Carbofuran Hydrolase—Purification and Properties†

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The enzyme that hydrolyzes the carbamate linkage of *N*-methylcarbamate insecticides in extracts of a carbofuran-degrading bacterium (*Achromobacter* sp.) was purified to homogeneity by high-performance liquid chromatography on TSK-DEAE and TSK-Phenyl columns followed by gel filtration chromatography on Bio-Gel A0.5m. The enzyme preparation was stabilized at all stages of the purification regimen by manganese ions. Carbofuran hydrolase cosedimented with yeast alcohol dehydrogenase in sucrose density gradient centrifugation, indicating a molecular size of 150 000 Da. SDS–PAGE of the purified enzyme indicated that it was composed of two subunits. Dialysis of the purified enzyme preparation against buffer containing EDTA resulted in the complete inactivation of the enzyme. Addition of $MnCl_2$ to the reaction mixture restored the activity to 70% of its original level. No other divalent cations could replace Mn as an activator. The purified carbofuran hydrolase had no urease activity.

INTRODUCTION

The microbial degradation of a pesticide molecule has a great effect on the environmental fate and efficacy of the compound. When a pesticide is degraded at a reasonable rate, it persists long enough to control pests but does not become a pollution problem. If a pesticide is degraded too rapidly, it may not adequately control target pests. Rapid microbial degradation of pesticides has been shown to contribute to the "problem" or "aggressive" soils phenomenon observed after repeated use of certain soil-incorporated pesticides (Felsot et al., 1981; Harris et al., 1984; Racke and Coats, 1988; Read, 1983; Wilson, 1984). Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate) is a soil-incorporated insecticide that has been shown to be adversely affected by accelerated microbial degradation (Felsot et al., 1981; Harris et al., 1984; Read, 1983). Carbofuran (trade name Furadan) is usually applied at the time of planting to control the corn rootworm (*Diabrotica* sp.) in corn. While this practice saves time, labor, and energy, it also requires that the carbofuran persist in the soil for 3–5 weeks after planting to control the feeding larvae.

In a previous paper we described the isolation and characterization of a bacterium (*Achromobacter* sp. WM111) that is capable of rapidly degrading carbofuran (Karns et al., 1986). Derbyshire et al. (1987) reported the detection, partial purification, and characterization of an enzyme from this bacterium that is able to hydrolyze the *N*-methylcarbamate linkage of carbofuran to yield products that no longer have insecticidal activity. In this paper we describe the purification of this protein to homogeneity and report some additional characteristics of the enzyme.

MATERIALS AND METHODS

Bacterial Strains. *Achromobacter* sp. WM111 was isolated from soil perfusion columns as described earlier (Karns et al., 1986). Starter cultures of the bacterium were grown in 50 mL of nitrogen-free basal salts supplemented with 20 mM glucose and 200 μg/mL carbofuran as described previously (Derbyshire et al., 1987). One entire starter culture was used to inoculate 1 L of basal salts medium containing 40 mM glucose, and the cultures were then incubated at 28 °C with shaking for 40 h. The cells were harvested by centrifugation (6000*g*, 15 min, 4 °C). The supernatant was decanted and the cell pellets were frozen at −20 °C until used.

Preparation of Cell-Free Extracts. The frozen cell pellet (12-g wet weight) was thawed and suspended in 24 mL of ice-cold 30 mM Tris-HCl (pH 7.2) containing 10% (w/v) glycerol, 10 mM $MnCl_2$, and 2 mM dithiothreitol, as well as RNase A and DNase I as previously described (Derbyshire et al., 1987). The cells were lysed by two passages through a chilled French pressure cell (15 000 psi), and whole cells and large debris fragments were removed by centrifugation (25000*g*, 20 min, 4 °C). The supernatant was subjected to ultracentrifugation (105000*g*, 2 h, 4 °C), and the supernatant from this treatment (27 mL, crude soluble fraction) was removed and used as a source of carbofuran hydrolase for further purification.

TSK-DEAE Column Chromatography. A portion (25 mL, 77.1 IU of activity) of the crude soluble fraction was pumped at 2 mL/min directly onto a 2.15 × 15 cm TSK-DEAE column (Waters, Milford, MA) that had been equilibrated with 10 mM Tris-HCl containing 1 mM $MnCl_2$, 10% (w/v) glycerol, and 1 mM dithiothreitol (buffer A). The column was washed with buffer A at 5 mL/min until all unbound protein was eluted and a stable baseline was obtained. A linear gradient of 0–0.25 M NaCl in buffer A was then run (5 mL/min) to elute bound material from the column. Fractions containing carbofuran hydrolase were pooled (15.3 mL) and used for further purification.

TSK-Phenyl Column Chromatography. A 14-mL portion of the pooled DEAE fractions containing 56.1 IU of carbofuran hydrolase activity was brought to 1 M $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ and pumped at 2 mL/min onto a 2.15 × 15 cm TSK-Phenyl column (HP-Genenchem, S. San Francisco, CA) that was equilibrated with 20 mM Tris-HCl (pH 7.2) containing 1 mM $MnCl_2$, 5% (w/v) glycerol, and 1 mM dithiothreitol (buffer B) containing 1 M $(NH_4)_2SO_4$. The column was washed with buffer B plus 1 M $(NH_4)_2SO_4$ until all unbound material was eluted and a stable baseline was obtained. A decreasing linear gradient of 1 to 0 M ammonium sulfate in buffer B was then run to elute the bound material from the column. Active fractions totaling 15.6 mL and 40.1 IU of activity were pooled.

Bio-Gel A0.5m Column Chromatography. Size exclusion chromatography was carried out as described previously (Derbyshire et al., 1987) except that the column was equilibrated and run with buffer A described above. The column was recalibrated by using protein standards run in buffer A.

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Sucrose Gradient Centrifugation. Sucrose density gradients of 5–20% sucrose in 25 mM Tris-HCl, 1 mM $MnCl_2$, and 1 mM dithiothreitol were prepared by layering 0.78-mL aliquots of sucrose solutions differing by 1% sucrose increments into a 13.5-mL Beckman Ultraclear centrifuge tube (14 × 95 mm) beginning with the 5% solution. Solutions were delivered to the bottom of the tube through a polyethylene capillary tube. The gradients were allowed to sit at 4 °C overnight before 0.5 mL of sample containing 154 μg of carbofuran hydrolase and 0.3 mg/mL each of ferritin (MW 450 000), alcohol dehydrogenase (from yeast, 150 000), bovine serum albumin (68 000), carbonic anhydrase (29 000), and cytochrome *c* (12 500) was layered onto the top of the gradient. The tubes were centrifuged for 24 h at 40 000 rpm ($\omega^2 t = 1.52 \times 10^{14}$ rad^2/s) in a Beckman SW40Ti rotor at 4 °C. The gradients were fractionated by puncturing the bottom of the tube and collecting 0.4-mL fractions. Ferritin and cytochrome *c* were detected by measuring the absorbance of each fraction at 400 nm. Yeast alcohol dehydrogenase was detected by measuring the reduction of 0.1 mM NAD in the presence of 1% ethanol spectrophotometrically at 340 nm.

Carbofuran Hydrolase Assays. Carbofuran hydrolase activity was assayed spectrophotometrically by using *o*-nitrophenyl dimethylcarbamate as substrate as described earlier (Derbyshire et al., 1987) except that the substrate was dissolved 200 mg/mL in dimethyl sulfoxide before addition to the reaction mixture at a final concentration of 1 mg/mL. The actual hydrolysis of the pesticides carbofuran and carbaryl (1-naphthyl *N*-methylcarbamate) was measured as previously (Derbyshire et al., 1987; Karns et al., 1986) except that the pH of the reaction mixture was adjusted to 8.5, the reactions were incubated at 37 °C, and $MnCl_2$ was added to the reaction mixture to a concentration of 1 mM. The hydrolysis of aldicarb [2-methyl-2-(methylthio)propionaldehyde *O*-(*N*-methylcarbamoyl)oxime] was measured as described previously (Derbyshire et al., 1987), with the changes noted above, except that the samples taken during the course of the reaction were immediately injected onto the HPLC column so that freezing was unnecessary.

Urease Assays. Urease activity was measured spectrophotometrically by measuring the oxidation of NADH by glutamate dehydrogenase in the presence of ammonia generated from urea by the action of urease. Reaction mixtures (1 mL) contained 10 mM urea, 10 mM 2-ketoglutarate, 0.24 mM NADH, and 10 IU of glutamate dehydrogenase. Carbofuran hydrolase was added to initiate the reaction, and the decrease of absorbance at 340 nm was measured. Jack bean urease was added to some reaction mixtures to test the efficacy of the system.

Polyacrylamide Gel Electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)–PAGE of proteins was performed according to the method of Laemmli (1970). The 1.5 mm thick slab gels were 8 or 12% resolving gels with 4% stacking gels. The proteins in the gels were fixed and stained in a solution of 0.25% Coomassie brilliant blue R in 40% methanol–7% acetic acid. The gels were destained in 40% methanol–7% acetic acid until the background was clear and were stored in 10% acetic acid. A mixture of proteins of known molecular weight were run as standards. The standards were phosphorylase B (95 500), bovine serum albumin (68 000), glutamate dehydrogenase (55 000), ovalbumin (43 000), lactate dehydrogenase (36 000), carbonic anhydrase (29 000), lactoglobulin (18 400), and cytochrome *c* (12 400).

Isoelectric Focusing. Isoelectric focusing was performed on precast IsoGel agarose isoelectric focusing plates (pH 3–10) (FMC Bioproducts, Rockland, ME) as described by the manufacturer. IsoGel p*I* markers were used as standards.

Protein Determinations. Protein concentrations in crude extracts were determined according to the method of Bradford (1976), using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). The concentration of protein in partially purified and purified enzyme preparations was determined according to the spectrophotometric method of Kalb and Bernlohr (1977).

RESULTS

Purification of Carbofuran Hydrolase. The results of the purification of carbofuran hydrolase using high-performance liquid chromatography are summarized in Table I. The use of the TSK-DEAE column allowed a very high loading capacity while excellent resolution (13-fold purification) and recovery (80%) were retained. Subsequent chromatography on a TSK-Phenyl column yielded a preparation that was 45-fold purified with over 50% recovery of the enzyme activity. Figure 1 shows the elution profiles for carbofuran hydrolase on these two columns. As seen previously (Derbyshire et al., 1987) the enzyme bound very tightly to the phenyl column, although ethylene glycol was not required for its elution. SDS–PAGE of the purified preparation showed that there were two polypeptide bands (M_r = 72 000 and 77 000) associated with the purified enzyme preparation (Figure 2). Further chromatography of the enzyme preparation on a Bio-Gel A0.5m column did not affect the relative ratios of the two bands in SDS–PAGE, indicating that both polypeptides are associated with carbofuran hydrolase. Comparison with samples purified in previous studies (Derbyshire et al., 1987) demonstrated that the HPLC-purified preparation was more highly purified (Figure 2).

Bio-Gel A0.5m column chromatography of the purified carbofuran hydrolase yielded an estimate of 170 000 for the molecular size of the native enzyme. This value was considerably different from our previous estimate of 105 000 (Derbyshire et al., 1987). Sucrose density gradient centrifugation was run to verify the molecular size estimate. Carbofuran hydrolase sedimented at the same velocity as yeast alcohol dehydrogenase (molecular size 150 000) (Figure 3), indicating that, when purified in the presence of manganese ion, the molecular size of the native enzyme is indeed on the order of 150 000. It is possible that the presence of Mn^{2+} stabilizes the enzyme in its globular form,

Table I. Purification of Carbofuran Hydrolase

extract	sp act.,[a] μmol min^{-1} (mg of protein)$^{-1}$	recovery[b]	*x*-fold purifn
105000*g* supernatant	0.14		
TSD-DEAE	1.79	79.5	13.2
TSK-Phenyl	6.26	52.0	46.3
Bio-Gel A0.5M[c]	7.85		58.1

[a] Measured as rate of hydrolysis of *o*-nitrophenyl dimethylcarbamate. [b] 25 mL of 105000*g* supernatant having a total of 77.1 IU of carbofuran hydrolase activity and 570 mg of protein was initially applied to the TSK-DEAE column. [c] Only a small portion of the TSK-Phenyl-purified material was subjected to Bio-Gel column chromatography; 37% of the activity applied was recovered.

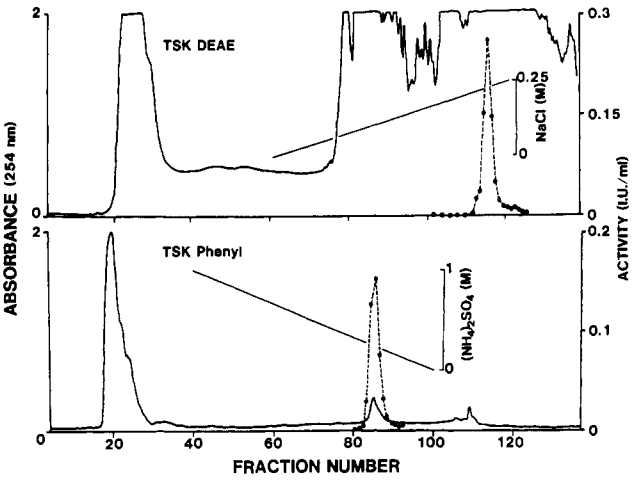

Figure 1. High-performance liquid chromatography of carbofuran hydrolase. The solid lines represent the absorbance at 254 nm as measured by a UV column monitor. The solid circles joined by dashed lines represent carbamate hydrolysis activity in column fractions as measured by the *o*-nitrophenyl dimethylcarbamate assay. The insets are graphic representations of the gradient programs run during column development.

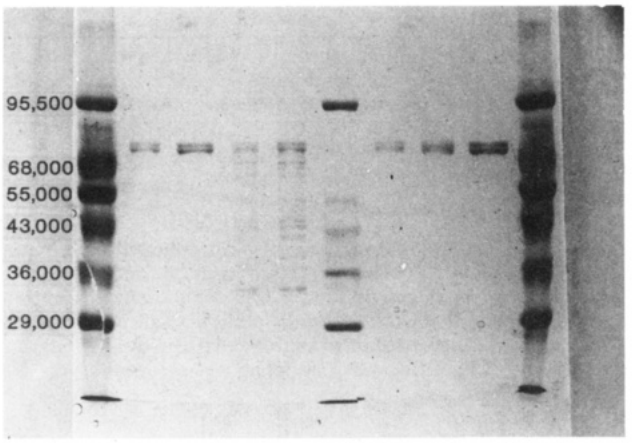

Figure 2. SDS–PAGE of purified carbofuran hydrolase preparations. Lanes 1, 6, and 10 contain a mixture of protein standards (described in the text). Lanes 2 and 3 contain 1 and 2 μg, respectively, of a carbofuran hydrolase preparation purified by HPLC as described in the text. Lanes 7, 8, and 9 contain 0.5, 1, and 2 μg, respectively, of the preparation in lanes 2 and 3 after further purification on Bio-Gel A0.5m. Lanes 4 and 5 contain 2.4 and 4.8 μg, respectively, of a carbofuran hydrolase preparation that was purified according to the method in ref 2. Numbers indicate the molecular weights and mobilities of protein standards.

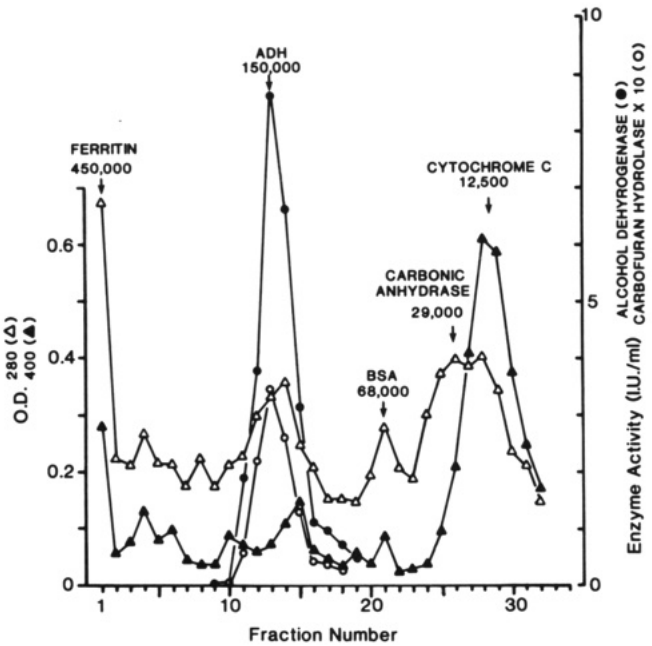

Figure 3. Sedimentation of carbofuran hydrolase and standard proteins in sucrose density gradients. Symbol identification is given in the figure.

allowing a better estimate of its size by the methods employed. The purification method employed here uses less NaCl (0 vs 170 mM) and dithiothreitol (1 vs 5 mM) than was previously used, and the enzyme prepared in this manner was very stable at all stages of the purification procedure, unlike the previous preparations. Regardless of the reason, the data presented here (estimation of molecular size of the native enzyme by two methods and the relationship of subunit sizes to the size of the native enzyme) conclusively demonstrate that the native carbofuran hydrolase has a molecular size in the range of 150 000–170 000.

Effects of Divalent Cations on Carbofuran Hydrolase Activity. Dialysis of purified carbofuran hydrolase against buffer containing 10 mM EDTA resulted in a preparation that had no carbamate hydrolysis activity

Table II. Effect of EDTA and Divalent Cations on Carbofuran Hydrolase Activity

dialysis treatment[a]	addition to reaction[b]	rate ofhydrolysis,[c] μmol min^{-1} (mg of protein)$^{-1}$
none	none	4.42
none	$MnCl_2$	4.52
10 mM EDTA	none	0.12
10 mM EDTA	$MnCl_2$	4.49
10 mM EDTA	$MgCl_2$[d]	0.12
1 mM $MnCl_2$	none	6.59
1 mM $MnCl_2$	$MnCl_2$	6.22
EDTA–none	none	0.12
EDTA–none	$MnCl_2$	3.96
EDTA–$MnCl_2$	none	5.88
EDTA–$MnCl_2$	$MnCl_2$	5.70

[a] Purified enzyme (46-fold) was dialyzed overnight against 1000 volumes of buffer [20 mM Tris-HCl, 5% (w/v) glycerol, 1 mM dithiothreitol] containing the listed additives. Where two additives are listed, the enzyme was dialyzed against buffer containing the first additive as above and then against buffer containing the second additive. [b] The chemicals listed were added to the reaction mixture to give a final concentration of 1 mM. [c] Measured as hydrolysis of *o*-nitrophenyl dimethylcarbamate. [d] Other divalent cations (Cu, Co, Ca, Fe, Ni, Pd, Sn, and Zn) as chlorides or sulfates were also tested with results identical with those obtained with Mg.

(Table II). The addition of 1 mM $MnCl_2$ or $MnSO_4$ to the reaction mixture prior to the addition of substrate restored the activity to approximately 70% of its original level, indicating that Mn^{2+} is required for enzyme activity. No other divalent cation tested (Mg, Ca, Co, Cu, Fe, Ni, Pd, Sn, or Zn) restored any activity to the enzyme when added to a concentration of 1 mM. Redialysis of the inactivated preparation against buffer containing Mn^{2+} restored activity to the same 70% level. Dialysis of the original enzyme preparation against buffer containing no divalent cation or $MgCl_2$ also yielded preparations having hydrolase activity at 70% of the original level, while redialysis of the EDTA inactivated enzyme against buffer containing no divalent cations did not restore any activity. These data suggest that Mn^{2+} is tightly bound by the enzyme and is only removed by the presence of a chelating agent and that no Mn^{2+} was present in the water or salts used to prepare buffers.

p*I* of Carbofuran Hydrolase. Upon isoelectric focusing in agarose gels the purified carbofuran hydrolase focused at the same point as the ovalbumin in the standard, indicating a p*I* of 4.8 for carbofuran hydrolase (data not shown).

Substrate Specificity. The purified enzyme preparation was able to hydrolyze carbofuran [turnover = 3570 μmol min^{-1} (μmol of enzyme)$^{-1}$], carbaryl [2490 μmol min^{-1} (μmol of enzyme)$^{-1}$], aldicarb [480 μmol min^{-1} (μmol of enzyme)$^{-1}$], and *o*-nitrophenyl dimethylcarbamate [915 μmol min^{-1} (μmol of enzyme)$^{-1}$], confirming that a single enzyme (and hence one or two genes) was responsible for the ability of *Achromobacter* sp. strain WM111 to hydrolyze a wide range of *N*-methylcarbamate insecticides (Karns et al., 1986). The K_m of the purified carbofuran hydrolase for carbofuran was 63 μM, which agrees with the K_m of 56 μM determined with the less pure preparation (Derbyshire et al., 1987). In addition, a K_m of 2700 μM was determined for *o*-nitrophenyl dimethylcarbamate.

The characterization of the carbofuran degrading *Achromobacter* sp. demonstrated that it was a urease-positive strain (Karns et al., 1986). When plated onto urease indicator agar (made up as in the Difco manual), the red color indicative of the hydrolysis of urea was seen before

bacterial growth was evident on the plates (J. Karns, unpublished observation), indicating that the organism produced a highly active urease. Since urea and *N*-methylcarbamates both have amide moieties, it seemed possible that the hydrolysis of carbofuran might be catalyzed by the urease produced by this organism. To determine if carbofuran hydrolysis in *Achromobacter* sp. WM111 was due to a cross-reactive urease, the highly purified enzyme preparation was tested for the ability to catalyze the hydrolysis of urea. The purified carbofuran hydrolase preparation did not hydrolyze urea at any detectable rate (data not shown). Since previous studies demonstrated that there was little, or no, carbofuran hydrolase activity associated with the membrane fraction of cell extracts (Derbyshire et al., 1987) and the loss of activity observed during the purification of the enzyme was not unreasonable, it seems unlikely that carbofuran hydrolysis is due to urease activity in *Achromobacter* sp. WM111 but rather that the reaction is catalyzed by the specific *N*-methylcarbamate hydrolase described here.

DISCUSSION

The characterization of pesticide degradative enzymes is of interest for two reasons. First, such enzymes may prove to be very useful for the simple and inexpensive detoxification and elimination of agrochemical wastes. Parathion hydrolase, an enzyme capable of degrading a large number of organophosphorus insecticides, has been successfully employed for this purpose (Kearney et al., 1986; Munnecke, 1976). Second, knowledge of the mechanisms by which such enzymes function may lead to the development of methods to inhibit their action for specific periods of time. This would help to prevent the economic hardships caused by loss of crops due to pesticide failure in "problem" soils and may allow the use of smaller amounts of soil-incorporated pesticides. An understanding of carbofuran hydrolase could contribute in both areas. Carbofuran and other *N*-methylcarbamate insecticides are acetylcholinesterase inhibitors and thus are toxic to mammals (and birds) to some degree (LD_{50} for carbofuran 8 mg/kg orally in rats; refer to Merck Index, 9th ed.). The use of an enzyme such as carbofuran hydrolase to clean equipment and containers and to treat leftover pesticide would greatly reduce the acute toxicity of the material, as the cleavage of the carbamate linkage destroys the activity of the compound. This same hydrolytic inactivation of carbofuran may also play a major role in the problem soils phenomenon, in which soil microbes degrade carbofuran rapidly so that the pesticide does not persist at levels high enough to control corn rootworms. Recently, Turco and Konopka (1990) have shown that ^{14}C label was rapidly released from the carbonyl position of carbofuran in soils characterized as having rapid carbofuran degradation relative to soils that had not been previously treated with carbofuran, while there was no significant difference in the release of ^{14}C from ring-labeled material in these same soils. These data suggest that hydrolysis of the carbamate linkage is the primary mechanism of carbofuran inactivation in these soils. An understanding of the mechanisms by which carbofuran hydrolase cleaves the carbamate linkage of carbofuran may lead to the ability to design specific inhibitors that would prevent degradation of the compound long enough for adequate pest control. At present it is impossible to determine whether this enzyme acts as an esterase or as an amidase because the products from either mechanism would be the same. A test was run to determine if this enzyme could hydrolyze benzamide, which would allow us to classify it as an amidase, but the enzyme was unable to cleave this substrate (unpublished results).

Some question remains as to the architecture of the native enzyme. SDS–PAGE of purified preparations always yielded two bands (77 000 and 72 000) with the smaller band always staining more intensely (see Figure 2). With an estimated molecular size of 150 000 for the native protein there is some question of whether the enzyme consists of two dissimilar subunits or whether one of the apparent subunits is derived from the other. Genetic evidence has shown that the amount of DNA encoding an active carbofuran hydrolase is too small to encode two dissimilar subunits in the 70 000 range unless the proteins are encoded in overlapping reading frames and has also shown that both these polypeptide bands are present in extracts of *Pseudomonas putida* containing this cloned gene (Tomasek and Karns, 1989). Thus, it seems most likely that carbofuran hydrolase from *Achromobacter* sp. WM111 is composed of two similar subunits and that modification or proteolytic cleavage is responsible for the appearance of two bands on SDS–PAGE gels.

Since, according to current dogma, genes encoding enzymes with new degradative capabilities must arise from existing genes through mutation and natural selection, there is always some question as to the origin of pesticide degradation genes in soil microorganisms. It is possible that microbial enzymes such as urease could be responsible for cometabolism of pesticide molecules with certain chemical structures. The present study has shown that carbofuran hydrolase has little or no urease activity; however, comparative DNA or protein sequence analysis will be required to determine if a urease gene was the progenitor of carbofuran hydrolase. Such comparative studies may lead to an understanding of how pesticide degradation genes arise and spread in the soil environment.

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Registry No. Mn, 7439-96-5; carbofuran hydrolase, 110141-85-0; carbofuran, 1563-66-2; *o*-nitrophenyl dimethylcarbamate, 3373-86-2.