APPLIED MICROBIOLOGY, May, 1965
Vol. 13, No. 3
Printed in U.S.A.
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Microbial Degradation of Isopropyl-*N*-3-Chlorophenylcarbamate and 2-Chloroethyl-*N*-3-Chlorophenylcarbamate

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Received for publication 15 January 1965

ABSTRACT

KAUFMAN, D. D. (Agricultural Research Service, Beltsville, Md.), AND P. C. KEARNEY. Microbial degradation of isopropyl-*N*-3-chlorophenylcarbamate and 2-chloroethyl-*N*-3-chlorophenylcarbamate. Appl Microbiol. **13**:443–446. 1965.—Microbial degradation of isopropyl-*N*-3-chlorophenylcarbamate (CIPC) and 2-chloroethyl-*N*-3-chlorophenylcarbamate (CEPC) was observed in a soil perfusion system. Degradation in perfused soils, and by pure cultures of effective bacterial isolates, was demonstrated by the production of 3-chloroaniline and the subsequent liberation of free chloride ion. Identified isolates effective in degrading and utilizing CIPC as a sole source of carbon included *Pseudomonas striata* Chester, a *Flavobacterium* sp., an *Agrobacterium* sp., and an *Achromobacter* sp. Identified isolates, effective in degrading and utilizing CEPC as a sole source of carbon, included an *Achromobacter* sp. and an *Arthrobacter* sp. CIPC-effective isolates degraded CEPC more slowly than CIPC, whereas CEPC-effective isolates degraded CIPC more rapidly than CEPC. Both CIPC- and CEPC-effective isolates degraded isopropyl *N*-phenylcarbamate (IPC) more rapidly than either CIPC or CEPC.

With continued use of pesticides in agriculture, it has been important to determine the fate of these chemicals in both soils and crop produce. Microbial degradation is an important factor affecting the persistence of many pesticides in soils. Little is known concerning the effect of soil microorganisms on the phenylcarbamate pesticides. Evidence obtained by many workers (DeRose, 1946; Newman, DeRose, and DeRigo, 1948; Freed, 1951; Stevens and Carlson, 1952; Ogle and Warren, 1954; Dubrovin, 1962) indicates that soil microorganisms are responsible for degradation of phenylcarbamate herbicides. Dubrovin (1962) observed that 4-chloro-2-butynyl-*N*-3-chlorophenylcarbamate (Barban) disappeared more rapidly in nonautoclaved soil than in autoclaved soil. Freed (1951) and Newman et al. (1948) obtained similar results with isopropyl-*N*-phenylcarbamate (IPC). Environmental factors which favor microbiological activity adversely affect the persistence of both IPC and isopropyl-*N*-3-chlorophenylcarbamate (CIPC) in soil (Newman et al., 1948; Freed, 1951; Stevens and Carlson, 1952; Ogle and Warren, 1954; Dubrovin, 1962). However, DeRose (1951) found CIPC to be more persistent in soil than IPC. The purpose of this investigation was to determine whether or not soil microorganisms can indeed degrade phenylcarbamate herbicides, and, if so, to isolate and identify those organisms responsible.

MATERIALS AND METHODS

The soil perfusion technique was used to enrich a muck soil with microbial populations capable of degrading either 2-chlorethyl-*N*-3-chlorophenylcarbamate (CEPC) or CIPC.

The soil perfusion apparatus used was similar to that of Lees (1949). Each perfusion unit initially contained 10 g (air dry) of muck soil treated with 0.5 g of either CEPC or CIPC and 250 ml of distilled water. An untreated perfused soil served as a blank. Degradation was measured in two ways: (i) production of 3-chloroaniline, and (ii) liberation of all chloride ion. Both 3-chloroaniline and chloride ion determinations were made daily. The 3-chloroaniline content of the perfusate was determined according to the procedure of Pease (1962); chloride ion determinations were made by the procedure of Iwasaki, Utsumi, and Ozawa (1952). After the initial lag phase and degradation period, a second application of the chemical was made to the perfused soils. These soils were then perfused with fresh distilled water, and the rate of degradation was determined again.

Isolation of pure cultures of effective organisms

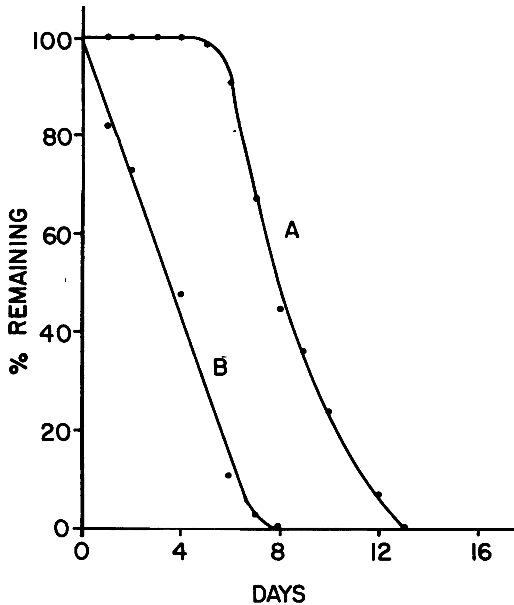

FIG. 1. *Disappearance of CIPC in (A) perfused soil treated with CIPC, and (B) same perfused soil after a second treatment with CIPC.*

from the perfused soils was accomplished via a soil dilution plate method. Serial dilutions were prepared with the enriched soil from the perfusion units. A set of 10 petri plates was prepared from each dilution series. The plating medium contained the following basal salts: K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4$, 0.1 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.001 g; $(NH_4)_2SO_4$, 5.0 g; agar, 20 g; and distilled water, 1,000 ml. CEPC and CIPC were supplied as a sole source of carbon for organisms obtained from perfused soils treated with these chemicals. The chemicals were introduced to sterile empty petri plates in chloroform solutions. The agar medium, containing the diluted suspension of organisms, was then added to the plates after evaporation of the chloroform and crystallization of the chemicals. Plates prepared in this manner were incubated for 1 to 3 weeks at 24 C. Dissolution of the crystals and subsequent formation of clear zones surrounding certain of the colonies was considered indicative of CIPC or CEPC degradation and utilization. Stock cultures of those organisms utilizing either CIPC or CEPC were isolated, purified, and maintained on the basal medium described above with CIPC or CEPC as the sole source of carbon. All effective cultures were identified, at least to genus, according to the seventh edition of *Bergey's Manual*.

Preliminary investigations were made to determine the substrate specificity of the effective isolates obtained. These studies were conducted under pure culture conditions with washed bacterial cells.

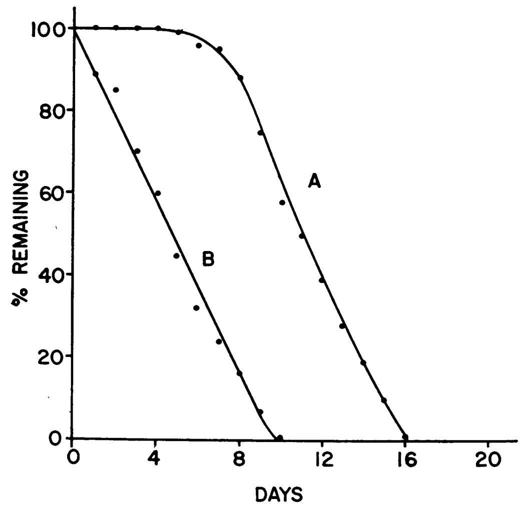

FIG. 2. *Disappearance of CEPC in (A) perfused soil treated with CEPC, and (B) same perfused soil after a second treatment with CEPC.*

RESULTS

Microbial degradation. Both CIPC and CEPC were degraded by soil microorganisms in perfused muck soil. The production of 3-chloroaniline was detected in both CIPC- and CEPC-treated soils. In CIPC-treated soils, 3-chloroaniline was first detected on day 2, and increased in content until day 4 when it reached a level of 0.75 μg/ml of perfusate, or 0.06% of the possible maximum (1.24 mg/ml). In CEPC-treated soils, 3-chloroaniline was also detected on day 2 but increased until day 5 when it reached a maximum of 1.5 μg/ml of perfusate, or 0.12% of the possible maximum. In both instances, 3-chloroaniline rapidly disappeared as chloride ion content of the perfusate increased.

Liberation of chloride ion from both compounds was first observed after 5 days (Fig. 1 and 2), indicating a lag period of approximately 4 days before complete degradation took place. Degradation of CIPC, once initiated, proceeded more rapidly than did degradation of CEPC; degradation of CIPC was complete within 13 days, whereas degradation of CEPC was completed (liberation of all bound chloride) only after 16 days. Both CIPC and CEPC were degraded more rapidly when reapplied to the enriched soils and perfused for the second time. No chloride or 3-chloroaniline was detected in either CIPC- or CEPC-treated soils perfused with distilled water containing 10 ppm of the microbiological inhibitor potassium azide.

Isolation and identification of effective organisms. Although numerous organisms effective in de-

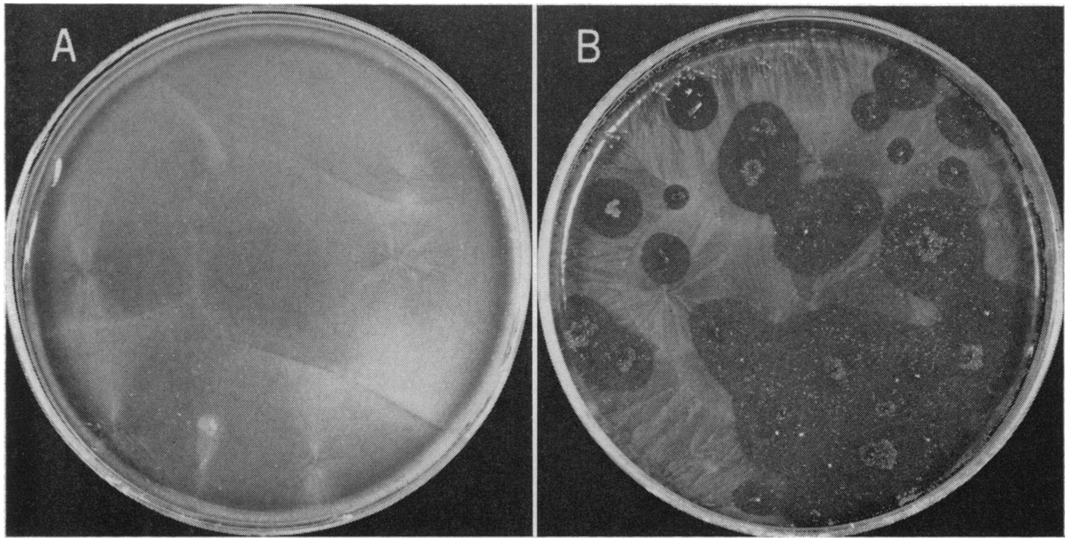

FIG. 3. *Isolation of effective organisms. (A) Sterile petri plate and medium. (B) Colonies of effective organisms surrounded by clear zones.*

grading either CEPC or CIPC (effective isolates designated as H and I, respectively) were isolated from soil dilution plates (Fig. 3), biochemical and morphological studies revealed that many of these organisms were merely different strains of the same organism. Representative isolates of each group are described herein.

Isolates I-1, I-2, I-6, I-7, and H-1 were gram-negative rods. Isolates I-2 and I-7 were motile by means of polar flagella, whereas I-1, I-4, and H-1 were nonmotile. Isolate H-2 was a motile, nonspore-forming, gram-positive rod. It produced large, slightly curved rods in 24-hr nutrient broth cultures, which ultimately developed shorter and smaller rods or cocci in older cultures. Glucose, lactose, maltose, or mannitol was not fermented; starch was not hydrolyzed; and indole was not produced by any of the isolates. Gelatin was liquified by I-7, but not by the others. Nitrates were reduced only by isolates I-2, I-6, and I-7. The effect on litmus milk was variable, with alkalinity being produced by I-2; no reaction by I-1, I-6, H-1, and H-2; and alkalinity and peptonization accompanied by reduction of the litmus by I-7. Isolates I-2, I-7, and H-2 were catalase-positive, whereas I-1, I-6, and H-1 were catalase-negative. Isolate I-1 produced yellow, translucent beaded growth on nutrient agar slants, and was placed in the genus *Flavobacterium*. Isolate I-2 produced filiform, whitish growth on nutrient agar slants, and was placed in the genus *Agrobacterium*. Isolates H-1 and I-6 produced white filiform growth on nutrient agar slants, and were placed in the genus *Achromobacter*. These two isolates, although isolated and effective on different carbamate substrates, appeared to be identical. Isolate I-7 produced yellow, opaque, filiform growth on nutrient agar slants and was classified as *Pseudomonas striata* Chester. Isolate H-2 produced cream-colored, translucent, filiform growth on nutrient agar slants, and was placed in the genus *Arthobacter*.

Substrate specificity. All effective isolates were tested for their ability to actively degrade analogues of CIPC and CEPC. CEPC isolates readily metabolized CIPC, and, conversely, CIPC isolates metabolized CEPC, although much more slowly than the former, never liberating more than 50% of the organically bound chloride. All isolates degraded IPC more rapidly than either CIPC or CEPC. Low concentrations of 3-chloroaniline were also readily metabolized by all effective isolates. Only one isolate (*P. striata*) was effective in degrading 3-(*p*-chlorophenyl)-1,1-dimethylurea (monuron). However, this isolate became effective only after prolonged exposure to monuron in a yeast extract-monuron-basal salts medium. All isolates lost their ability to produce 3-chloroaniline or free chloride ion from either CIPC or CEPC when maintained on Difco nutrient broth for several days. Readaptation occurred only when the isolates were placed in sterile soil solutions containing CIPC or CEPC.

DISCUSSION

The results of this investigation lend support to the suggestions of Ogle and Warren (1954) and Stevens and Carlson (1952), that the rapid loss of CIPC from soil is due, at least in part, to microbial decomposition. The kinetics involved in the disappearance of both CIPC and CEPC are consistent with the hypothesis that, in the presence of these substances, certain microorganisms undergo an adaptation and become capable of utilizing them as a source of carbon. Figures 1 and 2 illustrating the decomposition of CIPC and CEPC reflect the proliferation of suitably adapted organisms.

That the detection of an aniline-type compound should occur prior to detection of chloride ion is not surprising. Kearney and Kaufman (1965) recently demonstrated that a *Pseudomonas* sp. (further identified here as *P. striata*) produced an enzyme which liberated 3-chloroaniline from CIPC. Their identification of 3-chloroaniline was established through cochromatography. Although only small amounts of 3-chloroaniline were observed at any one time in this investigation, it is believed that larger amounts were actually produced. Other work not reported here has revealed that 3-chloroaniline is strongly adsorbed by muck soils, approximately 50% being adsorbed over a period of 24 hr.

The time interval between the initial detection of 3-chloroaniline and the detection of chloride ion is also of interest. Because the analytical tests for both compounds are in the same range of sensitivity (0.1 μg/ml), it would appear that the adaptation of organisms effective in degrading the chlorophenylcarbamate herbicide does not necessarily follow the principles of simultaneous adaptation (Stanier, 1947). In the present investigation, a lag period of several days was observed between the appearance of 3-chloroaniline and free chloride ion. However, all of the effective organisms isolated produced 3-chloroaniline and subsequently chloride ion. All cultures were unable to release either 3-chloroaniline or chloride from CIPC or CEPC after growing for several days on nutrient broth (Difco). It would appear, therefore, that more than one adaptive enzyme is required before an organism can completely degrade a chlorophenylcarbamate type of compound, and that a lag period may exist between the development of these enzymes.

It has been shown elsewhere (Alexander and Aleem, 1961) that slight changes in structural characteristics can significantly alter resistance of phenoxyalkyl carboxylic acid herbicides. The results of specificity studies reported herein, however, are too limited to draw accurate conclusions concerning the effect of structure on the resistance of phenylcarbamate herbicides to microbiological decomposition. That all effective isolates were able to degrade IPC more rapidly than either CIPC or CEPC reemphasizes the effect of chlorine substitution on microbial degradation processes.

ACKNOWLEDGMENTS

We acknowledge the cooperation of the Chemical Division of Pittsburgh Plate Glass Co., who donated chemicals for this study.

LITERATURE CITED

ALEXANDER, M., AND M. I. H. ALEEM. 1961. Effect of chemical structure on microbial decomposition of aromatic herbicides. Agr. Food Chem. **9**:44–47.

DEROSE, H. R. 1946. Persistence of some plant growth-regulators when applied to soil in herbicidal treatments. Botan. Gaz. **107**:583–589.

DEROSE, H. R. 1951. Crabgrass inhibition with o-isopropyl *N*-(3-chlorophenyl)carbamate. Argon. J. **43**:139–142.

DUBROVIN, K. P. 1962. Carbyne disappears rapidly in the soil. Crops Soils **14**:26–27.

FREED, V. H. 1951. Some factors influencing the herbicidal efficacy of isopropyl *N*-phenylcarbamate. Weeds **1**:48–60.

IWASAKI, I., S. UTSUMI, AND T. OZAWA. 1952. New colorimetric determination of chloride using mercuric thiocyanate and ferric ion. Bull. Chem. Soc. Japan **25**:226.

KEARNEY, P. C., AND D. D. KAUFMAN. 1964. Enzyme from soil bacterium hydrolyzes phenylcarbamate herbicides. Science **147**:740–741.

LEES, H. 1949. The soil percolation technique. Plant Soil **1**:221–239.

NEWMAN, A. S., H. R. DEROSE, AND H. T. DERIGO. 1948. Persistence of isopropyl *N*-phenylcarbamate in soils. Soil Sci. **66**:393–397.

OGLE, R. E., AND G. F. WARREN. 1954. Fate and activity of herbicides in soils. Weeds **3**:257–273.

PEASE, H. L. 1962. Separation and colorimetric determination of monuron and diuron residues. J. Agr. Food Chem. **10**:279–281.

STANIER, R. Y. 1947. Simultaneous adaptation: a new technique for the study of metabolic pathways. J. Bacteriol. **54**:339–48.

STEVENS, L. F., AND R. F. CARLSON. 1952. The effects of chloro-IPC on various crops and its residual properties in various soils. Weeds **3**:257–273.