BBA 66333

PARTIAL PURIFICATION AND PROPERTIES OF A SOIL ENZYME THAT DEGRADES THE INSECTICIDE MALATHION

L. W. GETZIN AND I. ROSEFIELD

Western Washington Research and Extension Center, Washington State University, Puyallup, Wash. 98371 (U.S.A.)

(Received January 18th, 1971)

SUMMARY

1. An unusually stable esterase which catalyzes the hydrolysis of malathion to its monoacid was extracted from irradiation-sterilized soil and non-irradiated soil with 0.2 M NaOH and partially purified by $MnCl_2$ treatment, $(NH_4)_2SO_4$ precipitation, dialysis and ion exchange chromatography. A 240-g sample of Chehalis clay loam yielded 28 mg of final preparation. It contained 9.8 mg of protein and hydrolyzed 31 μmoles of malathion in 4 h at pH 7.0 and 37°. Lineweaver–Burk plots obtained for two concentrations of the enzyme gave identical K_m values of $2.12 \cdot 10^{-4}$ M. The enzyme was optimally active around pH 7.0. The enzyme was very stable, being denatured only at temperatures above 70° and by 24-h exposures at pH < 2.0 and pH > 10.0. Lyophilization partially destroyed the activity but no loss of activity occurred during extended storage of enzyme solutions at 4° or frozen at −10°.

2. When partially purified enzyme was applied to soil, activity was detected for the duration of the experimental period (8 weeks). Its existence as a stable, cell-free soil enzyme is postulated from experimental evidence based on the persistence and adsorptive characteristics of the partially purified enzyme in soil. This esterase should be an excellent tool for investigating enzymatic biological transformations in soil.

INTRODUCTION

Since certain biological reactions in soil are catalyzed by exocellular enzymes, it is possible that cell-free enzymes also degrade pesticides in soil. Information on the existence and properties of stable, exocellular enzymes in soil is sparse because it is difficult to extract active enzymes from soil and even more difficult to establish whether the catalyzed reactions are due to intracellular enzymes, short-lived exocellular enzymes or stable exocellular enzymes[1].

Recently, evidence was obtained suggesting that soil enzymes contribute to the breakdown of some organophosphorus insecticides[2]. We demonstrated that malathion (diethylmercaptosuccinate, *S*-ester with *O,O*-dimethylphosphorodithioate), mevinphos (methyl-3-hydroxy-α-crotonate, dimethylphosphate), dichlorvos (2,2-di-

chlorovinyl dimethylphosphate) and crotoxyphos (α-methylbenzyl-3-hydroxycrotonate dimethylphosphate) degraded more rapidly in radiation-sterilized soil than in heat-sterilized soil and subsequently, extracted a heat-labile fraction from irradiated soil which degraded malathion. An organic composition of the active substance was indicated because it was removed with methods commonly used to extract organic matter from soils. The nature of this malathion-degrading substance from soil has been examined in greater detail. This paper includes a method for partial purification of the substance and presents evidence that it is a stable, cell-free soil enzyme which hydrolyzes malathion to its monoacid.

MATERIALS AND METHODS

Materials

Chehalis clay loam was used as a source of the enzyme. This soil was chosen because alkali extracts of it provided a maximum amount of active material with a minimum amount of interference from contaminating organic matter. The soil was collected from a cultivated area in western Washington and stored in a moist condition in concrete bins. It had a pH of 5.5 to 6.0 and contained 8.1% organic matter and 30% clay. When sterile soil was required, Chehalis clay loam was given 4 mrads of gamma radiation in a Mark II cobalt-60 unit.

^{14}C Malathion (8.69 μC/mg), labeled at the 2 and 3 positions of the succinyl radical, and ^{32}P]malathion (29.2 μC/mg upon receipt) were obtained from Nuclear-Chicago, Chicago, Ill. The reported purity of each was greater than 98%. Radiograms of the two samples developed with thin-layer chromatography on Eastman Chromagram silica gel sheets with chloroform–acetone (9:1, by vol.) contained single radioactive spots whose R_F values corresponded to that of malathion (R_F 0.62). Nonradioactive malathion (98.5% purity) and its phosphorus-containing degradation products were obtained from American Cyanamid Company, Princeton, N.J.. Diethyl malate was purchased from Eastman Chemical Co., Rochester, N.Y. and diethyl mercaptosuccinate was synthesized in our laboratory by refluxing thiosuccinic acid with ethanol in the presence of NaOH. Hexane (Skellysolve B) was purified by distillation following refluxing in the presence of NaOH.

Analytical procedures

Malathion concentrations were determined by gas–liquid chromatography and radioassay. Malathion was incubated with 5 or 10 ml of buffered enzyme solution in 25-ml screwcap culture tubes and subsequently extracted with 10 ml of hexane by shaking for 30 min. One-ml aliquots of the hexane phase were pipetted into planchets for assay of radiolabeled compounds and 10 mg of shellac in 0.2 ml of chloroform were added to each planchet to reduce volatilization of the insecticide. 1 to 2 h after the solvents evaporated, the radioactivity in the residue was counted. Aqueous samples were pipetted into ring planchets, dried for 24–30 h and then counted.

A Varian Aerograph 600-D gas chromatograph equipped with a phosphorus detector and a 5-foot × $\frac{1}{8}$-inch borosilicate glass column packed with 5% QF-1 on 60 80-mesh Gas Chrom Q was used for quantitative assay of nonradioactive insecticides and for verification of results obtained by radioassay. The flow rate of the

carrier gas (helium) was 18 ml/min and the hydrogen flow rate was adjusted between 20 and 25 ml/min depending upon the sensitivity of the detector. The temperatures of the injector column and oven were 250° and 240°, respectively. The retention time for malathion was 2.10 min.

Protein was determined by the method of LOWRY *et al.*[3] using bovine serum albumin as a standard. All soil extracts were first dialyzed to remove Mn^{2+} which interfered with the analysis. Total nitrogen was determined by a micro-Kjeldahl method. Carbohydrate analyses of fractions taken from Bio-Rex 70 chromatographic columns was determined by a modified Molisch test[4].

Enzyme assay

The mixture used to assay for activity consisted of 1.5 μmoles of malathion and desired amounts of enzyme in 5 or 10 ml of 0.075 M Tris–HCl buffer (pH 7.0). Controls included substrate and soluble fractions from soil that had been autoclaved to destroy its malathion-degrading activity. Samples were incubated for 4 to 6 h at 37° in 20-mm × 125-mm screw-cap culture tubes on a water bath shaker. Under these conditions substrate was not limiting and the reaction proceeded with zero order kinetics. Unless specified, enzymatic activity data presented in this paper were obtained under conditions for zero order kinetics.

One enzyme unit is defined as the amount of soil enzyme required to catalyze the degradation of 0.2 μmoles of malathion in 4 h at pH 7.0 and 37°. The selection of a unit with a low level of activity was necessary for zero order reaction rates because of the low water solubility of malathion (145 parts per million). Units were not defined in terms of protein content because active solutions did not give the characteristic spectrophotometric peak for proteins at 280 nm unless the enzyme was first subjected to hydrolysis in 6 M HCl. Furthermore, a protein content based upon the Lowry method may not have been accurate because the color produced by the soil extract differed slightly from that of the bovine serum albumin standard.

RESULTS

Purification of the enzyme

Extraction

All operations were carried out at room temperature. Initially, 120 g air-dried equivalents of moist Chehalis clay loam were placed in each of two, 1000-ml Erlenmeyer flasks with 600 ml of 0.2 M NaOH, and shaken for 30 min. The soil suspension was dispensed into 250-ml centrifuge bottles and centrifuged at 16 000 × *g* for 10 min in a Model SS-3 Sorvall centrifuge. The supernatant containing the enzyme was filtered through a Buchner funnel and immediately adjusted to pH 7.9 with 3 M HCl.

$MnCl_2$ treatment

20 ml of a 20% solution of $MnCl_2$ were added per 100 ml of the supernatant to precipitate the humates and other contaminating organic materials. After 30 min the insoluble materials were removed by centrifugation at 2000 × *g* for 5–10 min and the yellowish-brown supernatant containing the enzyme was collected. Excess Mn^{2+} and additional amounts of contaminating organic material were then removed by raising the pH of the solution to 8.0 with 4 M NaOH during constant stirring.

The slurry was centrifuged and filtered to separate insoluble $Mn(OH)_2$ and some organic contaminants from the straw-colored supernatant.

$(NH_4)_2SO_4$ precipitation

The supernatant was acidified to pH 4.6 with 3 M HCl and brought to 65% saturation with solid $(NH_4)_2SO_4$ to precipitate the enzyme. After a few hours much of the supernatant was removed by suction and the precipitate which floated on the top was collected by centrifugation at 23 000 × *g* for 10 min. The precipitate was dissolved in 40 ml of water and dialyzed against running tapwater.

Column chromatography

After dialysis, the enzyme was further purified on a column of Bio-Rex 70 cation-exchange resin which was first washed with 0.5 M NaOH, then with 0.5 M HCl and finally equilibrated in distilled water. A 12-g soil equivalent of enzyme in 5 ml of water was applied to a 16-mm × 65-mm column of resin and eluted with 100 ml of water and 200 ml of 0.3 M phosphate buffer (pH 8.0). Fractions were assayed for enzyme activity and protein content (Fig. 1).

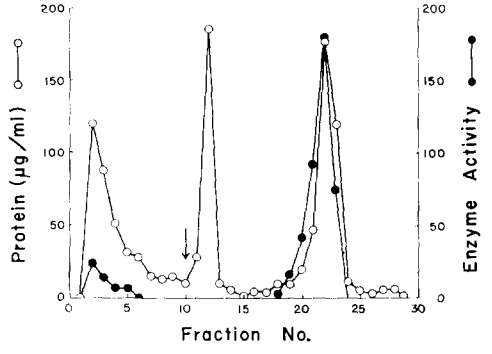


Fig. 1. Chromatography of malathion-degrading fraction on Bio-Rex 70 exchange resin. The column was first washed with 100 ml of distilled water and the enzyme was eluted with 200 ml of 0.3 M sodium phosphate buffer (pH 8.0). The arrow marks the point at which the eluting solvent was changed from distilled water to the phosphate buffer. The eluant was collected in 10-ml fractions with a flow rate of 50–70 ml/h.

Non-irradiated soil was used as a source of enzyme after it was established that the active fractions from non-radiated soil and radiation-sterilized soil were similar in behavior. Four to five times more activity was recovered from non-irradiated soil than from irradiated soil. Aqueous solutions of the enzyme also lost their activity when they were exposed to radiation.

The purification procedure was scaled up to accumulate enzyme for subsequent studies. Alkali extracts from 240-g samples of soil were purified, pooled and stored at 4° or frozen for months with no loss of activity. However, a 50–75% loss of activity occurred when the enzyme was lyophilized.

Table I gives data from the purification procedure obtained with 240 g of Chehalis clay loam. A 1-g soil equivalent of the original alkali extract contained approx. 2 units of enzyme. Recoveries following purification on Bio-Rex 70 varied from 0.5 to 1.6 enzyme units/g of soil. The steps following the alkaline extraction of soil represent a 100-fold decrease in total dry wt., a 67-fold decrease in total

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR MALATHION-DEGRADING ENZYME EXTRACTED WITH 0.2 M NaOH FROM 240 G OF CHEHALIS CLAY LOAM

Fraction	*Total vol. (ml)*	*Total dry wt. (mg)*	*Total nitrogen (mg)*	*Total protein (mg)*	*Total activity**	*Specific activity***	*Purification*	*Recovery (%)*
Soil extract	975	2810	85.3	661.0	95.5	0.14	1.0	100.0
$MnCl_2$ treatment	1000	144	11.3	58.8	78.5	1.33	9.3	82.2
$(NH_4)_2SO_4$ precipitate	40	259	3.6	33.7	59.7	1.77	12.3	62.5
Bio-Rex 70	25	28	1.4	9.8	30.6	3.10	21.6	32.0

* μMoles of malathion degraded in 4 h.
** μMoles of malathion degraded in 4 h per mg of protein.

protein and only a 3-fold decrease in total enzyme activity. The final product weighed 28 mg, of which 9.8 mg was protein. The remainder was primarily carbohydrates which upon acid hydrolysis yielded a mixture of pentoses and hexoses.

Electrophoresis

Attempts were made to further purify the enzyme from the Bio-Rex column with a model CP Beckman Continuous-Flow Paper Electrophoresis Cell. Since preliminary experiments indicated the enzyme migrated a sufficient distance for separation only in a highly alkaline medium, 0.01 M carbonate buffer (pH 10.5) was used as the electrolyte. Constant voltage (700 V) was maintained across the continuous flow cell with a current that ranged between 60 and 80 mA. For fractionation, the Bio-Rex-purified enzyme from 240 g of soil was prepared in 80 ml of the carbonate buffer. The sample applicator was positioned one-third of the way from the negative edge of the paper and the sample flow rate adjusted to 4 ml/h. After completion of the run, the fractions were assayed for activity and the most concentrated fractions were pooled and analyzed for protein content. Subsequently, the enzyme was concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed for strip electrophoresis.

The major portion of the enzyme subjected to continuous flow electrophoresis was collected in a 6-cm band centered approx. 5 cm away from the point of application. A 15–20% loss in protein content was associated with a 55–65% loss of activity. Gradual denaturization of the enzyme in the alkali buffer was primarily responsible for this loss of activity. While this step did not increase the specific activity, significant amounts of contaminating material were removed which enabled us to utilize the concentrated enzyme solution for further electrophoretic studies.

Strip electrophoresis was performed according to standard procedures on Gelman Sephraphore III cellulose acetate. The electrophoresis was carried out in 0.02 M carbonate buffer (pH 10.5) at 3 mA per strip for 90 min. After completing the run, each strip was cut in half longitudinally. One strip was immediately fixed in 5% trichloroacetic acid for 5 min and then stained with nigrosin. The other half was cut into 1-cm sections and each piece was placed in 5 ml of 0.075 M Tris–HCl buffer (pH 7.0) and assayed for enzyme activity.

The activity was detected in the sections 2 and 3 cm removed from the point of application. The nigrosin produced a weakly stained 1-cm band centered 3 cm from the point of application.

Identification of the reaction products

The degradation products formed during the incubation of ^{14}C- and ^{32}P-labeled malathion with soil enzyme were identified by thin-layer chromatography. The methods used for extraction, separation and chromatography of malathion and its breakdown products were modified slightly from those employed by BIGLEY AND PLAPP[5].

Two 50-ml flasks, each containing 0.6 μmole of malathion were incubated with 1.5 units of enzyme in 20 ml of 0.075 M Tris buffer (pH 6.8). One flask contained 1.75 μC of [^{14}C]malathion, the other contained 5.8 μC of [^{32}P]malathion. Comparable controls minus the enzyme were included. After incubating the mixtures for 6 h each solution was extracted with 40 ml of hexane in a 60-ml separatory funnel to remove undegraded malathion, malaoxon and most of any diethyl mercaptosuccinate which may have been formed. The aqueous phase was then acidified to pH 2.0 with 3 M HCl and stabilized with 2 ml of Sörenson's buffer (pH 2.0). The aqueous phase was extracted twice with 30-ml portions of chloroform to remove carboxylic acid derivatives of malathion, its *O*-desmethyl analog and diethyl malate. The hexane and chloroform phases were dried over anhydrous Na_2SO_4 and the aqueous phase was centrifuged to remove the remaining chloroform. After assaying the hexane, chloroform and aqueous phases for total radioactivity, the organic solvent phases were evaporated and the residues were dissolved in 1 ml of acetone for thin-layer chromatography.

10–20 μl of each unknown were spotted 15 mm from the bottom of commercially prepared silica gel sheets. Non-radioactive analytical standards of malathion, *O*-desmethyl malathion, and the mono- and dicarboxylic acid derivatives of malathion were individually cochromatographed with each of the unknowns. The plates were developed to a height of 15 cm with a solvent system consisting of a 85:15:1 (by vol.) ratio of acetonitrile, water and NH_4OH. After development, the chromatograms were thoroughly dried and exposed to bromine vapours for 30 sec, exposed to the air for 1 min, and sprayed lightly with a 0.5% solution of fluorescein in ethanol. Under shortwave ultraviolet light (2537 Å), malathion (R_F 0.95), *O*-desmethyl malathion (R_F 0.87), the malathion monoacid (R_F 0.57) and the dicarboxylic acid (R_F 0.22) appeared as quenched purple spots against the fluorescent yellow background. Sheets of Kodak Royal Blue X-ray film were exposed to the chromatograms for 24 to 48 h and developed to locate the radioactive spots which were then compared with the chromogenic non-radioactive standards located by the ultraviolet light.

The bromine-fluorescein technique was not suitable for detecting diethyl mercaptosuccinate, diethylmalate and malaoxon. Diethyl mercaptosuccinate (R_F 0.83) appeared as a pink spot after spraying with 1.5% sodium nitroprusside in methanol. Diethyl malate was located on thin-layer plates using the chromogenic method for quantitative determination of carboxyl esters as developed by HESTRIN[6]. The plates were first sprayed with the alkaline hydroxylaminer eagent (1 M hydroxylamine in 1.75 M NaOH) almost to the point of saturation. After 3 min the plates were sprayed lightly with 4 M HCl and then with 0.37 M $FeCl_3$ in 0.1 M HCl. Diethylmalate (R_F 0.90) appeared as a purple spot against a yellow background. The minimum detectable level was 10 μg. Malaoxon was neither sensitive to the bromine fluorescein chromogenic indicator nor separable from malathion by the above solvent system. To facilitate its isolation and detection, samples were chromatographed on

TABLE II

NATURE OF RADIOACTIVE COMPOUNDS CHARACTERIZED BY SOLVENT PARTITIONING AND THIN-LAYER CHROMATOGRAPHY AFTER INCUBATING [^{14}C]MALATHION AND [^{32}P]MALATHION WITH SOIL ENZYME FOR 6 H IN TRIS–HCl BUFFER (pH 6.8) AT 37°

Treatment	*Percent radioactivity detected*			
	Hexane phase	*Chloroform phase*		*Water solubles*
	Malathion	*Monoacid*	*Unidentified*	
Enzyme *plus* [^{32}P] malathion	19.5	74.0	2.0	4.5
Enzyme *plus* [^{14}C]malathion	21.3	73.5	2.7	2.5
[^{32}P]Malathion alone	97.5	0	0.3	2.2
[^{14}C]Malathion alone	98.5	0	0.9	0.6

silica gel with chloroform–acetone (9:1, by vol.) and detected by the chromogenic test of RAGAB[7]. The R_F-values for malathion, malaoxon and the malathion monoacid were 0.62, 0.50 and 0.05, respectively.

The percentages of radioactivity found in the hexane, chloroform and aqueous phases following the incubation of [^{32}P]malathion and [^{14}C]malathion with and without soil enzyme are shown in Table II. Approx. 20% of the radioactivity partitioned into the hexane phase from the water phase for each of the enzyme treatments. Thin layer chromatographic separation of these hexane residues revealed the presence of single radioactive spots with R_F values similar to that of malathion. Quantitative gas chromatographic assay confirmed that the radioactivity in the hexane phases was malathion. Similarly, all of the radioactivity which partitioned into the hexane phase of the controls was identified as malathion. Less than 3% of the malathion in the controls was degraded over the 6-h incubation period as compared to 80% degradation of the insecticide in the presence of the soil enzyme.

A major portion of the radioactivity from the malathion incubated with enzyme was extracted with chloroform from the acidified aqueous phase after the hexane partition. Thin-layer chromatography of the chloroform residues from the ^{14}C- and ^{32}P-labeled malathion treatments revealed the presence of one major spot for each. The R_F values were identical which suggested that the breakdown product contained phosphorous and the 2 and 3 carbon atoms of the succinyl group. On the basis of solvent partitioning properties and R_F values from thin-layer chromatography, the enzyme-catalyzed radioactive degradation product of malathion was identified as the monoacid.

Properties of the enzyme

Effect of pH, enzyme concentration and incubation time

Enzyme activity was determined in 0.05 M citrate buffer at pH 3.1, 3.8, 4.6, 5.4, 6.2 and 6.8, in 0.05 M phosphate buffer at pH 6.8 and 7.8, and in 0.075 M Tris–HCl buffer at pH 7.0, 7.8, 8.5 and 8.8. Two units of enzyme were incubated with 0.1 μmole of [^{32}P]malathion in 5 ml final volume for 2 h. Controls containing no enzyme were included at each pH level for the three buffers.

Maximum enzyme activity was obtained above pH 6.8 (Fig. 2). Quantitative enzyme activity above pH 7.8 could not be determined because the substrate was

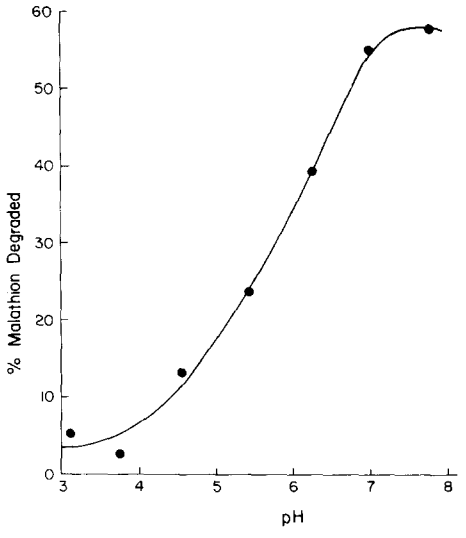


Fig. 2. Relative activity of malathion-degrading enzyme at various pH levels buffered with citrate (pH 3.1–6.8) phosphate (pH 6.8 and 7.8) and Tris·HCl (pH 7.0–7.8).

unstable under alkaline conditions. After the 2-h incubation period, hydrolysis of malathion in the controls was less than 3% below pH 7.5, and 8, 25, and 64% at pH 8.0, 8.5 and 8.8, respectively. The values presented in Fig. 2 are corrected for non-enzymatic degradation of malathion.

Although activity was negligible at low pH values, acidification as low as pH 3.0 did not denature the enzyme permanently since exposure of enzyme solutions for 24 h in buffer at various pH levels, followed by assays for activity at pH 7.0 did not result in loss of activity. However, the enzyme was irreversibly denatured below pH 3.0 and above pH 11.0. Enzyme solution exposed to HCl–KCl buffer at pH 2.0 lost 50% of its activity in 2 h and 80% in 24 h. The crude 0.2 M NaOH extract from soil retained most of its activity for several hours after extraction but following purification on Bio-Rex 70, the enzyme was completely denatured in 0.1 M NaOH within 8 h.

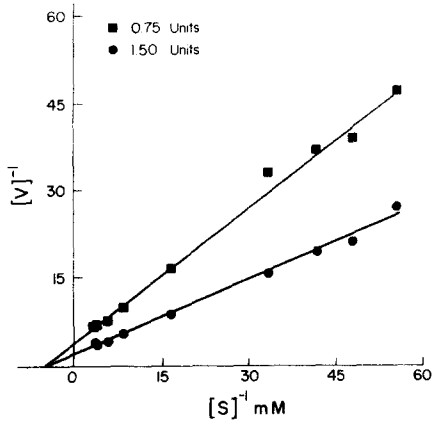
A direct relationship between enzyme concentration and the amount of malathion degraded was obtained with 1.5 μmoles of substrate and various amounts of enzyme ranging from 0.4 to 3.6 units in 5 ml of 0.075 M Tris·HCl buffer (pH 7.0). The rate of substrate degradation in relation to time was also linear up to 11 h when 0.5 unit of enzyme was incubated with 1.5 μmoles of malathion in 5 ml of buffer.

Effect of substrate concentration

Double reciprocal plots of initial velocities for various substrate levels with 0.75 and 1.5 units of enzyme are shown in Fig. 3. The data conformed to typical Michaelis–Menten kinetics and identical K_m values were obtained for both enzyme levels. The apparent K_m for malathion was $2.12 \cdot 10^{-4}$ M and v_{max} was 0.28 and 0.58 μmoles of malathion degraded per 4 h for 0.75 and 1.5 units of enzyme, respectively.

Thermal stability

Tubes containing two units of enzyme in 2 ml of water were subjected to 15-min



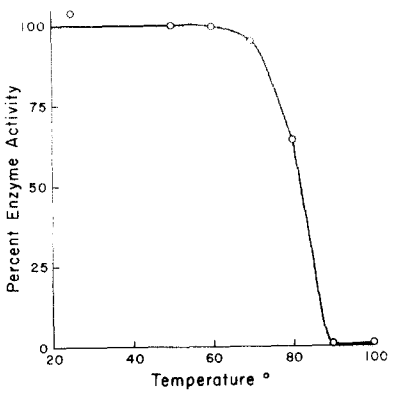


Fig. 3. Lineweaver–Burk plots for two concentrations of enzyme. The $K_m = 2.12 \cdot 10^{-4}$ M for both enzyme levels. The v_{max} was 0.28 and 0.58 moles per 4 h for the low and high enzyme levels, respectively.

Fig. 4. Thermal stability of malathion-degrading enzyme. Enzyme solutions were subjected to 15-min exposures at various temperatures and subsequently assayed for activity at 37°.

exposures at various temperatures, immediately chilled in an ice bath and subsequently assayed for activity in 5 ml total volume of 0.075 M Tris–HCl buffer (pH 7.0) at 37° (Fig. 4). The enzyme was stable following exposure at 60°, partially inactivated at 70° and completely destroyed at 90°. The temperature inactivation curve of the partially purified enzyme approximated the inactivation temperatures obtained for the enzyme in soil suspensions which were reported in an earlier paper[2].

Stability of the enzyme in soil

Soils were treated with partially purified enzyme preparation to determine its stability in a cell-free state. For this study 240-g soil equivalents of partially purified enzyme in 30 ml of deionized water were sprayed onto 150 g of Chehalis soil which had been steamed to destroy its original malathion-degrading activity. Prior to application of the enzyme, the soil was inoculated with 3 g of non-sterile soil to provide a source of microorganisms normally found in the Chehalis clay loam. A similar amount of enzyme was applied to a second soil (Felida fine silt) which, in its natural state, contained a small amount of the malathion-degrading enzyme. This soil was not autoclaved and contained an undisturbed microbial population. The treated soils were placed in pint jars and incubated at their moisture equivalent at 25°. Controls included autoclaved Chehalis loam *minus* enzyme and samples of both soils that were neither autoclaved nor supplied with enzyme. After 0, 1, 2, 4, and 8 weeks, 5-g samples were extracted with alkali and assayed for enzyme activity. Zero-day soil samples were also extracted with Tris–HCl buffer (pH 7.0) to determine if the added enzyme could be extracted more easily than that which occurred naturally.

Cell-free enzyme was stable in both soils for the duration of the experiment (Table III). The initial recoveries were equal to 65 and 50% for the Chehalis clay loam and Felida silt, respectively, and active fractions were recovered from both soils for the duration of the experiment (8 weeks). No activity was detected in alkali

TABLE III

RECOVERY OF MALATHION ESTERASE FROM TWO SOILS TREATED WITH PARTIALLY PURIFIED ENZYME SOLUTIONS

Weeks after treatment	*Units of enzyme recovered per 2 cm³ of soil**			
	Autoclaved Chehalis loam plus enzyme	*Autoclaved Chehalis loam without enzyme*	*Felida silt plus enzyme*	*Felida silt without enzyme*
0	3.00	0.05	2.83	0.76
1	3.18	0.02	3.03	0.86
2	2.98	0.03	2.93	0.83
4	2.81	0.04	2.82	0.75
8	2.88	0.05	2.75	0.81

* Enzyme activity determined from amount of malathion hydrolyzed per 2 cm³ soil equivalent of 0.2 M NaOH extract after incubation of soil extract with 0.3 mM malathion in Tris–HCl buffer (pH 7.0) for 6 h at 37°. One unit equals the amount of enzyme necessary to hydrolyze 0.2 μmoles of malathion in 4 h.

extracts of autoclaved Chehalis soil which was not treated with enzyme. The Felida silt control contained some malathion degrading factor because the soil was not autoclaved. Differences in the level of activity between the treated and control soil are from the addition of the partially purified cell-free material. Poor initial recovery from both soils was due to the destructive effect of the alkali extractant upon the enzyme and incomplete recovery of the enzyme from soil. Because similar levels of activity were detected throughout the 8-week period, it is unlikely the enzyme was destroyed in soil immediately following its application. The stability of the enzyme in Felida Silt loam indicates it is not easily destroyed by soil microorganisms normally present in that soil. Failure to recover the activity from treated soils by extraction with Tris–HCl buffer (pH 7.0) indicates the enzyme was quickly adsorbed onto soil.

DISCUSSION

These studies offer substantial evidence that Chehalis clay loam contains a stable, heat-labile enzyme which catalyses the hydrolysis of malathion to its monoacid. The ester hydrolysis of malathion in the presence of partially purified soil substance exhibited typical Michaelis–Menten kinetics.

Although the kinetic data would apply to non-enzyme-catalyzed reactions as well as enzyme-catalyzed reactions, there is sufficient evidence to attribute the malathion-degrading activity to an organic entity. The substance was extracted from soil by a method commonly used for removing organic matter and it was partially purified by procedures employed for isolating proteins. The active substance was heat-labile, non-dialysable and susceptible to denaturation in weak acid and alkali.

Enzymes of various kinds are continually being released into soil through physiological activities of living organisms and through the release of protoplasm which accompanies cellular disruption. Although most of these enzymes are rapidly denatured or quickly metabolized by other living organisms, it has been speculated that stable enzymes may exist in an active state for prolonged periods[8]. The malathion

esterase isolated from Chehalis clay loam is unusually stable and possesses many of the characteristics necessary for prolonged activity in a cell-free state in soil. It is less heat-labile than most enzymes, loses little or no activity upon prolonged storage and survives desiccation in soils.

Soluble fractions with catalytic activity have been extracted from soils[9,10] but the removal of cell-free enzymes is usually a difficult task because they are generally tightly adsorbed or complexed in physical or chemical associations with soil constituents. Drastic treatments are required to remove soil-bound enzymes and consequently, they do not often retain their catalytic activity when attempts are made to extract them. Perhaps even more difficult than demonstrating the presence of cell-free soil enzymes is the determination of their stability in soil. More than likely, most cell-free enzyme activity is temporal and dependent upon the existence of viable cells.

There is evidence that, under natural conditions, the malathion esterase exists as a stable cell-free entity in soil for extended periods. First, to remove the malathion esterase from soil the nonsoluble residues must be separated from the alkaline extract prior to neutralization with HCl; otherwise, the active fraction is adsorbed out of solution and can only be recovered by the addition of more alkali. If the sole purpose of the alkali extraction was to free the enzyme from intact cellular materials, it is unlikely the active fraction would respond to subsequent pH adjustment in the manner that it does. Additional support for this thesis is based upon the experiment which demonstrated that partially purified enzyme was highly stable in non-autoclaved Felida silt loam and in autoclaved Chehalis clay loam subsequently inoculated with non-autoclaved Chehalis soil. Certainly the enzyme is not easily destroyed by soil microorganisms or denatured by other components of these soils. Following its application to soil, the partially purified enzyme is adsorbed rapidly and cannot be extracted by neutral buffer. The necessity of using an alkaline solvent to extract the cell-free enzyme, suggests it may be bound in a condition similar to that which exists in the natural state. Soil clays adsorb proteins[11–13] and it is probable the malathion esterase exists in an adsorbed complex with clays.

The origin of the enzyme is unknown. Most likely it originates from microorganisms or higher plants both of which contain carboxylesterases capable of degrading malathion to its monoacid[14,15]. MATSUMURA AND BOUSH[14] recovered carboxylesterase products of malathion from several soils treated with the insecticide and subsequently isolated a fraction with carboxylesterase activity from cultures of *Trichoderma viride*, a common soil fungus with high capabilities for degrading malathion. Their carboxylesterase differs from the enzyme isolated in the present studies in that the activity of the fungus-derived enzyme was blocked by $1 \cdot 10^{-6}$ M diisopropyl fluorophosphate (DFP). We were not able to inhibit the action of the esterase from Chehalis clay loam with 10^{-5} M DFP (L. W. GETZIN AND I. ROSEFIELD, unpublished data).

Alkali-extractable, heat-labile substances capable of accelerating the degradation of malathion are present in many soils of western Washington. During the course of these investigations soils from 15 locations in western Washington and 8 locations in eastern Washington have been extracted with 0.2 M NaOH. Heat-labile substances capable of degrading malathion were found in extracts of 9 soils west of the Cascade mountains and none of the soils located east of the mountains. Activity

was present in both cultivated and non-cultivated soils, including forest soils. Presumably, these active components are similar or identical to the enzyme in Chehalis clay loam, but it was not established that they are catalysts or that they degrade malathion to the monoacid.

The malathion esterase is of interest because it represents a heretofore unknown mechanism for the degradation of pesticides in soil. In addition, this stable esterase could serve as a useful tool for investigations on the behavior of extracellular enzymes in soil. We are continuing with the purification and characterization of this enzyme because of its unusual properties.

ACKNOWLEDGEMENTS

Scientific Paper 3611, College of Agriculture, Washington State University, Pullman. Investigation supported in part by Public Health Service Research Grant FD 00247, from the National Institutes of Health, Bethesda, Md.. Appreciation is extended to R. B. Warwick, G. W. Beckendorf and Clara Bednorz for technical assistance, H. S. Cheng for total nitrogen analyses, William Miller for strip electrophoresis and T. Satyanarayana for review of manuscript.

REFERENCES

1 J. J. Skujins, in A. D. McLaren and G. H. Peterson, *Soil Biochemistry*, Marcel Dekker, New York, N.Y., 1967, Chapter 15.
2 L. W. Getzin and I. Rosefield, *J. Agr. Food Chem.*, 16 (1968) 598.
3 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
4 Z. Dische, *Mikrochemie*, 7 (1929) 33.
5 W. S. Bigley and F. W. Plapp, *J. Insect Physiol.*, 8 (1962) 545.
6 S. Hestrin, *J. Biol. Chem.*, 180 (1949) 249.
7 M. T. H. Ragab, *Bull. Environ. Contamin. Toxicol.*, 2 (1967) 279.
8 J. J. Skujins and A. D. McLaren, *Enzymologia*, 34 (1968) 213.
9 M. H. Briggs and L. Segal, *Life Sci.*, 2 (1963) 69.
10 M. Martin-Smith, *Nature*, 197 (1963) 361.
11 L. E. Ensminger and J. E. Gieseking, *Soil Sci.*, 53 (1942) 205.
12 A. D. McLaren, *J. Phys. Chem.*, 58 (1954) 129.
13 A. D. McLaren, *Soil Sci. Soc. Am. Proc.*, 18 (1954) 170.
14 F. Matsumura and G. M. Boush, *Science*, 153 (1966) 1278.