Table V. Residues of Insecticides Endosulfan and Ethion Found in Anjou Pears Harvested Following Normal Time Limitation from Last Application to Harvest

Sample	Concentration of insecticide, ppm			
	α-Endosulfan	β-Endosulfan	Endosulfan sulfate	Ethion
(1) 6 lb/acre Thiodan 50 WP (14 days application to harvest)				
A	0.36	1.13	0	0
B	0.33	0.89	0	0
(2) 6 lb/acre Thiodan 50 WP + 8 lb/acre Ethion 25 WP (20 days application to harvest)				
A	0.45	0.57	0.06	0.62
B	0.43	0.58	0.04	0.61
(3) Control	0	0	0	0

period. Results on pear leaves may be compared with the degradation rates on grape leaves in Table IV. While the grapes received a much lower treatment rate than the pears, the higher final residues on the pear leaves may be due in part to the heavily waxed surface of the latter. Final or hard residues would tend to be trapped in this wax layer and therefore would be less vulnerable to physical or chemical attack.

Anjou pears sampled 14 days after treatment with endosulfan and 20 days after treatment with endosulfan–ethion were found to have insecticide residues within tolerances. Results are reported in Table V. While residues of each of these materials individually are considered acceptable, questions of possible potentiation and of acceptable total pesticide residues should be given careful consideration in the future.

The greater persistence of ethion on pears sprayed in July than on those sprayed 20 days prior to harvest may be due in part to the irrigation practices followed. Residues on the pears in the block under trickle irrigation tended to be higher than in the other blocks, as the influence of sprinklers washing off deposits was removed. Results could also have been influenced by the fact that the Anjou pears were sprayed later in the season than those in the first four test blocks by a different operator using a second sprayer of the same make (Turbo-Mist) as that used in the initial application. The effect of various irrigation practices on residue persistence is a subject requiring further study.

LITERATURE CITED

"Analytical Methods for Pesticide Residues in Foods", The Department of National Health and Welfare, Food and Drug Directorate, Tunney's Pasture, Ottawa 3, Canada, 1969.
Cassil, C. C., Food Machinery Corp., Niagara Chemical Division Research and Development Department, Richmond, Calif., unpublished report, 1960.
Gunther, F. A., Blinn, R. C., Carman, G. E., *J. Agric. Food Chem.* **10**, 224 (1962).
Hinden, E., Bennett, P. J., *Wash. State Univ., Coll. Eng., Res. Div., Bull.* **No. 317**, 45 pp (1970).
Maier-Bode, H., *Residue Rev.* **22**, 1 (1968).

Received for review April 14, 1975. Accepted August 21, 1975. Contribution No. 412.

Effect of Phosphatases on the Persistence of Organophosphorus Insecticides in Soil and Water

Bruria Heuer,* Yehudith Birk, and Bruno Yaron

The degradation of some organophosphorus insecticides by phosphatases was studied in water, soil, and soil extract. There is an obvious effect of the phosphatases in solution, but no effect of the enzymes was noted on the insecticides which were adsorbed on the soil surface, even after multiple enzyme applications.

The degradation of organophosphorus insecticides consists of chemical and biological processes (Lichtenstein and Schulz, 1964; Menzer and Dauterman, 1970). The chemical degradation of parathion, guthion, and pyrimiphos-methyl was recently studied in our laboratory (Yaron et al., 1974; Yaron, 1975; Mingelgrin and Yaron, 1973), but very little information is available on the biological decomposition of these insecticides. It is assumed that the decomposition stems from the secretion of microbial enzymes, and more specifically phosphatases, into the soil (Skujins, 1967; Kaufman, 1970). The establishment of phosphatase activity was shown only indirectly by determining the breakdown products which result from incubation of soil samples with different phenyl phosphate substrates (Kramer, 1957; Halstead, 1964; Ramirez-Martinez and McLaren, 1966; Tabatabai and Bremner, 1969). The aim of our study was to determine whether and to what extent one can attribute the biological degradation to the phosphatases by examining directly the effect of different phosphatases on three common organophosphorus insecticides. Since the phosphatases are the most likely enzymes to affect the organophosphorus metabolism, it was interesting to establish which of them are the more effective, and on which substrate.

Division of Soil Residues Chemistry, Institute of Soils and Water, Agricultural Research Organization, Bet Dagan, Israel (B.H., B.Y.), and the Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel (Y.B.).

MATERIALS AND METHODS

The soil used throughout the experiments was a loamy loessial sierozem (from the Gilat Regional Experiment Station) with an organic matter content of less than 1%,

pH 8.4, and a cation exchange capacity of 13.4 mequiv/100 g. The soil was sterilized before use. Sterile soil was obtained by irradiating subsequent samples of the initial soil in a JS-6000 irradiator (Nahal Soreq Atomic Research Institute) provided with a cobalt-60 radiation source. The radiation dose was 3 Mrad.

The following insecticides were used.

Guthion (*O,O*-Dimethyl *S*-[4-Oxo-1,2,3-benzotriazin-3(4*H*)-yl]methyl Phosphorodithioate). Pure guthion was synthesized in our laboratory from the commercially available methyl anthranilate at an overall yield of 35% (Weissenberg, 1972). The intermediate and final products were identified by NMR spectroscopy and by comparison with reported physical data.

Parathion (*O,O*-Diethyl *O*-*p*-Nitrophenyl Phosphorothioate). Pure parathion was obtained from Analabs Inc. (North Haven, Conn.).

Pyrimiphos-methyl (PP 511) [2-Diethylamino-6-methylpyrimidin-4-yldimethyl Phosphorothionate). The pure insecticide was obtained from ICI (Bracknell, Berkshire, England).

The phosphatases employed were: acid phosphatase, type II from potato, lyophilized powder, 0.4 unit per mg of solid (Sigma, St. Louis, Mo., Catalog No. P. 3752); alkaline phosphatase, type I from calf intestinal mucosa, 1–3 units/mg of solid (Sigma, St. Louis, Mo., Catalog No. P. 3877).

The acid phosphatase was assayed according to Hofstee (1954) and Brandenberger and Hanson (1953). The alkaline phosphatase was assayed according to Garen and Levinthal (1960).

The effect of the phosphatases on the insecticides was studied in water, soil, and soil extract.

The soil extract was prepared according to the United States Salinity Laboratory Staff's (1954) instructions, taking air-dried soil and distilled water in a ratio of 3:1. The pH of the extract was above 7.6.

One gram of sterile soil was shaken with 2 ml of insecticide solution of suitable concentration (25 ppm of parathion in hexane, 25 ppm of guthion in chloroform, and 40 ppm of pyrimiphos-methyl in acetone) for 30 min at 25 °C. The solvent was then evaporated under a fan and 1 ml of enzyme solution (20 μg/ml for alkaline phosphatase and 32 μg/ml for acid phosphatase) was added either by a single addition or by multiple additions at 2-h intervals. In the controls, enzyme solution was replaced by water. The experiment was conducted in duplicates. Samples were withdrawn at different time intervals as stated in the Results section. The three insecticides are extracted by a different extraction medium.

Guthion was extracted by mechanically shaking the soil with a 1:2 mixture of chloroform–methanol (9:1) and water. In the case of parathion and pyrimiphos-methyl, hexane was used for extraction. The soil was separated from the solvents by brief centrifugation (400 rpm) and the aqueous phase was removed from the organic solvent in a separatory funnel. The organic phase was injected and tested by gas chromatography using a Packard gas chromatograph with a flame ionization detector. The conditions for the GLC were: glass column, 180 cm × 3 mm i.d., filled with 10% DC 200 + 15% QF 1 on Gas-Chrom Q, 80–100 mesh; inlet and column temperature, 225–245 °C; detector temperature, 200 °C; carrier gas flow, 60–70 ml/min. We were obliged to maintain the detector temperature lower than the column temperature since we used the gas chromatograph equipment simultaneously for EC and FI determinations. Two microliters of insecticide solution at a known concentration was injected as a standard after every two injections of samples. Peak height was used for quantification.

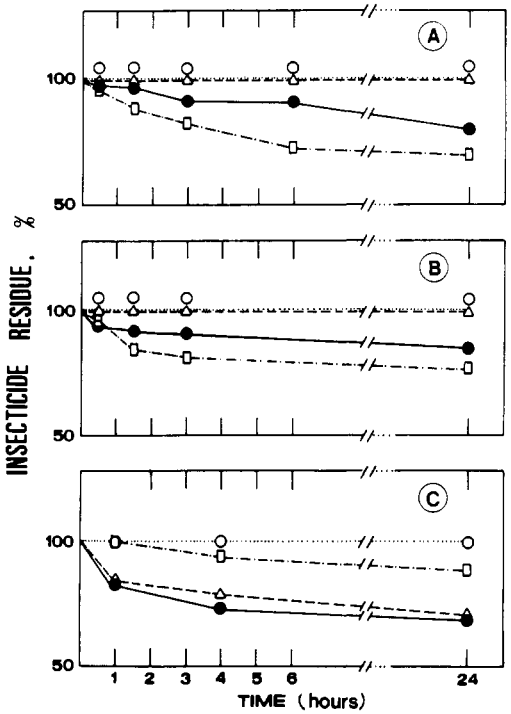

Figure 1. Effect of different enzyme concentrations on the extent of insecticide degradation in water solution. (A) 10 ppm of parathion + alkaline phosphatase (ppm): (□) 5; (●) 20; (△) 50; (○) 100. (B) 30 ppm of guthion + alkaline phosphatase (ppm): (□) 15; (●) 30; (△) 50; (○) 100. (C) 10 ppm of PP 511 + acid phosphatase (ppm): (□) 2; (●) 4; (△) 6; (○) 10.

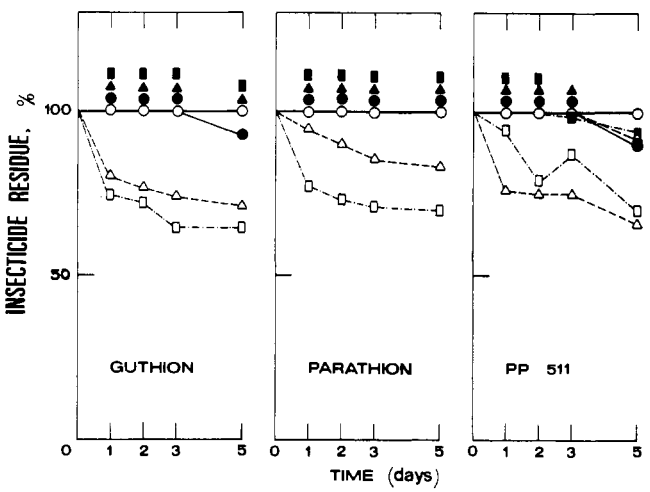

Figure 2. The persistence of three organophosphorus insecticides in soil water and soil extract after a single application of the enzyme: in soil, (○—○) enzyme; (●—●) water; water solution, (△- - -△) enzyme; (▲—▲) water; soil extract, (□- - -□) enzyme; (■—■) water.

In the experiment with multiple applications of enzyme, the amount of insecticide that had remained in the medium was calculated after each extraction, and according to this we applied the suitable concentration of the enzymes in the next application. The samples were extracted and tested as described above.

RESULTS AND DISCUSSION

In order to determine the relative affinity of the phosphatases for the three different insecticides, a preliminary experiment was carried out (Table I). From

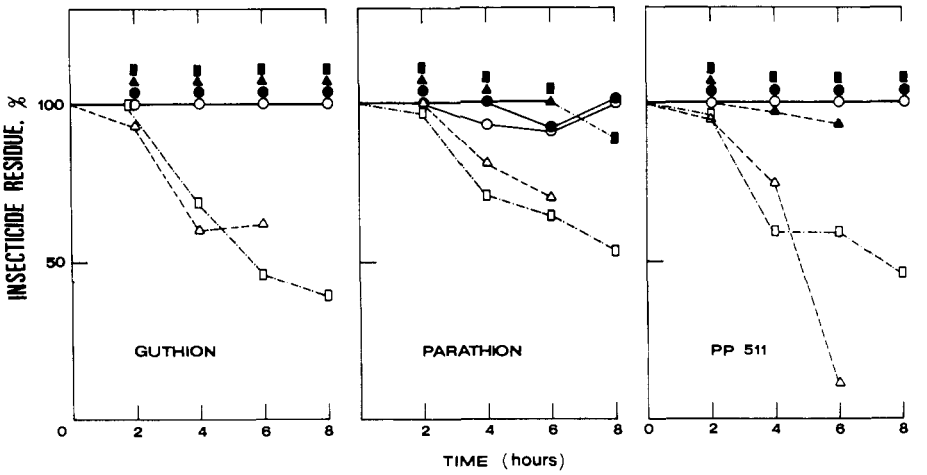

Figure 3. Organophosphorus residue in soil, water, and soil extract after multiple enzyme application, at 2-h intervals: in soil, (○—○) enzyme; (●—●) water; water solution, (△- - -△) enzyme; (▲—▲) water; soil extract, (□- - -□) enzyme; (■—■) water.

Table I. Comparative Effect of Acid and Alkaline Phosphatases on Three Insecticides in Water Solution (in Percent Residual Concentration of Substrate)

Time of reaction, h	Residual % concentration of Parathion	Guthion	Pyrimiphos-methyl
	Acid Phosphatase		
0	100	100	100
0.5	94	100	80
1.5	94	100	78
	94	100	70
	Alkaline Phosphatase		
0	100	100	100
0.5	98	92	100
1.5	93	85	100
3	80	81	100

Table I it can be seen that the acid phosphatase affects mainly pyrimiphos-methyl and the alkaline phosphatase has the greatest effect on parathion and guthion.

The effect of different enzyme-to-substrate ratios on the extent of insecticide degradation is shown in Figure 1. Since the optimal ratio was found in the range of 1:2–3, it was decided to employ a 1:2.5 enzyme:substrate ratio throughout the experiments.

The effect of a single application of the enzymes on the three organophosphorus insecticides is shown in Figure 2. No effect of the enzyme was noted when the insecticides were adsorbed on the soil surface. It seems that the lack of activity results from the adsorption of the enzymes on the clay minerals which are present at a concentration of 13% in Gilat soil (Mortland and Gieseking, 1952).

In aqueous solutions a certain activity of the phosphatases was noted. In soil extracts the activity was more pronounced, especially on guthion and parathion. Thus, a 30% loss of parathion after 5 days was noted in soil extracts as compared with a 14% loss in water solution.

The data in Figure 2 also indicate that the breakdown of the insecticides is only partial after 5 days of incubation. This may result from a decrease in enzyme activity with time. A series of preliminary experiments with the phosphatases showed that indeed they maintain their initial activity for a period of about 3 h. An experiment was then performed with multiple enzyme application, namely, the enzymes were applied to the insecticides at a concentration to maintain the ratio of enzyme to substrate constant throughout the incubation period. The results of this experiment are given in Figure 3.

Under these conditions a higher percent of degradation, both in the water solution and soil extract, was achieved. Only 11% recovery of the initial pyrimiphos-methyl was obtained after 6 h of multiple application of acid phosphatase in water. Even in this experiment there is no effect on the pesticides on the soil surface, so it can be assumed that the adsorption of the enzymes is very strong and rapid.

It should be emphasized that the phosphatase activity assayed in all the experiments resulted only from the phosphatase added and not from soil phosphatases, since irradiated soil was used throughout, and precautions to maintain sterility were taken.

From the results obtained we can conclude the following. (1) The persistence of organophosphorus insecticides in nature is affected by their susceptibility to phosphatases. Each enzyme works on the insecticide according to its preferential affinity. (2) The enzymes applied on sterile soil do not act on the adsorbed insecticides on soil surfaces either after one or after multiple applications. (3) In soil solution and water multiple enzyme application provides a constant supply of enzymes, and therefore the effect is much stronger than in the case of a single application. (4) Assuming that the enzymes do not act when adsorbed on soil surfaces, it is suggested that the interaction between them and the organophosphorus insecticides occurs only when both components are not adsorbed.

LITERATURE CITED

Brandenberger, H., Hanson, R., *Helv. Chim. Acta* **36**, 900 (1953).
Garen, A., Levinthal, C., *Biochim. Biophys. Acta* **38**, 470 (1960).
Halstead, R. L., *Can. J. Soil Sci.* **44**, 137–144 (1964).
Hofstee, B. H. J., *Arch. Biochem. Biophys.* **51**, 139 (1954).
Kramer, M., *Naturwissenschaften* **44**, 13 (1957).
Kaufman, D. D., *Pestic. Soil: Ecol., Degradation Movement, Int. Symp., 1970*, 73–85 (1970).
Lichtenstein, E. P., Schulz, K. R., *J. Econ. Entomol.* **57**, 618–627 (1964).
Menzer, R. E., Dauterman, W. C., *J. Agric. Food Chem.* **18**, 1031 (1970).
Mingelgrin, U., Yaron, B., *Bull. Environ. Contam. Toxicol.* **10**, 285–290 (1973).
Mortland, M. M., Gieseking, J. E., *Soil Sci. Soc. Am. Proc.* **17**, 10–13 (1952).
Ramirez-Martinez, J. R., McLaren, A. D., *Enzymologia* **30**, 243–253 (1966).
Skujins, J. J., *Soil Biochem.*, 371–414 (1967).
Tabatabai, M. A., Bremner, J. H., *Soil Biol. Biochem.* **1**, 301–307 (1969).
United States Salinity Laboratory Staff, "Diagnosis and Improvement of Saline and Alkali Soils", Richards, L. A., Ed., Washington, D.C., 1954.

Weissenberg, M., in A10-SWC-71 Second Annual Report, Institute of Soils and Water, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, 1972.
Yaron, B., *Soil Sci. Soc. Am. Proc.* **39**, 639–643 (1975).
Yaron, B., Heuer, B., Birk, Y., *J. Agric. Food Chem.* **22**, 439–442 (1974).

Received for review January 17, 1975. Accepted December 2, 1975. Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, 1974 Series, No. 252-E.

Some Chemical and Functional Characteristics of a Fiber-Free Coconut Protein Extract Obtained by the Enzymic Chemical Process

Mario R. Molina,* Paul A. Lachance,[1] and Ricardo Bressani

The coconut protein extract obtained through the enzymic chemical process contained 32.4% protein and 42.6% of nitrogen-free extract. It also had a high total sugar content (39.2%) and a relatively small amount of reducing sugars (6.2%). Hydrolysis and paper chromatography were used to determine the composition of the total sugars. Column chromatography showed that 66% of the protein had a molecular weight higher than 5000. Only 41.3% of the protein proved to be nondialyzable, suggesting a molecular weight higher than 12 000. Such a protein fraction was electrophoretically homogeneous. The protein showed minimum solubility at pH 7.0 and 34.2% was heat coagulable at 120 °C. The extracted product was highly dispersible with an emulsifying capacity of 24.8 ml/g and did not form a gel at 15 and 30% levels. Preliminary tests showed possible practical applications of the extracted product in drinks and bakery products formulations.

Recently an enzymic chemical technique was developed to effect the protein extraction of coconut meal (Molina and Lachance, 1973). This technique was found to be equally effective for those meals obtained through a solvent or through an expeller oil-extraction process. The fiber-free coconut protein extract obtained has been found to have a higher nutritive value than the original coconut meal (Lachance and Molina, 1974).

More recent work has shown the enzymic chemical technique to be applicable to cottonseed meals which have undergone partial protein denaturation through the oil-extraction process (Childs, 1975). In addition, the same author (Childs, 1975) indicates that the total costs of the extraction, as well as the time to effect it, can be substantially lowered by using ultrasonic energy to activate both the enzymic and the chemical stages. These findings undoubtedly will accelerate the implementation of the enzymic chemical protein extraction technology for a better utilization of protein-rich by-products as coconut and cottonseed meals.

The implementation of an enzymic chemical protein extraction technology in Latin America would be advisable since most oilseed meals (coconut, cottonseed, and the like) are produced through dry oil-extraction processes which generally result in a partial denaturation of their protein.

Based on the above concepts it was thought of interest to define some chemical and functional characteristics of the coconut protein extract obtained through the original enzymic chemical technique as described by Molina and Lachance (1973).

MATERIALS AND METHODS

The coconut protein extract sample used was an aliquot of the freeze-dried extract obtained by Molina and Lachance (1973) and used for the nutritional evaluation studies (Lachance and Molina, 1974).

Moisture, nitrogen, ash, oil, and crude fiber were determined according to AOAC (1970). Protein determinations were carried out by the macro biuret colorimetric test as described by Bailey (1967) using bovine serum albumin fraction V (Sigma Chemicals Co., St. Louis, Mo.) as standard. Total sugars were determined by the phenol–sulfuric method (Dubois et al., 1956). Determinations of total reducing sugars were performed by the neocuproine-HCl method (Dygerts et al., 1965). Both total sugars and total reducing sugars were expressed as glucose. Total free amino acids were determined by the ninhydrin colorimetric method as described by Rosen (1957) using leucine as a standard. Glucose and galactose were determined using the glucostat and galactostat enzymatic reagent (Worthington Biochemicals Corp., Freehold, N.J.), respectively, and following the methods detailed in the Worthington Biochemicals Corp. Publications No. 4-68 and 4-66 for each of the reagents. All determinations were carried out in triplicate.

The dialysis experiments were done with a cellulose dialyzer tubing (1.59 cm diameter) retaining materials with a molecular weight of 12 000 or higher. In all cases the dialysis was carried out for 48 h at room temperature (25 °C) against running water using chloroform as a preservative. The sample was 100 ml of a 10% aqueous suspension of the material.

The identification of simple sugars was performed by descending paper chromatography using Whatman No. 1 chromatographic paper. The chromatogram was developed for 22 h at room temperature (25 °C) using the upper layer of an ethyl acetate–water–pyridine (2:2:1) mixture as solvent, as recommended by Jermyn and Isherwood (1949) for the separation of simple sugars. The sugars were visualized by the method described by Pintauro (1967) using aniline oxalate. Reagent grade samples of glucose, galactose, arabinose, xylose, and mannose were used as standards; the samples utilized for this test were both an aqueous suspension of the protein extract and an aliquot of this aqueous suspension which had been treated with

Division of Agricultural and Food Sciences, Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala, Central America.
[1]Present address: Food Science Department, Cook College, Rutgers—The State University, New Brunswick, New Jersey 08903.