Properties of a Stable Cell-Free Esterase from Soil†

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ABSTRACT: The properties of a stable, extracellular phenyl esterase which hydrolyzes the organophosphorus insecticide, malathion, to its monoacid were investigated following its isolation from soil by 0.2 N alkali extraction and 560-fold purification by $MnCl_2$ treatment, protamine sulfate treatment, and QAE Sephadex A-50 chromatography. Phosphonate and phenyl thiophosphate anticholinesterase insecticides were potent competitive inhibitors of soil esterase activity. Inhibition was also observed with mono- and dithiols, but not with diisopropyl fluorophosphate or sulfhydrol compounds. The esterase was not susceptible to enzymatic proteolysis nor easily inactivated by metal ions. The characteristic ultraviolet 280-nm absorption peak for proteins was not present unless the enzyme was first hydrolyzed in 6 N HCl or digested with testicular hyaluronidase. Hyaluronidase digestion increased the esterase activity almost twofold, but it also decreased the stability of the enzyme. On the basis of its chemical composition and response to hyaluronidase treatment the enzyme is classified as a glycoenzyme. The proposed carbohydrate–protein complex may account for the enzyme's unusual stability and persistence in soil as an extracellular entity.

A heat-labile component has been isolated from soil which degrades the insecticide, malathion (diethyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate), to its monoacid, and evidence was presented that the substance is a stable, cell-free enzyme (Getzin and Rosefield, 1968, 1971). It was partially purified by procedures employed for isolating proteins and exhibited typical Michaelis–Menten kinetics. It was electrophoretic, filterable, heat-labile, nondialyzable, and susceptible to denaturation by acid and alkali. Its existence as a stable, cell-free enzyme was postulated from evidence based upon the persistence and adsorptive characteristics of the partially purified material in soil.

Soil enzymes must possess certain characteristics to ensure their existence in the cell-free state for extended periods. The malathion esterase has some of these properties (Getzin and Rosefield, 1971). It is not easily destroyed by heat, resists microbial attack, loses little or no activity upon prolonged storage, and is resistant to desiccation in soil. Perhaps most significant, it withstands the comparatively drastic alkali treatment necessary for freeing it from soil and thus enables one to examine its properties *in vitro*. The present investigations were designed to further purify and characterize the enzyme and to examine some of the properties that contribute to its unusual stability in soil.

Experimental Section

Materials. Chehalis clay loam (pH 5.5–6.0) was collected from a cultivated area in western Washington and used as a source of the enzyme. It contained 8% organic matter and 30% clay. [^{14}C]Malathion (14.0 μCi/mg), labeled at the 2 and 3 positions of the succinyl moiety, was purchased from Amersham/Searle Corp., Chicago, Ill. Nonradioactive malathion (98.5% purity) and malaoxon were obtained from American Cyanamid Co., Princeton, N. J. Other organophosphorus insecticides used in the study were obtained from City Chemical Corp., New York, N. Y., or from their representative manufacturers.

QAE Sephadex A-50, protamine sulfate (grade I), hyaluronidase (type I), and most of the proteases, enzyme inhibitors, and other organic chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., or Eastman Chemical Co., Rochester, N. Y. Metal salts and other normal laboratory chemicals (reagent grade) were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J., or Mallinckrodt Chemical Works, St. Louis, Mo.

Analytical Methods. Malathion concentrations were determined by radioassay with a Nuclear-Chicago Model D-47 gas flow detector in combination with a Model C-110B automatic sample changer and Model 181B scaler. Combinations of nonradioactive and [^{14}C]malathion were incubated with 5 ml of buffered enzyme solutions in 25-ml screw cap culture tubes and subsequently extracted in 7 ml of hexane (Skellysolve B). One-milliliter aliquots of the hexane phase were pipetted into planchets and the radioactivity in the residue was counted after the solvent evaporated.

Cellulose acetate electrophoresis was conducted on Sepraphore III 5.5 × 12.5 cm strips in a Gelman Sepra Tek chamber for 30 min. Ionic strengths of desired buffers were adjusted to obtain current flows of 6–16 mA with a stabilized potential of 250 V. After electrophoresis, the strips were sectioned longitudinally. One longitudinal portion was cut into 0.5-cm sections and each piece was placed in 5 ml of buffer and assayed to locate the enzyme. The remaining strips were immediately fixed in 5% trichloroacetic acid and stained with Coomassie Blue, nigrosin, periodic acid Shiffs reagent, or Amido Black.

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Enzyme Assay. The amount of nonhydrolyzed malathion remaining after desired incubation periods was measured to determine soil esterase activity. The assay mixture contained either 0.3 or 1.5 μmol of malathion in 5 ml of 0.075 M Tris-HCl buffer (pH 7.0). Samples, including appropriate controls, were incubated for 4–6 hr in 20 mm × 125 mm screw cap culture tubes in a water bath at 37°, and the intact malathion

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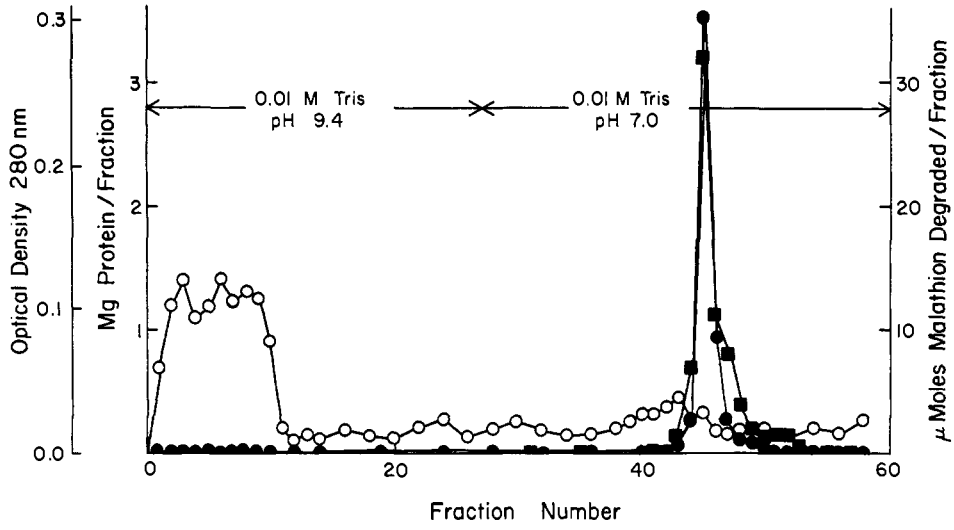

FIGURE 1: Chromatography of malathion esterase on QAE Sephadex A-50. Seventy milliliters of dialyzed protamine sulfate supernatant in 0.01 M Tris-HCl buffer (pH 9.4) was applied to the column (2.5 × 35 cm) and washed with 250 ml of the buffer. The enzyme was eluted with 0.01 M Tris-HCl buffer (pH 7.0). Ten-milliliter fractions were collected at a flow rate of 1 ml/min. Fractions were assayed for enzyme activity (●) and protein content by the Lowry method (○) and by spectrophotometric absorption at 280 nm after acid hydrolysis (■).

was determined quantitatively by the procedure described above. One enzyme unit is defined as the amount of esterase required to catalyze the degradation of 0.2 μmol of malathion in 4 hr at pH 7.0 and 37°.

Results

Purification of the Enzyme. All operations were performed at room temperature. The initial steps involving 0.2 N NaOH extraction of soil, $MnCl_2$ treatment, and $(NH_4)_2SO_4$ precipitation were identical with those reported previously (Getzin and Rosefield, 1971) except that Bio-Rex 70 column chromatography was omitted because it provided poor yields of enzyme with little increase in specific activity. Instead the enzyme was further purified by the following procedure.

Protamine Sulfate Treatment. Seven milliliters of 4% protamine sulfate solution were added to each 63 ml of dialyzed ammonium sulfate fraction from the previous purification step. After 5 min of stirring, the suspension was centrifuged and the precipitate was discarded. The clear supernatant was dialyzed against running tap water in a cold room for 12–15 hr. A slight precipitate which formed during dialysis was centrifuged and discarded.

QAE Sephadex A-50 Chromatography. The clear supernatant from the previous step was adjusted to pH 9.4 in 0.01 M Tris-HCl buffer and transferred to a 2.5 × 35 cm column of QAE Sephadex A-50 equilibrated with 0.01 M Tris-HCl buffer (pH 9.4). The column was washed with 200 ml of the same buffer. After washing, the buffer was changed to 0.01 M Tris-HCl buffer (pH 7.0) to elute the enzyme. Fractions of 10 ml were collected at a flow rate of 10–15 ml/15 min. The elution profile is shown in Figure 1. Lowry protein in the active fractions was barely detectable. However, when the fractions were treated with 6 N HCl in a boiling water bath for 5 min 280-nm spectrophotometric responses were obtained which parallelled enzyme activity. The active fractions from several runs were pooled and stored at 4° for later use without further purification.

The results of a typical purification procedure for 240 g of soil are given in Table I. The enzyme was purified 560-fold with 23% recovery of the overall activity.

General Characteristics of the Enzyme Preparation. The enzyme preparation did not show the characteristic ultraviolet peak at 280 nm unless the enzyme was first treated with 6 N HCl in a boiling water bath for 5 min. Amino acid analysis of 0.44 mg of QAE Sephadex A-50 purified material yielded significant amounts (0.15–0.30 μmol) of aspartic acid, threonine, serine, glycine, alanine, valine, tyrosine, histidine, and arginine, intermediate amounts (0.05–0.15 μmol) of glutamic acid, proline, isoleucine, leucine, and histidine, and trace amounts of methionine, phenylalanine, and lysine. No analyses were made for cysteine and tryptophan. The QAE Sephadex A-50 purified enzyme gave a positive reaction for reducing sugars with the method of Dische (1929), a positive reaction for acetylated and amino sugars as determined by Dische and Borenfreund (1950), and a negative reaction for uronic acid by the carbazole method (Dische, 1947). The positive responses were too low for quantitation.

Cellulose acetate strip electrophoresis and subsequent staining procedures revealed that the QAE Sephadex A-50 preparation was impure. Portions of the electrophoresis strips containing the activity did not stain with Coomassie Blue, nigrosin, Amido Black, or periodic acid Schiffs reagent. How-

TABLE I: Purification Procedure Summary for Malathion Esterase Extracted with 0.2 N NaOH from 240 g of Chehalis Clay Loam.

Fraction	Total Lowry Protein (mg)	Total Activity[a]	Sp Act.	% Yield
Soil extract	615.00	167	0.27	100
$MnCl_2$ treatment	54.50	123	2.26	74
$(NH_4)_2SO_4$ precipitate	25.90	110	4.24	66
Protamine sulfate	7.30	91	12.45	54
QAE Sephadex A-50	0.25	38	152.00	23

[a] Micromoles of malathion degraded in 4 hr.

TABLE II: Comparative Hydrolysis of Carboxy Esters by Malathion Esterase.[a]

Substrate[b]	% Hydrolysis
Phenyl acetate	99
Phenyl propionate	72
Malathion	46
p-Nitrophenyl acetate	40
p-Nitrophenyl butyrate	30
Phenyl isobutyrate	10

[a] Reaction mixture containing 10 μmol of substrate and 2 units of enzyme in 5 ml of 0.075 M Tris-HCl buffer (pH 7.0) with 0.01% Triton X-100 was incubated for 16 hr at 37°. [b] Less than 5% hydrolysis was observed with diethyl malate, tributyrin, methyl butyrate, ethyl acetate, diethyl succinate, diethyl mercaptosuccinate, and triacetin.

ever, these reagents produced a lightly stained area which generally trailed the active band.

Properties of Malathion Esterase. The reaction kinetics of the QAE Sephadex A-50 purified preparation were similar to those previously reported for the Bio-Rex 70-purified material (Getzin and Rosefield, 1971). The enzyme exhibited optimum activity around pH 7.5 in 0.075 M Tris-HCl buffer and 0.05 M sodium phosphate buffer. Enzyme activity was proportional to enzyme concentration and a linear relationship between activity and time was obtained. The apparent K_m for malathion with this preparation was 6.06×10^{-5} M as compared to a K_m of 2.12×10^{-4} M with the Bio-Rex 70-purified material in the previous study.

Substrate Specificity. The relative activity of the soil esterase against a number of possible substrates was measured by the Hestrin procedure for quantitative determination of carboxyl esters (Hestrin, 1949). Comparative determinations for malathion were obtained by radioassay. A surfactant (Triton X-100) was included with the incubation mixture to emulsify insoluble substrates. Controls contained substrate without enzyme.

Phenyl acetate was almost completely hydrolyzed within the 16-hr incubation period (Table II). In addition to malathion, the enzyme was active against phenyl propionate, *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, and phenyl isobutyrate, but it did not catalyze the hydrolysis of aliphatic esters. In a separate experiment the soil esterase did not hydrolyze the glycerides of olive oil while a purified control lipase gave a positive reaction as determined by pH titration.

Organophosphorus Insecticide Inhibitors. Soil esterase partially purified by Bio-Rex 70 column chromatography (Getzin and Rosefield, 1971) was used for these inhibitor studies. Serial dilutions of selected organophosphorus insecticides were incubated with 2 units of enzyme and 1.5 μmol of malathion for 6 hr. The amount of malathion remaining was determined and the I_{50} calculated from activity *vs.* inhibitor concentration curves.

A number of organophosphorus insecticides inhibited the enzyme (Table III). Phosphonates (dichlovos, crotoxyphos, mevinphos, and *O*-Zinophos) and phenyl thiophosphates (parathion, V-C 13, EPN, and ronnel) were the most effective inhibitors. In addition, malaoxon was highly inhibitory. Gas chromatographic analyses indicated that none of these compounds was hydrolyzed by the esterase.

TABLE III: Inhibitory Effect of Some Organophosphorus Insecticides on Activity of Soil Esterase against Malathion.

Compound	I_{50}[a]
Malaoxon (diethyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorothioate)	<0.1
Dichlorvos (2,2-dichlorovinyl dimethyl phosphate)	<0.1
Parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate)	<0.1
Crotoxyphos (α-methylbenzyl 3-hydroxy-crotonate dimethyl phosphate)	0.1
V-C 13 (*O*-2,4-dichlorophenyl *O,O*-diethyl phosphorothioate)	0.1
EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphorothioate)	0.3
Mevinphos (methyl 3-hydroxy-α-crotonate, dimethyl phosphate)	0.3
Ronnel (*O,O*-dimethyl *O*-2,4,5-trichlorophenyl phosphorothioate)	0.5
O-Zinophos (*O,O*-diethyl *O*-2-pyrazinyl phosphate)	0.8
Diazoxon (*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphate)	7.4
Dicrotophos (3-hydroxy-*N,N*-dimethyl-*cis*-crotonamide, dimethyl phosphate)	16.3
Diazinon (*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate)	16.5
Zinophos (*O,O*-diethyl *O*-2-pyrazinyl phosphorothioate)	20.0

[a] Micromoles of compound necessary for 50% inhibition of enzyme with 1.5 μmol of malathion.

Double reciprocal plots of initial velocities for serial substrate levels of malathion with two concentrations of malaoxon and representative phosphonate (mevinphos) and phenyl thiophosphate (parathion) inhibitors showed that the compounds are competitive inhibitors. Graphical presentation of the data for one of the inhibitors is shown in Figure 2. The K_i values were 1.96×10^{-5} M, 1.61×10^{-5} M, and 1.66×10^{-5} M for malaoxon, mevinphos, and parathion, respectively.

Effect of Inhibitors. Enzyme activity was determined in the presence of 12 mM concentrations of metal ions following dialysis of purified enzyme against 10 μM EDTA for 24 hr and distilled H_2O for 24 hr. Among these, Ag^+, Hg^{2+}, Pb^{2+}, and Cu^{2+} showed inhibitions of 58, 53, 27, and 24%, respectively. The Hg^{2+} also catalyzed the hydrolysis of malathion which interfered with inhibition estimations. None of the other ions affected substrate stability or enzyme activity to a significant extent. Likewise, the chelating agents EDTA, 8-hydroxyquinoline, 1,10-phenatroline, and α,α′-dipyridyl, at 10 mM, had no affect upon enzyme activity.

The enzyme was not inhibited by any of the sulfhydryl reagents listed in Table IV. Diisopropyl fluorophosphate, a potent inhibitor of most esterases at 10^{-5} and 10^{-6} molar concentrations, had little effect upon the malathion esterase at 8 mM. The enzyme was inhibited by sodium dodecyl sulfate, Na_2SO_3, sodium arsenite, monothiols, and dithiols. Substrate inhibition ranged from 26 to 78% when concentrations of β-mercaptoethanol were varied from 8 to 36 mM. Levels of Na_2SO_3 from 6 to 36 mM gave 31–83% inhibition of the ester-

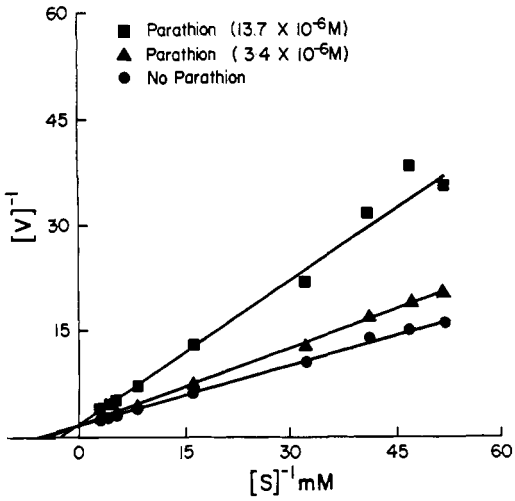

FIGURE 2: Kinetics of soil esterase inhibition by organophosphorus insecticides. Double reciprocal plots of malathion concentrations and velocity in presence and absence of parathion.

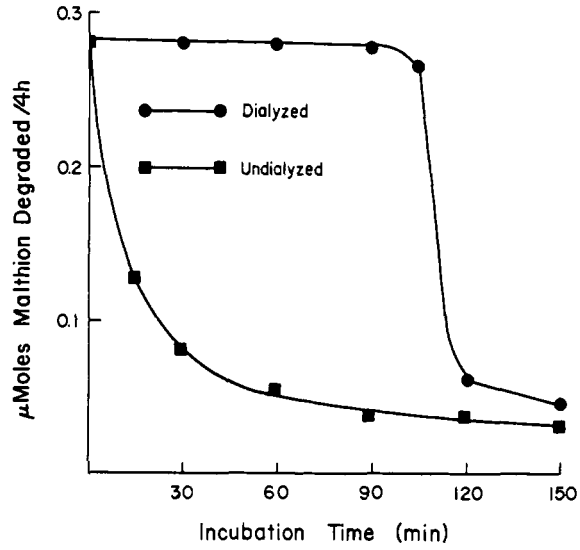

FIGURE 3: Effect of dithiothreitol on malathion esterase activity. Esterase was incubated with dithiothreitol in 0.075 M Tris-HCl buffer (pH 7.0). At the times indicated, an aliquot was immediately assayed for enzyme activity. A second aliquot was dialyzed against 1 mM Na_2CO_3 buffer (pH 10.0) for 16 hr followed by dialysis against 1 mM Tris-HCl buffer (pH 7.0) for 2 hr and then assayed for activity.

ase. The data from detailed examination of esterase behavior in the presence of dithiothreitol and reduced lipoic acid are given in Figures 3 and 4.

Thermal Stability of Malathion Esterase. The enzyme was inactivated in a stepwise manner when buffered solutions (pH 7.0) were subjected to 15-min exposures at various temperatures (Figure 5). The enzyme was stable to 60°, partially inactivated between 60 and 75°, and rapidly destroyed by exposures above 80°.

Effect of Proteases and Other Enzymes. The malathion esterase was not destroyed when enzyme solutions were exposed to varying concentrations (up to 1-mg quantities) of pectinase, trypsin, chymotrypsin, papain, bromelain, Pronase, and thermolysin for 12–24 hr under optimum conditions for the respective proteases.

Since previous data suggested the possibility of a protein–carbohydrate linkage, the malathion esterase was incubated with several carbohydrate splitting enzymes (hyaluronidase, snail gut enzyme, β-glucosidase, chitinase, β-galactosidase, lysozyme, collagenase, neuramindase, heparinase, and β-glucuronidase) and subsequently assayed for activity. Among these only testicular hyaluronidase, which is known to split *N*-acetylhexosamine bonds, had an effect on esterase activity. When an aliquot of the esterase was incubated with 20 mg of testicular hyaluronidase (340 NSF units/mg) for 18 hr under toluene in 0.075 M Tris-HCl buffer (pH 7.0) and subsequently assayed for activity, the hyaluronidase-treated enzyme was 1.7 times more active than the untreated enzyme. Similar results were observed with highly purified hyaluronidase (3000 NSF units/mg). Hyaluronidase alone did not affect the hydrolysis rate of the substrate. In addition to possessing more activity the hyaluronidase-digested enzyme mimicked

TABLE IV: Effect of Inhibitors on Enzyme Activity.

Inhibitor[a]	μmol of Malathion Degraded/4 hr	% Inhibition
Control	0.23	
Diisopropyl fluorophosphate	0.19	17
Sodium arsenite	0.18	22
Sodium dodecyl sulfate (5 mM)	0.05	78
β-Mercaptoethanol	0.17	26
Sodium sulfite (6 mM)	0.16	31
2,3-Dimercaptopropanol	0.06	75
Dithiothreitol	0	100

[a] All inhibitor concentrations were 8 mM except when noted otherwise. The inhibitors were preincubated with the esterase for 5 min at 37° prior to addition of the substrate. Less than 10% inhibition was observed with *p*-hydroxymercuribenzoate, iodoacetate, iodoacetamide, thiomalic acid, *N*-ethylmaleimide, cysteine, and methylmercury chloride.

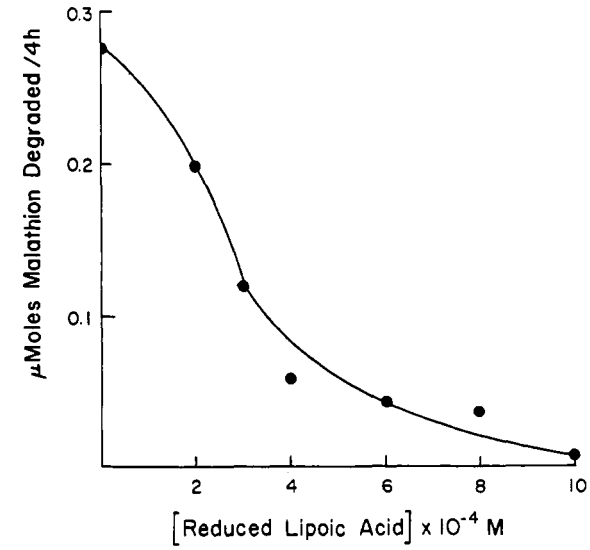

FIGURE 4: Effect of reduced lipoic acid on malathion esterase activity. The inhibitor was preincubated with the enzyme for 10 min at 37° prior to assay for activity.

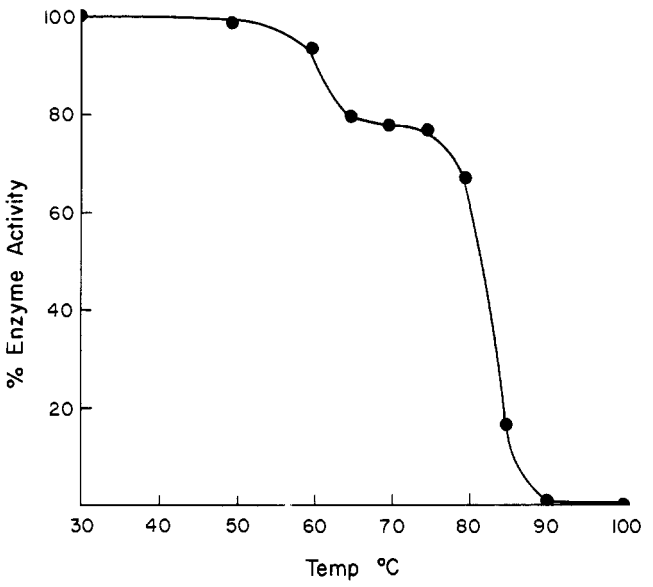

FIGURE 5: Thermal stability of QAE Sephadex A-50 purified malation esterase. One unit of enzyme in 2 ml of 0.075 M Tris-HCl buffer (pH 7.0) was subjected to 15-min exposures at various temperatures, immediately cooled to room temperature, and then assayed for activity at 37°.

the spectrophotometric characteristics of the 280-nm peak previously observed with the acid treatment (Figure 6) and exhibited less pH stability than the nondigested enzyme (Table V). Attempts to separate the esterase from the hyaluronidase digest with alumina, calcium phosphate gel, and hydroxylapatite chromatography were unsuccessful. Also, the hyaluronidase-digested esterase was not susceptible to proteolysis by Pronase.

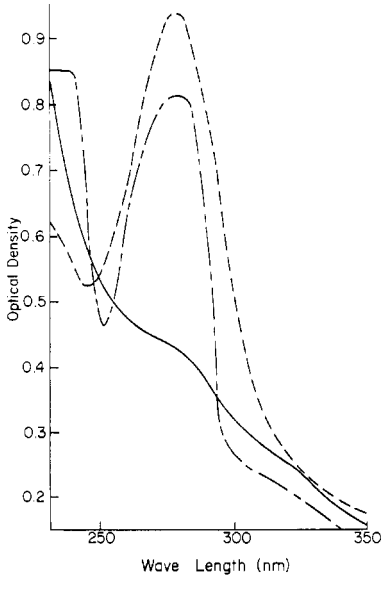

FIGURE 6: Ultraviolet spectra of nontreated enzyme (—), acid-hydrolyzed enzyme (- - -) and hyaluronidase-treated enzyme (— · —). For acid hydrolysis 1 ml of concentrated HCl was added to 1 ml of enzyme, heated for 15 min in a boiling water bath and diluted to 3 ml for reading. For hyaluronidase digestion 3 ml of enzyme was incubated with 5 mg of testicular hyaluronidase (340 NSF units/mg) for 2.5 hr in 0.075 Tris-HCl buffer (pH 7.0). The control contained hyaluronidase without enzyme. Data were obtained with a Hitachi Perkin-Elmer Model 124 double beam spectrophotometer.

TABLE V: Comparative pH Stability of Nontreated and Hyaluronidase-Digested Soil Esterase.[a]

pH	μmol of Malathion Degraded/4 hr	
	Nontreated	Hyaluronidase-Treated
3.0	0.12	0
4.0	0.13	0.18
7.0	0.13	0.23
9.1	0.11	0.14
9.6	0.12	0.11
10.1	0.12	0.09

[a] Nontreated and hyaluronidase-digested esterase (0.5 unit) was exposed to buffers at the above pH levels for 15 min at 37° and subsequently assayed for activity at pH 7.0.

Discussion

The isolation of this stable, cell-free enzyme from soil is significant because (1) it identifies a mechanism by which a pesticide may be degraded in soil in addition to nonbiological chemical processes and microbial metabolism, and (2) it demonstrates that certain biological reactions in soil are catalyzed by stable, cell-free enzymes. A number of enzymes have been extracted from soil but it is difficult to determine whether they existed in a nonephemeral cell-free state or were simply isolated from living microbes during the extraction process (Skujins, 1967).

The malathion esterase has all of the properties that an enzyme must possess for prolonged cell-free existence in soil where an organic entity is subject to inestimatable interplay of biological, chemical, and physical factors. The enzyme has a relatively high temperature threshold, requires no demonstratable cofactors, and is not easily inactivated by metal ions or inhibited by many common enzyme inhibitors. It tolerates extreme variations in pH and, although optimumly active around pH 7.0, it catalyzes reactions at pH 5.0 and below. Furthermore, it resists enzymatic proteolysis. Proteins are subject to microbial attack and, consequently, it is unlikely that an enzyme could exist in the cell-free state for extended periods unless it was protected from proteolysis. Burns *et al.* (1972) isolated a stable urease from soil which also resists enzymatic proteolysis, and they postulated the urease is associated with an organomineral complex which acts as a shield against proteolytic enzymes.

On the basis of substrate specificity the enzyme which hydrolyzes malathion should be classified as an aromatic esterase because it hydrolyzed phenyl esters but had little or no effect upon aliphatic esters. Earlier, Haig (1955) suggested that soils contain cell-free esterases when he demonstrated the catalytic hydrolysis of phenyl acetate in nonautoclaved Yolo fine sandy loam which had been stored air-dry for over 15 years. There are striking similarities between the intact activity of the Yolo loam described by Haig and the esterase isolated from the Chehalis clay loam with regard to substrate specificity, thermal inactivation, pH optima, and metal ion sensitivity. However, we were not able to demonstrate malathion-degrading activity in 0.2 N NaOH extracts of fresh Yolo fine sandy loam (provided through the courtesy of A. D. McLaren, University of California).

A number of enzymes, primarily aliesterases, have been

isolated from insects and mammals, which like the soil esterase, hydrolyze malathion to its monoacid and are inhibited by malaoxon, parathion, EPN, and related organophosphorus insecticides (Yip and Cook, 1959; Main and Braid, 1962). However, the substrate specificity of these aliesterases differs from that of the soil enzyme which degrades malathion. Animal aliesterases readily hydrolyze aliphatic esters and triesters such as tributyrin and triacetin; the soil esterase only hydrolyzed aromatic esters in these tests. Additional enzymes which degrade aromatic esters have been isolated from various animal systems, but again they differ from the soil esterase on the basis of substrate specificity, inhibitor action and other properties (Main, 1960; Aldrich, 1953a,b, 1954; Main *et al.*, 1961).

Most of the esterases studied which degrade malathion to its monoacid have originated from animals. The soil esterase is probably derived from microorganisms or higher plants but little information is available on the properties of plant esterases which hydrolyze malathion. A carboxylesterase which degrades malathion has been isolated from the common soil organism *Tricoderma viride*, but unlike the soil esterase, the enzyme from *T. viride* is inhibited by diisopropyl fluorophosphate (Matsumura and Boush, 1966). We obtained soil samples similar to those used by Matsumura and Boush and from these obtained an alkali-extractable, heat-labile fraction which degrades malathion. However, no attempt was made to establish if the substance was identical with the soil esterase or even that it degraded malathion to its monoacid. We have been unsuccessful in our attempts to determine the origin of the soil esterase which degrades malathion.

The malathion esterase was unusually stable in the presence of metal ions and most of the common enzyme inhibitors. The heavy metals, Ag, Hg, and Pb, caused some inhibition at high concentrations. The activity of the soil esterase in the presence of diisopropyl fluorophosphate is especially surprising in contrast to its effect on most other esterases. Monothiol and dithio compounds exhibited varying degrees of inhibition. Among these, dithiothreitol, 2,3-dimercaptopropanol, reduced lipoic acid, sodium sulfite, and β-mercaptoethanol were potent inhibitors. The enzyme activity was also inhibited by sodium dodecyl sulfate which is a well known denaturant for several oligomeric proteins (Weber and Kulter, 1971). However, the sodium dodecyl sulfate inhibition was readily reversible by simple dialysis at alkaline pH (9.4) contrary to the reports where it was not possible to reverse the sodium dodecyl sulfate inhibition or denaturation of several proteins by simple dialysis.

The effect of dithiothreitol and other dithiols has been considered from three points of view. One point of view is that dithiothreitol and other dithiols could inhibit the reaction by saturating the double bond between carbohydrate and protein linkages created by alkali extraction of the enzyme from soil. It is known that in several glycoproteins the amino acid serine is linked to the carbohydrate chain which when treated with alkali is broken and the serine residue is transformed to dehydroalanine (Hunt, 1970). This explanation is not likely for the malathion esterase because alkali extracts of the soil were as active as soil suspensions. Secondly, the possibility that the dithiols inhibit enzyme activity by acting as chelating agents is ruled out because none of the chelating agents showed any effect even at 10^{-3} M in the assay mixture. Finally, the dithiols could act by rupturing S–S bonds in the protein. This is the most logical explanation for the inhibitory action of dithiol compounds because of the following reasons. The inhibition by dithiothreitol was reversible only during the 90-min preincubation period. However, after 120 min the inhibition was irreversible. If the enzyme had many S–S bonds, disruption of a few of them might not affect the activity of the enzyme while the disruption of a crucial one could irreversibly inactivate the enzyme. The diphasic thermal denaturation curve (Figure 5) and the sigmoidal lipoic acid inhibition curve (Figure 4) might also suggest varying stability based upon the numbers of intact S–S bonds. A less attractive explanation, that two enzymes are present, is unlikely in view of the data obtained from kinetic and other inhibitor experiments.

Several lines of indirect evidence suggest the malathion esterase is a glycoprotein. First, amino acids only constituted 65% of the sample identified by the amino acid analyzer and QAE Sephadex A-50 purified enzyme gave positive reactions for carbohydrate residues. The action of hyaluronidase upon enzyme activity and spectrophotometric response provides the best evidence as to the nature of the esterase structure. That hyaluronidase acts directly upon the enzyme molecule is shown by its enhanced catalytic activity. Hyaluronidase probably loosens the carbohydrate shield, allowing the protein core to gain easy access to the substrate and thereby increasing the enzymatic activity.

The 280-nm peak observed following hyaluronidase digestion suggests the carbohydrate–protein linkage occurs through *N*-acetylhexosamine–tyrosine bonds. Furthermore, the absence of a 280-nm peak with the nontreated enzyme indicates the tyrosine residues are masked since this amino acid was identified by amino acid analysis. The presence of masked phenolic amino acid residues was also supported by 6 N HCl digestion. The significance of the proposed protein–carbohydrate complex is of considerable interest since it probably accounts for the unusual stability of the esterase in soil. Others have demonstrated that the carbohydrate moieties of glucoproteins protect the protein fraction against proteolysis and function as stabilizers for the enzymatic portion of the molecule (Coffey and deDuve, 1968; Pazur *et al.*, 1970). The remarkable stability of the malathion esterase under a variety of conditions is compatible with this explanation.

Acknowledgments

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Factors Affecting Tetramer Dissociation of Rabbit Muscle Lactate Dehydrogenase and Reactivity of Its Sulfhydryl Groups†

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ABSTRACT: Studies of the molecular size of rabbit muscle lactate dehydrogenase in 0.1 M phosphate buffers indicated that the tetramer undergoes a slow, concentration-dependent dissociation to a dimer. Dissociation is slight above 4–5 mg/ml at 20° and pH 7, but pronounced below 1 mg/ml. Increased dissociation occurs with storage of dilute solutions for periods up to 1 month. Reactivity of enzyme sulfhydryl groups could also be related to a slow, concentration-dependent dissociation. The number of groups reacting increased either with time after dilution with thiol reagent or simply with time following dilution. Dissociation in dilute solutions was prevented by NADH (0.5 μM) as evidenced by its effect in sedimentation equilibrium and sulfhydryl group reactivity studies. Storage for several days in dilute solutions resulted in oxidation to disulfide. Dithiothreitol protected against storage inactivation of dilute solutions but more concentrated solutions did not lose activity upon storage in either its presence or absence. Extensive modification of enzyme carboxyl groups or a large increase in net charge (pH 2) caused dissociation mostly to dimer but significant concentrations of monomer were detectable only after extensive disruption of structure. Dissociation and sulfhydryl group reactions may account for the previously unexplained sub-bands observed in gel electrophoresis experiments.

The quaternary structure of L-lactate dehydrogenase (EC 1.1.1.27) has been studied extensively during the past several years. Although an octameric structure has been proposed (Millar *et al.*, 1969), recent evidence has favored a tetrameric structure (Huston *et al.*, 1972). Several investigations have suggested a rapid dissociation equilibrium between protomers and the tetramer for both bovine heart (Millar, 1962) and rabbit muscle lactate dehydrogenases (Griffin and Criddle, 1970). However, conflicting observations have been made by others (*e.g.*, see Jaenicke and Knof, 1968). Recent studies of hybridization in dilute solution (Millar *et al.*, 1971) have supported the case for dissociation.

A suitable explanation for the sub-bands occurring in the region of each isozyme band upon gel electrophoresis has not yet been found although reaction of sulfhydry groups has been implicated (F itz and Jacobson, 1965). A thorough study of the reactivity of sulfhydryl groups of the isozymes from various species has been reported (Fondy *et al.*, 1965); however, studies of the rabbit muscle enzyme were not included.

We report here evidence suggesting that rabbit muscle lactate dehydrogenase tetramer dissociates slowly upon dilution in 0.1 M phosphate buffers, at a rate which is dependent on the preparation. This dissociation appears to be correlated with the reactivity of the enzyme's sulfhydryl groups.

Experimental Section

Materials. Three lots (type II, No. 00C-9530, 90C-9520, and 51C-9580) of crystallized rabbit muscle lactate dehydrogenase were obtained from Sigma Chemical Co.[1] and used for this study. A crystallized preparation of the bovine heart enzyme (type III, lot No. 98B-0911) was also obtained from Sigma. Concentrations of the rabbit muscle enzyme were determined spectrophotometrically using an absorbancy index of 1.44 cm^2/mg at 280 nm (Jaenike and Knof, 1968). NADH[2] (grade III) and dithiothreitol were also obtained from Sigma.

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[1] The use of trade names in this publication does not imply endorsement of the product names by either the U. S. Department of Agriculture or the North Carolina Agricultural Experiment Station, nor criticism of similar ones not mentioned.