Purification and Characterization of an *N*-Methylcarbamate Pesticide Hydrolyzing Enzyme

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An enzyme that hydrolyzes the carbamate linkage of carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate) was detected in crude cell-free extracts of a carbofuran degrading *Achromobacter sp.* Activity was labile in crude French pressure cell extracts but was partially stabilized by ammonium sulfate fractionation. The activity was stable after further purification by phenyl–Sepharose chromatography. The hydrolase was stable in extracts prepared by a combination treatment of Triton X-100/osmotic shock/lysozyme/alumina. It was soluble with a molecular size of 105 000 Da as estimated by gel filtration chromatography. No cofactor requirement could be demonstrated in crude or partially purified extracts. The optimum pH was broad (9.0–10.5), and the optimum temperature was between 45 and 53 °C. K_m values of 56, 15, and 2800 μM were determined for carbofuran, carbaryl (1-naphthyl *N*-methylcarbamate), and aldicarb (2-methyl-2-(methylthio)propionaldehyde *O*-(*N*-methylcarbamoyl)oxime), respectively. A product of carbofuran hydrolysis was identified as 2,3-dihydro-2,2-dimethyl-7-benzofuranol. A product of carbaryl degradation cochromatographed with 1-naphthol. The enzyme was unable to hydrolyze parathion (*O,O*-diethyl *O*-(*p*-nitrophenyl) phosphorothioate), EPTC (*S*-ethyl dipropylthiocarbamate), or chlorpropham (isopropyl *m*-chlorocarbanilate).

Degradation of pesticides by chemical processes and microorganisms in soils reduces the persistence of these compounds in the environment. Current agricultural practice is to apply preemergence soil insecticides at planting to save labor and energy. Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate) has been used extensively to control corn rootworm (*Diabrotica sp.*). Carbofuran is a plant systemic insecticide and must persist in the soil for at least 3–5 weeks postplanting until the emergence of the feeding larvae. Although there is some controversy (Siddaramappa et al., 1978; Ahmad et al., 1979; Gorder et al., 1980), enhanced microbial degradation is thought to play a role in "problem" or "aggressive" soils in which soil-incorporated pesticides are ineffective in controlling the pest (Felsot et al., 1981; Harris et al., 1984). A number of soil microorganisms have been isolated that degrade *N*-methylcarbamate insecticides [carbofuran: Williams et al. (1976), Kandasamy et al. (1977), Felsot et al. (1981), Venkatswarlu and Sethunathan (1985), Karns et al. (1986)], [carbaryl (1-naphthyl methylcarbamate): Tewfik and Hamdi (1970), Liu and Bollag (1971), Sud et al. (1972), Rodriguez and Dorough (1977), Gupta et al. (1975), Karns et al. (1986)], [aldicarb (2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime): Jones (1976), Karns et al. (1986)], and [propoxur (2-isopropoxyphenyl *N*-methylcarbamate): Gupta et al. (1975), Karns et al. (1986)].

The bacterium *Achromobacter sp.* WM111 (Karns et al., 1986) is one microorganism able to rapidly degrade *N*-methylcarbamate insecticides. When this organism was introduced as a 5% inoculum into a nitrogen-free basal salts medium containing glucose as a carbon source and 200 μg/mL carbofuran as the sole nitrogen source, it grew with a doubling time of about 4 h at 30 °C. The carbofuran was degraded to undetectable levels (less than 2 μg/mL) within 42 h. Enzymes that degrade carbamate pesticides have been described [reviewed by Munnecke et al. (1982)]. One of the best characterized is the *N*-phenylcarbamate hydrolase described by Kearney (1965). It was extracted from a soil isolate of *Pseudomonas striata* that was able to use chlorpropham (isopropyl *m*-chlorocarbanilate) as a sole source of carbon and energy (Kearney and Kaufman, 1965). That enzyme catalyzed the degradation of several alkyl *N*-phenylcarbamates and acylanilides but was inactive against the *N*-methylcarbamate carbaryl. In this report we describe the purification and characterization of an *N*-methylcarbamate insecticide degrading enzyme from *Achromobacter sp.* WM111.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. The isolation and characterization of the carbofuran degrading bacterium *Achromobacter sp.* WM111 has been described (Karns et al., 1986). The culture was maintained on nitrogen-free basal salts medium (NFB) containing 40 mM glucose and 200 μg/mL carbofuran (Karns et al., 1986). For solid media, purified agar (Difco, Detroit, MI) was added to 1.5%. Colonies from solid medium were suspended in 5 mL of NFB–glucose–carbofuran medium in a 16 × 125 mm culture tube, and the cultures were shaken at room temperature (ca. 25 °C) for 7 days. The entire 5-mL culture was added to 100 mL of fresh NFB–glucose–carbofuran medium in a 500-mL Erlenmyer flask, and agitation at room temperature was continued. After 7 days the cells were aseptically collected by centrifugation (6000*g*, 10 min, 25 °C), washed by suspension in one culture volume of sterile 50 mM potassium phosphate (pH 7.0), and centrifuged as above. The washed cell pellets were added to 1 L of modified basal salts medium (BSM) (Hylemon and Phibbs, 1972; Karns et al., 1983) supplemented with 40 mM glucose in a 2.8-L Fernbach flask. The cultures were incubated at room temperature with shaking for 40 h. The cells were harvested by centrifugation (6000*g*, 10 min, 0 °C), washed by suspension in one culture volume of pH 7 phosphate buffer, and recentrifuged. The washed cell pellets were blotted briefly on Whatman No. 1 filter paper to remove excess buffer and were then frozen in liquid nitrogen and stored at −70 °C until used.

Preparation of Crude Cell-Free Extracts. All steps in the preparation of extracts were carried out between 0 and 4 °C. For some preparations an Aminco French

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pressure cell was used to accomplish cell lysis. Cell pellets were thawed and suspended in 2 mL of 50 mM Tris-HCl (pH 7.2) containing 170 mM NaCl and 5 mM dithiothreitol (buffer I) per gram of blotted wet weight of cells. $MgCl_2$ was added from an 800 mM stock to give a final concentration of 2 mM. Ribonuclease A (Sigma Chemical Co., St. Louis, MO) was added to give 1 Kunitz unit/mL, and deoxyribonuclease I (Boehringer Mannheim, Indianapolis, IN) was added to give 30 Kunitz units/mL. The preparation was passed twice through a chilled French pressure cell (15000 psi) then centrifuged (20000*g*, 30 min). the supernatant was taken as a source of crude cell-free extract. These extracts were stored at −30 °C. For studies on the cellular location of enzyme activity, a 2-mL extract was thawed and subjected to ultracentrifugation (105000*g*, 2 h). The supernatant (1.8 mL) was removed and labeled "soluble fraction", and the pellet was resuspended in 1 mL of buffer I and labeled "particulate fraction".

For purification studies, cell lysis was accomplished by adding 420 μL of Triton X-100, 42 μL of 2-mercaptoethanol, and 10.5 mL of glycerol to 42 g of thawed cells. This suspension was stirred vigorously for 30 min before 84 mL of 50 mM Tris-HCl (pH 7.2) containing 170 mM NaCl, 16 mM $MgCl_2$, lysozyme (11.5×10^3 units/mL), ribonuclease A (0.77 Kunitz units/mL), and deoxyribonuclease I (27 Kunitz units/mL) were added. Stirring was continued for 20 min after which 84 g of chilled alumina was added. After 10 min further stirring the slurry was centrifuged. This and all subsequent centrifugations in the purification of the enzyme were at 27000*g* for 20 min. The supernatant (56 mL) was taken as a source of crude extract for use in purification.

Protamine and Ammonium Sulfate Fractionations. A 1.25-mL portion of a 5.3% (w/v) solution of protamine sulfate (neutralized with NaOH) was added to 56 mL of crude extract in 25-μL lots with vigorous stirring. The preparation was stirred for 10 min after the final addition and centrifuged. Precipitated material was discarded, and solid ammonium sulfate was added to 53 mL of the supernatant to 25% saturation. The preparation was stirred for 20 min and centrifuged. The supernatant (53 mL) was brought to 85% saturation with ammonium sulfate, stirred for 20 min, and centrifuged. The pelleted material was suspended in 53 mL of buffer I.

Hydrophobic Interaction Column Chromatography. Solid ammonium sulfate was added to the suspended 25–85% precipitated material to give a final concentration of 1 M, and the preparation was applied to a 2.6 × 5.1 cm column of phenyl–Sepharose CL 4B (Pharmacia, Uppsula, Sweden) that had been equilibrated with buffer I containing 1 M ammonium sulfate. The column was then washed with 107 mL of the equilibration buffer. Bound proteins were eluted with a stepped gradient of decreasing ammonium sulfate concentration combined with an increasing ethylene glycol concentration in buffer I. Each step was 130 mL, and the flow rate was 45 mL/h throughout application and elution of the proteins: (1) 0.75 M ammonium sulfate, 12.5% (v/v) ethylene glycol; (2) 0.5 M ammonium sulfate, 25% (v/v) ethylene glycol; (3) 0.26 M ammonium sulfate, 37% (v/v) ethylene glycol. Fractions (3.8 mL) containing carbofuran hydrolase activity were pooled and concentrated by dialysis against 40 volumes of buffer I 85% saturated with ammonium sulfate. Precipitated material was collected by centrifugation and suspended in 2.5 mL of buffer I. Glycerol was added to a final concentration of 20% (v/v), and the preparation was frozen at −70 °C.

BioGel A0.5m Column Chromatography. The phenyl–Sepharose-purified material was thawed and applied to a 2.6 × 91.5 cm column of BioGel A0.5m that had been equilibrated with buffer I. The column was developed with buffer I at a flow rate of 23 mL/h. Fractions (3.3 mL) containing carbofuran hydrolase activity were pooled and concentrated by the addition of solid ammonium sulfate to 85% saturation. Precipitated material was collected by centrifugation and suspended in 1.45 mL of buffer I. Glycerol was added to a final concentration of 20% (v/v), and the preparation was stored at −70 °C until it was used as a source of partially purified enzyme for further studies.

The BioGel A0.5m column was calibrated by chromatographing a series of marker proteins under conditions identical with those used for the elution of carbofuran hydrolase. The marker proteins were β-amylase (200000 Da), yeast alcohol dehydrogenase (150000 Da), carbonic anhydrase (29000 Da), and cytochrome *c* (12400 Da).

Protein Determinations. Protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Enzyme Assays. Unless otherwise noted, all assays were run at 25 °C. Routine carbofuran assays contained 50 mM Tris-HCl (pH 8.3) and either 10 μL of crude enzyme, 3 μL of phenyl–Sepharose purified enzyme, or 2 μL of BioGel A0.5m purified enzyme in 990-μL total volume to which 10 μL of 10 mg/mL carbofuran in methanol was added. Samples (100 μL) of the assay were removed periodically over the course of 1 h, and the reactions were terminated either by dilution 1:1 with 100% (w/v) trichloroacetic acid or by the addition of 4 μL of concentrated sulfuric acid. The acid-treated reaction mixtures were stored at −30 °C. Carbofuran was resolved from the 7-phenol hydrolysis product (2,3-dihydro-2,2-dimethyl-7-benzofuranol) by high-performance liquid chromatography (HPLC) on a Waters RESOLVE C-18 radially compressed column (10 × 0.8 cm, 5-μm particle size) in a solvent system of 75% (v/v) water containing phosphoric acid (pH 2) and 25% (v/v) acetonitrile, at a flow rate of 3 mL/min. Injection volume was 20 μL. The absorbance of the column effluent was monitored at 210 nm. K_m values of carbofuran hydrolase for carbofuran, carbaryl, and aldicarb were determined with the BioGel A0.5m purified enzyme. Varying amounts of substrate were added in a constant 10 μL of methanol. Samples (100 μL) were removed at various intervals, and the reaction in each sample was stopped by the addition of 4 μL of concentrated H_2SO_4. The time course was monitored, and the frequency of sampling varied depending on the concentration of substrate. Reactions containing aldicarb were terminated by rapid freezing (liquid nitrogen) since both aldicarb and its hydrolysis product are destroyed by treatment with acid. Controls containing no enzyme were included to account for any chemical hydrolysis of the pesticides.

Carbaryl and its hydrolysis product, which cochromatographs with a 1-naphthol standard, were resolved in a solvent system of 65% water containing phosphoric acid (pH 2) and 35% acetonitrile. Aldicarb and its hydrolysis product were resolved in a solvent system of 77% water containing phosphoric acid (pH 2) and 23% acetonitrile.

[^{14}C]Carbofuran Degradation Studies. (*ring*-^{14}C)- (39.4 mCi/mM) and (*carbonyl*-^{14}C)- (14.4 mCi/mM) carbofuran were obtained from FMC Corp. Princeton, NJ. The 6-mL reaction mixture contained 50 mM Tris-HCl (pH 8.6), 100 μg/mL labeled carbofuran (total radioactivity 0.5 μCi), and either 10 or 50 μL of crude cell-free extract. Reaction mixtures containing boiled enzyme or no enzyme were included as controls. Samples (1 mL) were removed

periodically after the addition of enzyme and added to 1 mL of 0.1 N HCl and 1 mL of ethyl acetate to terminate the reaction and extract the reaction products. The ethyl acetate phase was removed, and the aqueous phase from each sample was extracted twice more with 2 mL of ethyl acetate. The three ethyl acetate phases from each sample were pooled, dried over sodium sulfate, and then evaporated to 0.5 mL under a stream of nitrogen gas. Duplicate 100-μL volumes from each sample were spotted onto 20 × 20 cm silica gel 60 F-254 precoated TLC plates (0.25 mm, E. Merck, Darmstadt, W. Germany). Standards of carbofuran and the 7-phenol metabolite of carbofuran were also spotted. The plates were developed in ethyl ether–hexane (3:1) to a height of 15 cm, air-dried, and exposed to NS 5T No Screen X-ray film (Eastman Kodak Co., Rochester, NY) in darkness for 11 days to locate radioactive spots. Each radioactive spot was scraped into a scintillation vial containing 10 mL of Redi-Solv scintillation fluid (Beckman, Palo Alto, CA) and counted.

Spectrophotometric Assay of Carbofuran Hydrolase. The most convenient method for the routine measurement of carbofuran hydrolase was a spectrophotometric assay monitoring the accumulation of *o*-nitrophenol due to the hydrolysis of *o*-nitrophenyl dimethylcarbamate (Sigma Chemical Co., St. Louis, MO). The appearance of *o*-nitrophenol was measured by following the increase in absorbance of a reaction mixture at 413.5 nm in a spectrophotometer. Reaction mixtures (1 mL) contained 400 μg of *o*-nitrophenyl dimethylcarbamate (added from a 100 mg/mL methanol stock solution). The rate of hydrolysis was calculated from a molar extinction coefficient of 4900 M^{-1} cm^{-1} for *o*-nitrophenol. The temperature and pH optima of the carbofuran hydrolase reaction were determined by assaying the hydrolysis of *o*-nitrophenyl dimethylcarbamate. To determine the optimum temperature, buffer that had been preheated to the desired temperature was added to cuvettes that were maintained at the same temperature in an electronically heated cuvette holder. Substrate and enzyme (5 μL of BioGel A0.5m purified) were added, and the temperature was measured before monitoring the change in absorbance over a 4-min time course. The optimum pH was determined at 25 °C. The pH of the entire reaction mixture was determined with a combination calomel electrode prior to monitoring the absorbance change.

Measurement of Parathion, Chlorpropham, and EPTC Hydrolysis. Hydrolysis of parathion was measured by monitoring the appearance of the *p*-nitrophenol hydrolysis product at 405 nm. Hydrolysis of EPTC and chlorpropham was monitored by HPLC of reaction mixtures. Chlorpropham was separated from its chloroaniline hydrolysis product in a solvent system of 75% acetonitrile and 25% H_3PO_4 (pH 2) at a flow rate of 3 mL/min. EPTC hydrolysis was monitored with a solvent system of 50% acetonitrile and 50% H_3PO_4. In all cases where HPLC was used to monitor reactions, the detection was at 210 nm.

RESULTS

Degradation of Carbofuran by Cell-Free Extracts. Crude extracts of the carbofuran degrading *Achromobacter sp.* WM111 catalyzed the rapid degradation of carbofuran (Figure 1). When ^{14}C-U-ring-labeled carbofuran was used as substrate, the 7-phenol metabolite was the only degradation product detected. The recovery of the labeled 7-phenol product was less than what was theoretically expected, probably because of loss of this more volatile compound during the evaporation of solvent. The rate of reaction was dependent upon the amount of extract added to the reaction mixture. No detectable degradation of

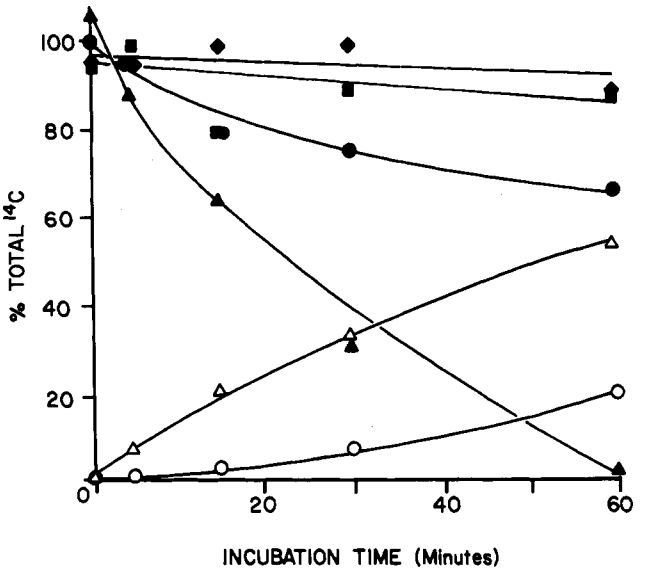


Figure 1. Degradation of ^{14}C-U-ring-labeled carbofuran by a crude extract of *Achromobacter sp.* WM111. The percentage of the original amount of ^{14}C present in the zones of a TLC plate identified as carbofuran (closed symbols) or 7-phenol (open symbols) was calculated and plotted. Key: (▲ and △) carbofuran and 7-phenol, respectively, in a reaction mixture containing 50 μL of crude extract; (● and ○) carbofuran and 7-phenol in a reaction containing 10 μL of crude extract; (■) carbofuran in a control containing 50 μL of extract that had been heated at 100 °C for 10 min prior to adding it to the reaction mixture; (♦) carbofuran in a control without extract.

carbofuran occurred in the absence of extract or when extract that had been heated to 100 °C for 10 min was added in lieu of active enzyme. No extractable intermediates or end products were detected when [^{14}C]-carbonyl-labeled carbofuran was used as substrate (data not shown). Neither dialysis nor additional purification of the enzyme affected its ability to convert carbofuran to 7-phenol, indicating that no soluble cofactors were required for activity. These data strongly suggest that crude extracts of *Achromobacter sp.* WM111 contain an enzyme that catalyzes the direct hydrolysis of the *N*-methylcarbamate insecticide carbofuran. Addition of 0.2 mM concentrations of NAD, NADH, NADP, NADPH, or FAD and the combination of NADH or NADPH with FAD had no effect on the rate of hydrolysis of *o*-nitrophenol dimethylcarbamate, further indicating that the mechanism of the reaction is hydrolysis rather than oxidation or reduction (data not shown).

The carbofuran hydrolase activity was very unstable in crude extracts prepared with a French pressure cell to accomplish cell lysis, having a half-life of 20 h at 0 °C. The enzyme was partially stabilized after protamine sulfate and ammonium sulfate fractionation, having a half-life of 35 h at 0 °C. Subsequent phenyl–Sepharose chromatography yielded a preparation that was very stable, showing no measurable loss of activity after 5 days at 0 °C. When crude extracts were prepared from the same cell preparation using a treatment of Triton X-100/osmotic shock/lysozyme/alumina, the resulting enzyme preparation was very stable, with no loss of activity over a 48-h period at 0 °C.

Enzyme Purification. The partial purification of carbofuran hydrolase from crude extracts of *Achromobacter sp.* WM111 is summarized in Table I. Little or no purification was accomplished by fractionation with protamine sulfate; however, a substantial amount of material, presumably nucleic acid, was removed by this procedure.

Table I. Purification of Carbofuran Hydrolase

fraction	total protein, mg	total act., μmol o-nitrophenol/min	sp act., nmol o-nitrophenol/min per mg protein	fold purif
crude extract	730	18.2	25	1
protamine sulfate fractionation	700	16.7	24	1
ammonium sulfate fractionation (25–85% satn)	293	9.4	36	1.3
phenyl–Sepharose	75	6.9	92	3.7
dialysis against ammonium sulfate (85% satn)	62	6.4	106	4.2
Biogel A0.5m	8.5	1.8	211	8.4
ammonium sulfate fractionation (85% satn)	8.5	1.5	182	7.3

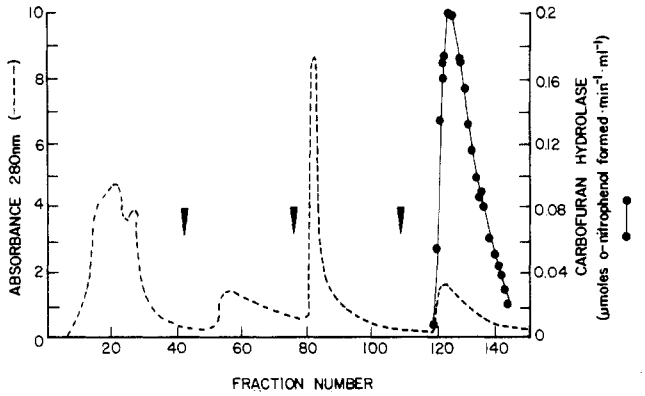


Figure 2. Phenyl–Sepharose CL 4B column chromatography of carbofuran hydrolase in an ammonium sulfate fractionated extract of *Achromobacter sp.* WM111. Arrows indicate the points at which the stepped gradient was changed, as described in Materials and Methods. Carbofuran hydrolase activity was detected using the *o*-nitrophenyl dimethylcarbamate hydrolysis assay and is expressed as micromoles of *o*-nitrophenol formed/minute per milliliter of each fraction.

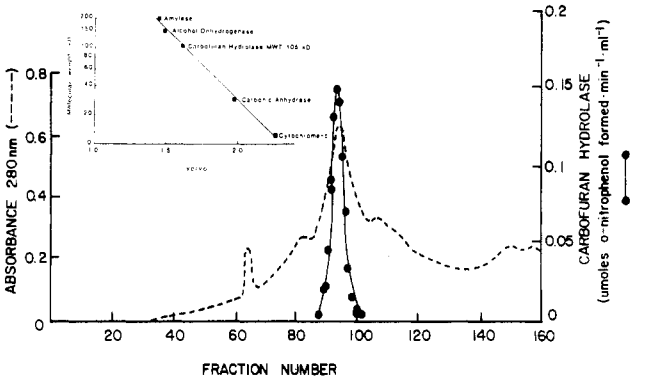


Figure 3. BioGel A0.5m column chromatography of carbofuran hydrolase. Enzyme activity was assayed as in the legend to Figure 2. Inset is a calibration curve for the column used and indicates the elution of carbofuran hydrolase relative to that of 4 molecular size standards.

Ammonium sulfate fractionation between 25% and 85% saturation resulted in a 1.3-fold purification of the enzyme. Only 58% of the active enzyme was recovered, presumably due to loss of enzyme trapped in the Triton X-100, which separated as a floating layer during this procedure. This poor recovery was balanced by the gain in stability achieved by this lysis procedure and by the ease in which large amounts of crude extract could be prepared.

Hydrophobic interaction chromatography using phenyl–Sepharose was very effective in purifying carbofuran hydrolase. The enzyme was very strongly bound to the column and eluted only when the organic component of the elution buffer was increased to 37% (Figure 2). Further purification, to a total of 8-fold, was achieved on a column of BioGel A0.5m. This procedure also yielded an estimate of 105 000 Da for the molecular size of carbofuran hydrolase (Figure 3). After concentration by ammonium sulfate precipitation, the final enzyme preparation was 7.3-fold purified and had a specific activity of 182 nmol of *o*-nitrophenol formed/min per mg of protein when *o*-nitrophenyl dimethylcarbamate was used as substrate. This same preparation, assayed under identical conditions with carbofuran as substrate, had a specific activity of 806 nmol of carbofuran hydrolyzed/min per mg of protein.

Solubility of Carbofuran Hydrolase. Upon ultracentifugation, 86.5% of the total carbofuran hydrolase activity in a crude French pressure cell extract of WM111 was contained in the supernatant fraction. The remaining 13.5% of the activity was contained in the unwashed membrane pellet. This result suggests that carbofuran hydrolase is either a soluble enzyme of the cytoplasm or periplasmic space or that it is very weakly associated with the cell membrane and the lysis procedure strips it from the membrane.

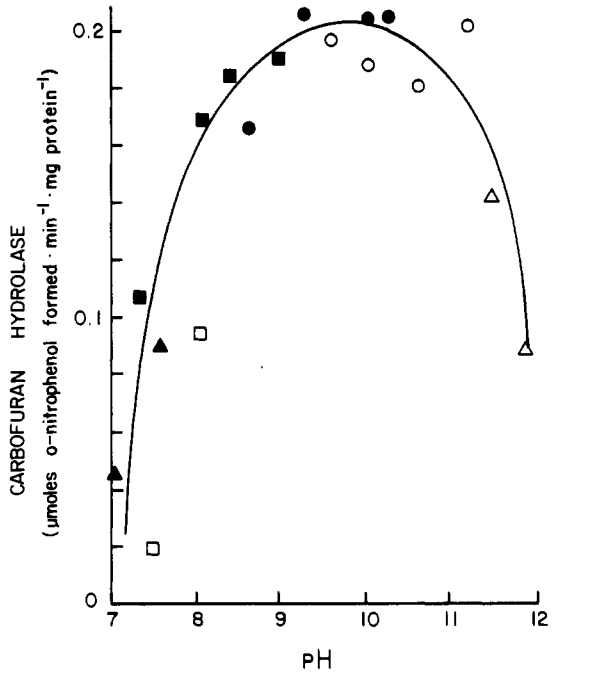


Figure 4. Effect of pH on the rate of the carbofuran hydrolase reaction. Enzyme activity was measured by the hydrolysis of *o*-nitrophenyl dimethylcarbamate. Buffers used: (■) 50 mM Tris-HCl; (□) 100 mM sodium phosphate; (▲) 50 mM 3-(*N*-morpholino)propanesulfonic acid [MOPS]–HCl; (△) 50 mM sodium phosphate–NaOH; (●) 50 mM glycine–NaOH; (○) 50 mM sodium carbonate–bicarbonate. The pH of the complete reaction mixture is plotted.

Optimal pH and Temperature. The partially purified carbofuran hydrolase had maximum activity over a broad alkaline range pH 9.0–10.5 (Figure 4). The effect of temperature on the rate of the reaction is shown in Figure 5. Peak carbofuran hydrolase activity was measured be-

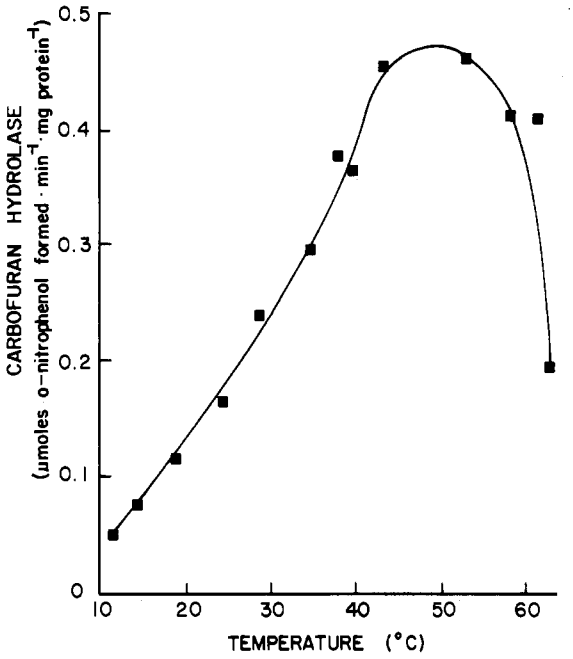


Figure 5. Effect of assay temperature on the rate of hydrolysis of *o*-nitrophenyl dimethylcarbamate by carbofuran hydrolase.

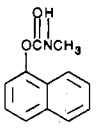
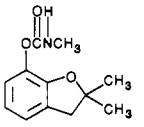
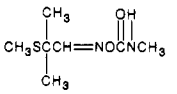
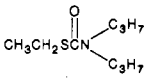
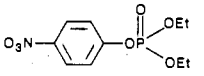
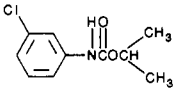
tween 45 and 53 °C. The pH of the reaction mixture varied slightly with temperature (data not shown); however, even if one allowed for the effect of pH on the rate of the reaction, the shape of the temperature curve and the point of optimum activity are unlikely to be significantly altered.

Substrate Specificity. The partially purified carbofuran hydrolase preparation was very active against the three *N*-methylcarbamate insecticides that were tested as substrates (Table II). The kinetics of the reaction obeyed the equations of Michaelis and Menton and the K_m and V_{max} of the enzyme for each substrate were calculated from Woolf plots (Dowd and Riggs, 1965). The maximum rates of hydrolysis of carbofuran and carbaryl were nearly identical, while the rate of hydrolysis of aldicarb was significantly lower.

The K_m values obtained for carbaryl and carbofuran as substrates were very low and indicated that the enzyme may have a slightly greater affinity for carbaryl than for carbofuran (Table II). The K_m obtained for aldicarb was significantly higher than that seen for either carbaryl or carbofuran. The lower V_{max} and higher K_m seen for aldicarb as a substrate indicate that the enzyme is more effective in hydrolyzing *N*-methylcarbamate insecticides that have aromatic moeities. This result mimics that seen in whole cells where aldicarb is degraded much more slowly than any of the aromatic *N*-methylcarbamate insecticides tested (Karns et al., 1986).

No enzyme-dependent hydrolysis of the organophosphate insecticide parathion, the thiocarbamate herbicide EPTC, or the *N*-phenylcarbamate herbicide chlorpropham could be detected when these compounds were incubated with the 8-fold purified enzyme preparation (Table II).

Table II. Michaelis-Menton Kinetic Constants for Carbofuran Hydrolase with Several Substrates

substrate	K_m μM	K_m μg/mL	V_{max}, nmol substrate/min per mg protein
carbaryl 	15	3	780
carbofuran 	56	12	810
aldicarb 	2800	530	270
EPTC 	nd[a]	nd	0[b]
parathion 	nd	nd	0
CIPC 	nd	nd	0

[a] nd = not determined. [b] A zero in this column means no activity could be detected.

DISCUSSION

Numerous investigators documented the degradation of pesticides by soil and water microorganisms. Except for the studies of Kearney (1965), Engelhardt et al. (1973), and Blake and Kaufman (1975) on the degradation of anilide herbicides, and those by Brown (1980) and Munnecke (1976) on the degradation of parathion and related organophosphate insecticides, few have examined the enzymes responsible for pesticide degradation. To our knowledge, this is the first description of a microbial enzyme that degrades *N*-methylcarbamate insecticides. Biological degradation of *N*-methylcarbamate insecticides to ineffective concentrations has been documented in numerous reports of "problem" or "aggressive" soils. The mechanism of enhanced *N*-methylcarbamate insecticide degradation by microorganisms in such soils is unknown. However, the mechanism by which *Achromobacter sp.* WM111 rapidly degrades these compounds may be a principal means by which these compounds are inactivated in problem soils.

The enzyme isolated from *Achromobacter sp.* WM111 catalyzes the cleavage of carbofuran to its 7-phenol metabolite (2,3-dihydro-2,2-dimethylbenzofuranol) and an unknown amine. The enzyme appears to be a hydrolase (E.C. 3) as no cofactor requirement could be demonstrated after numerous purification and dialysis steps. In fungal (Liu and Bollag, 1971) and insect (Casida, 1970; Gemrich, 1967) systems oxygenases (hydroxylases and epoxidases) have been shown to be effective in the detoxification of *N*-methylcarbamate insecticides. The enzymatic hydroxylation of xenobiotic compounds usually requires reduced pyridine nucleotides as cofactors. The lack of any demonstrable cofactor requirement for the carbofuran degrading enzyme from *Achromobacter sp.* WM111 indicates that the reaction proceeds by the direct hydrolysis of the insecticide as opposed to hydroxylation. We have, therefore, named this enzyme carbofuran hydrolase on the basis of the first activity we detected and on the ability of *Achromobacter sp.* WM111 to utilize carbofuran as a nitrogen source (Karns et al., 1986). It is unknown, at present, whether this enzyme is an esterase (E.C. 3.1; cleaving between the carbonyl group of *N*-methylcarbamic acid and the phenol) or an amidase (E.C. 3.5; cleaving between the carbonyl and amine moieties of *N*-methylcarbamic acid). In either case the products of the reaction would be identical due to the instability of *N*-methylcarbamic acid. The hydrolysis of an *N*-methylcarbamate

insecticide at either position totally destroys the insecticidal activity of the compound.

The data for several studies on the problem soils phenomenon have been interpreted as indications that some degree of "cross adaptation" can be seen. That is, that from repeated treatment of a soil with one pesticide the microorganisms in problem soils acquire the ability to degrade a number of other pesticides (Kaufman et al., 1985). As we have shown here, the carbofuran degrading *Achromobacter sp.* WM111 produces an enzyme that is capable of rapidly degrading several pesticides within the class of *N*-methylcarbamates. However, this cross adaptability does not extend beyond this one class of pesticides as chlorpropham (an *N*-phenylcarbamate), EPTC (a thiocarbamate), and parathion (an organophosphate) were not hydrolyzed by the purified enzyme preparation.

Carbofuran hydrolase appears to have a higher affinity for, and a higher turnover of, *N*-methylcarbamates with aromatic moeities (carbofuran, K_m 56 μM, rate 810 nmol/min per mg of protein; carbaryl, K_m 15 μm, rate 780 nmol/min per mg of protein) than it does for those with nonaromatic substitutions (aldicarb, K_m 2800 μM, rate 270 nmol/min per mg of protein). Although a K_m was not determined, the fact that a saturating amount of *o*-nitrophenyl dimethylcarbamate was hydrolyzed at a rate only 23% that of which carbofuran is cleaved indicates that alteration of the carbamate moiety of the substrate has a larger effect on the enzyme's ability to hydrolyze a substrate than do changes in the aromatic portion of the molecule. Similarly, Brown (1980) noted that the rate of parathion hydrolysis by parathion hydrolase was greatly affected by altering the alkyl esters attached to the thiophosphoric acid moiety, while Munnecke (1976) found that *O,O*-diethyl thiophosphorates with aromatic moieties quite different from the 4-nitrophenol of parathion can be hydrolyzed at rapid rates. In light of these observations, and those by Engelhardt et al. (1973), Blake and Kaufman (1975), and Kearney (1965), the ability of carbofuran hydrolase to cleave a number of different *N*-methylcarbamate compounds is not surprising. In general, pesticide hydrolyzing enzymes seem to be able to act on a range of compounds with similar chemical linkages rather than on specific compounds.

It is not presently known whether carbofuran hydrolase is involved in the "problem" or "aggressive" soils phenomenon. Given the apparent simplicity of the reaction, and the fact that hydrolysis completely destroys the pesticidal properties of carbofuran, it is possible that this (or a similar enzyme) may be at least partially responsible for carbofuran failures. In a more positive vein, it may be possible to utilize pesticide degrading enzymes for the safe elimination of waste pesticide solutions. Munnecke (1976) and Kearney et al. (1986) demonstrated that parathion hydrolase could be used for this purpose. The isolation and characterization of new pesticide degrading enzymes may lead to the development of cheap and effective systems for the disposal of agrochemical wastes.

Registry No. Carbofuran, 1563-66-2; aldicarb, 116-06-3; carbaryl, 63-25-2; carbofuran hydrolase, 110141-85-0.

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Extraction of Aminocarb and a Metabolite from Whole Fish and Derivatization for Electron-Capture Gas Chromatography

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A method is presented for the detection and measurement of residues of aminocarb and one metabolite in whole fish using electron-capture gas chromatography. The procedure, a combination of unrelated extraction and derivatization procedures, had a mean recovery for aminocarb of approximately 90% and a mean recovery for the metabolite MAM [4-(methylamino)-3-methylphenyl *N*-methylcarbamate] of approximately 88%. Detection limits for aminocarb and MAM were 0.02 and 0.3 μg/g, respectively. The extraction process was straightfoward. The derivatization process was rapid, requiring only 30 min at room temperature. Derivatized residues were stable for at least 7 days if stored under appropriate conditions.

A limited number of methods for gas chromatographic analysis of carbamate pesticides and their metabolites in biological tissue samples are described in the literature (Stanley and Delphia, 1981; Szeto and Sundaram, 1980; Sundaram and Szeto, 1979; Sundaram et al., 1976; Wong and Fisher, 1975; Lau and Marxmiller, 1970). Some use nitrogen-specific detectors that permit rapid, direct assessment of these residues, but problems can arise with the inherent instability of some carbamate molecules when exposed to thermal stress (Seiber, 1972). Those employing an electron-capture detector require derivatization (reacted with trifluoroacetic anhydride, heptafluorobutyric anhydride, etc.) of the carbamate molecule to permit detection. Derivatized carbamates are more stable under heat stress (Seiber, 1972) and offer a high degree of sensitivity due to the addition of several electron-capturing components (halogens) to the molecule. However, derivatization generally requires time-consuming procedures for cleanup or for the derivatization reaction itself (Stanley and Delphia, 1981; Sundaram et al., 1976; Wong and Fisher, 1975; Lau and Marxmiller, 1970). The purpose of this paper is to describe a method used in this laboratory to evaluate aminocarb [4-(dimethylamino)-3-methylphenyl *N*-methylcarbamate] and one of its metabolites [4-(methylamino)-3-methylphenyl *N*-methylcarbamate] (MAM) in whole fish, using electron-capture gas chromatography. It combined separate, unrelated extraction and derivatization procedures found in the literature. These methods were successfully combined and provided a comparatively simple and quick analysis.

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EXPERIMENTAL SECTION

Apparatus. A Hewlett-Packard Model 5713A gas chromatograph fitted with a ^{63}Ni electron-capture detector was used. Operating conditions: injection port, 200 °C; oven, 175 °C; detector, 250 °C; carrier gas, 5% methane/95% argon; carrier gas flow rate, 50 mL/min. The column was glass, 1.8-m length, 2-mm inside diameter, packed with 3% OV-17 on Chromsorb W (HP) (Chromatographic Specialties Ltd.).

Reagents. A 0.1 M solution of trimethylamine (TMA) in benzene was used as a catalyst for the derivatization reaction. This TMA solution was prepared by adding cooled (0 °C) trimethylamine (Kodak Inc.) to cooled, tared benzene to produce a solution of 1 M. Of this solution 10 mL was diluted to 100 mL with benzene in a 100-mL volumetric flask to produce a solution of 0.1 M.

Procedure. *Extraction.* Brown bullhead (*Ictalurus nebulosus* Lesueur) of approximately 5 g were extracted according to the procedure. In a Sorval blender, fish were extracted according to Szeto and Sundaram (1980). Cleanup of the extract was achieved by extracting the aqueous phase of the procedure (phosphoric acid solution) three times with 25 mL of hexane, as done by Sundaram and Szeto (1979), and not by filtration. The extraction and cleanup were then completed according to Szeto and Sundaram (1980).

Derivatization. Fish extracts were derivatized according to the procedure of Lawrence (1976). To the residue in the vial was added 15 μL of heptafluorobutyric anhydride (HFBA) (Kodak Inc.) and 1.0 mL of the 0.1 M TMA solution. The vial was capped tightly, swirled, and allowed

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