 mL of acetone. The medium was inoculated with a suspension in sterile distilled water of a 5-day old culture of the carbofuran-degrading Arthrobacter sp. and then incubated at 35°C. At log phase, bacterial cells were harvested from 3-liter medium by centrifugation at 18,000 rpm for 15 min at 4°C in a Sorvall RC 5C refrigerated centrifuge, washed several times with 100 mM sodium phosphate buffer (pH 7.0) and finally resuspended in 70 ml of the phosphate buffer.

The degradation of carbofuran, carbaryl (1-naphthyl-N-methyl-carbamate), bendiocarb (2,2-dimethyl-1,3-benzdioxol-4-yl-Nmethyl carbamate) and carbosulfan [2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-n-butyl-aminosulfenyl) methylcarbamate] by the resting cells of Arthrobacter sp. was studied as follows : Twenty-milliliter portions of 100 mM sodium phosphate buffer (pH 7.0) containing 20 ug/mL of carbofuran, carbaryl, bendiocarb or carbosulfan were

Send reprint requests to N. Sethunathan at the above address.

inoculated with 1 mL suspension of the resting cells in phosphate buffer. The same reaction mixture, inoculated with 1 mL of resting cells but killed by heating at 100°C for 5 min served as control. At periodical intervals after incubation at 35°C, aliquots were removed from duplicate samples and then analysed by gas-liquid chromatography (glc) for respective insecticides after extraction with ethyl acetate.

To prepare cell-free extract (crude enzyme) from Arthrobacter sp., cells of Arthrobacter sp., harvested from 3 liters of the medium, were concentrated to 25 mL in phosphate buffer. The cell pellet obtained by centrifugation (15,000 rpm for 10 min) was washed thrice with 50 mM Tris-HCl buffer (pH 8.0) and centrifuged at 4°C at 15,000 rpm for 10 min. The resting cells, resuspended in 25 ml of Tris-HCl buffer (pH 8.0), were treated with 0.5 mL of 10% sodium dodecyl sulphate (SDS) and then incubated at 28°C for 1 h with intermittent shaking. Unbroken and lyzed cells were pelleted by centrifugation (4°C) at 18,000 rpm for 1 h and the clear viscous supernatant or crude enzyme was tested for its ability to degrade carbofuran, bendiocarb and carbaryl. The reaction mixture consisted of 20 mL of 100 mM sodium phosphate buffer (pH 7.0) and respective insecticide at 20 ug/mL concentration. The reaction was started by adding enzyme solution (0.5 mL) and the contents mixed by stirring. At 5-min intervals, 1 mL of the reaction mixture was removed from duplicate flasks, the residues extracted with ethyl acetate and analysed by glc.

The effect of pH on the chemical stability of carbosulfan was studied in a mineral salts medium incubated at 35°C. The mineral salts medium was adjusted with 0.1 N HCl or 0.1 N NaOH to obtain pH values of 4, 5, 6, 7 and 8. Carbosulfan dissolved in 0.1 ml ethanol was added to the medium of different pH to give a final concentration of 20 ug/mL of the medium. At periodical intervals, aliquots of the medium from duplicate flasks of each treatment were removed, residues of carbosulfan extracted with ethyl acetate and analysed by glc.

For the analysis of carbofuran, carbaryl, bendiocarb and carbosulfan in the samples, 1 to 2 mL of the medium were removed from each of duplicate flasks and then shaken with 2 to 4 mL of ethyl acetate for 20 min. The residues in ethyl acetate fraction were analysed by injecting 1 to 2 uL of the ethyl acetate fraction into the Varian gas chromatograph Model 3400 equipped with a thermionic nitrogen-phosphorus specific detector and 5% OV 101 stainless steel column. The operating conditions for carbofuran, carbaryl and bendiocarb were : argon (carrier gas) flow, 30 mL/min; hydrogen flow, 3 mL/min; air flow, 150 mL/min; column temperature, 190°C; injector temperature, 240°C and detector temperature, 250°C. Under these conditions, the retention times for carbosulfan, carbaryl and bendiocarb were 1.0, 1.5 and 0.6 min, respectively. For estimation of carbosulfan, column, injector and detector were maintained at 250, 250 and 275°C, respectively and the flow rates for argon, hydrogen and air were 30, 3 and 150 mL/min, respectively. Under these conditions, the retention time for carbosulfan was

Table 1. Degradation of carbamate insecticides by resting cells of Arthrobacter sp. in phosphate buffer[a] held at 35°C

Incubation (min)	Carbofuran[b]		Carbaryl[b]		Bendiocarb[b]		Carbosulfan[b]	
	Killed[c]	Living[d]	Killed	Living	Killed	Living	Killed	Living
0	19.5	20.0	19.0	18.5	20.0	19.2	15.8	16.0
20	19.5	11.5	18.5	8.5	19.0	3.0	15.8	15.6
40	19.0	6.6	18.5	2.5	18.0	0	15.6	15.6
60	19.0	0.8	18.5	0	18.0	0	15.8	15.6

[a] The sodium phosphate buffer was supplemented with 20 ug of the insecticide/mL.

[b] Insecticide recovered, ug/mL.

[c] Phosphate buffer was inoculated with resting cells killed by heating at 100°C for 5 min.

[d] Phosphate buffer was inoculated with living resting cells.

1.8 min. These insecticides were quantified using a calibration graph with 2 to 10 ng of the respective insecticide. The recovery by this procedure ranged from 93 to 100% for carbofuran, 96 to 100% for bendiocarb and 93 to 95% for carbaryl and 72 to 80% for carbosulfan. Variations within replications in all experiments ranged from 0.5 to 1 ug/mL.

RESULTS AND DISCUSSION

Resting cells of Arthrobacter sp. degraded carbofuran, carbaryl and bendiocarb with ease; but carbosulfan appeared to resist degradation by this bacterium (Table 1). Although this bacterium was isolated from a carbofuran-treated soil (Ramanand et al. 1988a) carbofuran was degraded at a rate slower than that of carbaryl and bendiocarb. Thus, carbofuran, carbaryl and bendiocarb almost completely disappeared in 60, 40 and 20 min, respectively after exposure to the resting cells. During the corresponding period, loss of these insecticides from the reaction mixture containing killed resting cells was negligible.

Cell-free preparation from Arthrobacter, like resting cells, degraded carbofuran, carbaryl and bendiocarb with ease. Specific activity of the crude enzyme in degrading the three insecticides followed the order : bendiocarb > carbaryl > carbofuran (Table 2). The specific activity of the crude enzyme used in this study in degrading carbofuran appears to be higher than that of Pseudomonas sp. and Flavobacterium sp. isolated by Chaudhry and Ali (1988).

Table 2. Degradation of carbofuran, carbaryl and bendiocarb in cell-free extract of Arthrobacter sp.

Insecticide	Activity[1] (units/mL)	Specific activity (U) (units/mg protein)	Relative[2] activity
Carbofuran	141.02	20.15	1.00
Carbaryl	199.00	28.43	1.41
Bendiocarb	236.36	33.77	1.68

[1] One unit of activity is defined as the disappearance of 1 nmol of the respective insecticide in 1 second at 30°C. One milliliter of the enzyme contained 7 mg of protein.

[2] Compared to carbofuran.

There are reports of cross adaptation of carbofuran-acclimatized soil or carbofuran degrading microorganisms to other related carbamate pesticides (Kaufman and Edwards 1983, Karns et al. 1986,

Table 3. Effect of pH on the chemical stability of carbosulfan in a mineral salts medium[a] held at 35°C

Incubation (days)	Compound recovered (ug/mL) at pH									
	4		5		6		7		8	
	CS[b]	CF[c]	CS	CF	CS	CF	CS	CF	CS	CF
0	15.5	1.0	14.6	0.5	15.0	1.0	15.0	0.8	15.4	0.8
3	5.0	7.0	11.4	2.0	14.5	1.0	15.0	0.5	15.6	0.8
6	0	9.5	7.0	3.8	14.0	1.5	15.6	0.8	15.0	0.8
10	0	9.0	3.0	7.0	12.5	1.5	14.5	0.8	14.5	0.5

[a]The mineral salts medium was supplemented with 20 ug of carbosulfan/mL dissolved in 0.1 mL ethanol.

[b]Carbosulfan recovered.

[c]Amount of carbofuran formed from chemical hydrolysis of carbosulfan.

Read 1987, Racke and Coats 1988). In the present study, of the four carbamate insecticides tested, Arthrobacter sp. could readily degrade bendiocarb, carbaryl and carbofuran, but not carbosulfan. All the four insecticides are N-methylcarbamates, but carbosulfan alone was characterised by the N-S-N linkage in the functional unit attached to the aromatic ring of carbofuran. Possibly, this N-S-N linkage in carbosulfan made it more resistant to bacterial degradation than carbofuran, carbaryl and bendiocarb. There is considerable concern over the phenomenon of accelerated degradation of carbofuran in carbofuran-retreated agricultural soils eventually leading to its decreased efficacy in pest control (Felsot et al. 1981, Read 1983, 1986, 1987, Chapman et al. 1986 a,b). Possibly, carbosulfan can be used as an effective substitute for carbofuran in problem soils with decreased efficacy of carbofuran in view of the recalcitrance of carbosulfan to degradation by carbofuran-degrading Arthrobacter sp.

Carbosulfan appeared to be more resistant to microbial degradation than carbofuran. Its chemical stability at different pH was studied. Carbosulfan was stable at pH of 6, 7 and 8 (Table 3); but at pH 5.0 and below, the concentration of carbosulfan decreased rapidly. Carbofuran accumulated in the medium at pH of 4 and 5 concomitant with rapid decrease in the concentration of carbosulfan. Chemical stability of carbosulfan at pH 6 and above can be of great advantage in flooded rice paddies. In flooded rice paddies especially with profuse growth of blue-green algae, the pH of the flood water increases from around 7.0 in the morning to as high as 8.5 to 9.5 at 2 or 3 p.m. (Siddaramappa et al. 1978, Siddaramappa and Seiber 1979; Ramanand et al. 1988b) due to CO_2 depletion by photosynthetic aquatic organisms like the dominant blue-green algae. At this pH, carbofuran would undergo rapid chemical hydrolysis (Siddaramappa et al. 1978, Siddaramappa et al. 1979) while carbosulfan is very stable. In such situations, carbosulfan has a greater advantage over carbofuran in the control of insect pests of rice paddies.

The data from this study show that carbosulfan is chemically stable under near neutral and alkaline conditions, hydrolyses rapidly at pH below 6.0 to carbofuran and resists degradation by carbofuran-degrading Arthrobacter sp.

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