Study of Parathion Biodegradation Using Gnotobiotic Techniques

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Bean roots grown under aseptic conditions were exposed either to parathion or parathion and the alga *Chlorella* for seven days. The only pesticide-related product detected in extracts from the aerial parts of plants exposed to the insecticide alone was parathion, whereas parathion and a sulfur-containing parathion metabolite were recovered from bean leaves and stems of plants whose roots were exposed to both parathion and the alga. These data suggest that pesticide metabolites found in plants may be a product of microbial metabolism rather than metabolism by the plant.

Microorganisms, such as bacteria, actinomycetes, algae, and yeast, metabolize organophosphate compounds, resulting in the degradation of the parent molecule (Ahmed and Casida, 1958; Gunner *et al.*, 1966; Lichtenstein and Schulz, 1964). Organophosphate compounds were also reported to break down within plant tissues, though whether by photochemical conversion or metabolic activity of the plant was not determined (El-Refai and Hopkins, 1966; Kansouh and Hopkins, 1968). In these latter experiments, microbes that could have contributed to the breakdown of the pesticide molecule were not excluded. To study the fate of organophosphate pesticides following application, the authors felt that control must be exerted over the biotic environment if the manner in which these chemicals break down and are detoxified is to be fully understood.

The present studies demonstrate the utility of, and often the requirement for, germ-free systems in the study of pesticide degradative pathways.

MATERIALS AND METHODS

Experimental Design. These experiments were conducted with bean plants (*Phaseolus vulgaris*) grown in a sterile root culture apparatus designed by Stotzky *et al.* (1962), and later used by Zuckerman *et al.* (1966), to study the translocation of parathion (*O,O*-diethyl *O-p*-nitrophenyl thionophosphate). In this experiment, the important features of this apparatus are: The plant can be grown for periods in excess of one month with its roots held under aseptic conditions and the pesticide and/or a pure culture of a microorganism can be introduced to the vicinity of the roots, while at the same time maintaining the rhizosphere free of microbial contaminants.

Chlorella pyrenoidosa proteose was the microorganism used, this alga having previously been shown by Ahmed and Casida (1958) to metabolize parathion readily. The alga used in the experiment was propagated in pure culture in a medium described by Stiller (1966).

The experiment consisted of four treatments, replicated twice. Parathion alone was added to the root chamber of a two-week-old bean plant. Each plant received 5.5 μc. of ^{35}S parathion (specific activity 1.9 mc. per mmole when first measured) and 100 mg. of unlabeled parathion (99.7% analytical standard) dissolved in 95% ethyl alcohol. After one week, the apparatus was opened and parathion and its metabolites were extracted separately from the water contained in the root chamber, from the roots, and from the aerial portions of the plant, using methods previously described by Zuckerman *et al.* (1966).

Parathion and a pure culture of the alga were added to the root chamber of a two-week-old bean plant.

Parathion was added to an algal culture growing in 100 ml. of Stiller's medium in an Erlenmeyer flask.

Parathion was added to an Erlenmeyer flask containing 100 ml. of Stiller's medium.

The latter two treatments served as controls for the root culture experiments. The parathion dosage, incubation time, and extraction procedures in all experiments were the same as in the first treatment.

Up to the time that the root culture apparatus was opened, sterile procedures were scrupulously followed. These procedures included sterility checks of the algal cultures before use and of the contents of the root chamber prior to the termination of the experiment.

Analytical Techniques. The pesticide and its breakdown products were separated, identified, and quantitated by standard chromatographic and radiological procedures.

THIN LAYER CHROMATOGRAPHY (TLC). Chromatoplates with silica gel G contained $CaSO_4$ binder; fluorescent silica gel HF 254. Solvents were chloroform with 0.7% ethyl alcohol and hexane-acetone (8 to 2). The two-dimensional system was employed to confirm identifications. Indicators were 0.4% DQC (2,6-dibromobenzoquinone-4-chloroimide) in glacial acetic acid; 0.5% palladium chloride in 2% hydrochloric acid; 5*N* sodium hydroxide.

GAS-LIQUID CHROMATOGRAPHY (GLC). Samples were analyzed in an Aerograph Model 204, using a flame ionization detector. The column was 5-foot × 1/8-inch stainless steel, packed with 5% SE-30 on 60/80 Chromosorb W, regular. Operating parameters were: column temperature, 170° C.; injector temperature, 200° C.; detector, 180° C.; nitrogen flow rate, 20 ml. per minute; hydrogen flow rate, 20 ml. per minute.

RADIOLOGICAL PROCEDURES. Labeled samples were counted using a Nuclear-Chicago low-background, Q-gas counting system, Model 4312. Counting efficiency of this instrument was 19%, as calculated with a barium carbonate standard. All samples were counted for adequate time periods to be statistically significant above background at the 5% level.

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Table I. The Distribution of Parathion and Its Sulfur-Containing Metabolites from the Root Chamber Liquid, Bean Plant Tops, and the Culture Medium as Separated by TLC, Detected by Autoradiography, and Quantitated by Radiological Analysis

		Bean Plant with *Chlorella*		Bean Plant without *Chlorella*			
	R_f	Extract from root chamber liquid, %	Extract from plant tops, %	Extract from root chamber liquid, %	Extract from plant tops, %	*Chlorella* Alone, Extract from the Medium, %	Extract from the Uninoculated Culture Medium, %
Parathion	0.95	24.3	96.3	100	100	26.1	100
Aminoparathion	0.45	66.0	...	...	...	57.0	...
Unknown metabolite	0.20	9.7	3.7	...	...	16.9	...

In earlier experiments in which scintillation counting of ^{35}S parathion samples was attempted, extremely low counting efficiencies were noted. A quenching curve plotted using a dilution series starting with 50 mg. of parathion showed that the pesticide was a strong quencher. For example, 97 μg. of parathion inhibited counts by 55%.

The ^{35}S parathion as obtained from Nuclear-Chicago contained two radiochemical impurities which amounted to 1.2% of total volume. The chemical was purified by TLC separation, followed by elution of the parathion from the gel with acetone. The recovered product was reexamined for purity prior to use by autoradiography, using Eastman No-Screen X-Ray film. When the labeled parathion was first received, concentrations as low as 20 picograms were detected by the autoradiographic procedure.

RESULTS

After seven days, 3% of the total radioactivity corrected for decay was recovered from the aerial portions of bean plants whose roots were exposed to parathion alone, and an additional 2% was recovered from the roots. Thus, a total of about 5 mg. of parathion had moved into the plant during the test period. All of the recovered material appeared to be parathion, since this was the only compound detected either by GLC or TLC followed by autoradiography. The liquid in the root chamber, which contained 86% on an average of the total initial radioactivity, also contained only parathion.

Extracts from the root chamber liquid in units exposed to both parathion and algal cells contained three labeled compounds. Two of these compounds were identified as parathion and aminoparathion. The third compound, though not identified, was characterized by reactions with specific indicators and on fluorescent silica gel as containing the P=S group and the benzene ring. Extracts from the plant tops contained both parathion and the unknown metabolite (Table I). Although 66% of the parathion contained in the root chamber had been converted to aminoparathion by algal activity during the test period, none of the aminoparathion moved into the plant. The total radioactivity recovered from the plant and the root chamber in these experiments averaged 90.5%.

The same degradation products in similar proportions were recovered from the pure algal cultures to which parathion was added (Table I). Where parathion was added to the medium alone, no sulfur-containing metabolites were detected.

In each experiment extracts were also examined by TLC and GLC for paraoxon, *p*-nitrophenol, and *p*-aminophenol. A trace amount of *p*-nitrophenol was detected by GLC in each test involving algae, but neither of the other compounds was found. Detection of paraoxon was also attempted with the cholinesterase method used by El-Refai and Hopkins (1965). The minimum level of sensitivity of this method is 0.025 nanogram for paraoxon. The results were negative, indicating that significant amounts of paraoxon were not produced within the gnotobiotic system.

DISCUSSION

The biological degradation of pesticides hitherto has been considered largely a function of individual species, be they higher plants or microorganisms. Little attention appears to have been directed to the possibility of a sequential attack on a pesticide molecule by successive populations, or species, in an ecosystem. The present work indicates that the unidentified metabolite recovered from the aerial parts of bean plants was the result of parathion degradation by the alga, and not by the plant. If plant enzymes actively participate in the decomposition of the thiono portion of the parathion molecule, as the work of El-Refai and Hopkins (1966) seems to indicate, then it must be assumed that the unknown metabolite has entered into the second phase of a degradative chain that will ultimately bring about the complete breakdown of the pesticide.

Since all organisms obviously do not attack pesticides in the same manner, the eventual understanding of biodegradative pathways would appear to require the use of a germ-free system at some stage of the investigation.

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