Increased Biological Hydrolysis of Diazinon after Repeated Application in Rice Paddies

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Repeated application of diazinon granules to the soil surface of rice fields affected the development of a biological factor that caused rapid degradation of diazinon. This factor was present in the paddy water, in rhizosphere soil of the rice plant, and in nonrhizosphere soils. Isotope studies showed that when incubated with water from diazinon-treated fields, diazinon was hydrolyzed to 2-isopropyl-6-methyl-4-hydroxypyrimidine in about 75 hr after incubation. Within the next 25 hr, this hydrolysis product was completely mineralized, apparently to CO_2. No other metabolite was recorded on the autoradiochromatogram. This rapid hydrolysis of diazinon in paddy water from diazinon-treated fields was of a microbial nature. This microbial factor developed only in diazinon-treated fields and degraded diazinon, but not other insecticides related to it. The addition of streptomycin to the incubation mixture prevented the breakdown of diazinon.

The application of diazinon granules to the paddy water at 2 kg/ha active ingredient (a.i.) every 20 days has effectively controlled common insect pests of rice (Pathak, 1968). This practice was used in seven consecutive crops during 3.5 yr at the International Rice Research Institute (IRRI) with highly satisfactory results. Later, such treatments became ineffective for controlling the brown plant hopper (*Nilaparvata lugens* Stål.) and several diazinon-treated plots suffered severe damage (IRRI, 1970).

Investigations undertaken to explain the decline in diazinon's effectiveness showed that incubation of water from fields that had previously received at least three applications of diazinon caused a complete breakdown of the chemical in 3 to 5 days. Sethunathan and Pathak (1971) and IRRI (1969) attributed the breakdown to a microbial factor, particularly to *Arthrobacter* sp., that developed in paddy water after repeated applications of diazinon. Recent studies have led to the identification of another bacteria, *Flavabacterium* sp., responsible for this degradation of diazinon (Yoshida, 1971). The buildup of this factor in paddy water increased with the frequency of diazinon treatments (Sethunathan *et al.*, 1971). This paper reports further results on this degradation factor, its specificity, and the metabolic pathway of diazinon degradation.

MATERIALS AND METHODS

Degrading Factor in Rice Field. A study was conducted to find out whether the rhizosphere soil as well as the nonrhizosphere soil of rice grown in diazinon-treated plots would degrade diazinon. The soil and water samples were collected from diazinon-treated and untreated 8- × 5-m field plots planted with the rice variety IR8. Granular diazinon was broadcast at 2 kg/ha a.i. every 20 days. Each treatment was replicated three times and samples were collected from each plot. Paddy water samples were collected 10 days after the fourth application of the insecticide. Five milliliters of this water was incubated with 5 ml of aqueous diazinon solution at room temperature (23 ± 2°C). The plots were drained and allowed to dry slightly and two rice plants were pulled from each plot. The soil clinging to the roots was gently shaken off and a 3-g sample was taken from the roots. The soil still adhering to the roots represented the rhizosphere sample. It was shaken with 100 ml of sterile distilled water and allowed to settle. Five milliliters of the solution was incubated with 5 ml of aqueous diazinon solution. Soil collected from between the hills served as the nonrhizosphere sample. Two grams of the soil were added to 5 ml of aqueous diazinon solution and 5 ml of distilled water. Aliquots from incubated samples of paddy water, rhizosphere soil, and nonrhizosphere soil were taken at desired intervals for residue analyses. Water samples were also collected from experiments conducted at three other locations in the Philippines and also from a field in each area which, as far as known, were never treated with any insecticide. These samples were tested for their diazinon-degrading ability.

Specificity of the Degrading Factor. Lindane (chlorinated hydrocarbon), carbofuran (carbamate), Phosvel, Sandoz-6626, Dursban, and diazinon (organosphosphates) granules were applied to the standing water of rice plots at 10, 40, and 70 days after transplanting. Each insecticide was applied in three replications. Water samples were collected from the plots 10 days after the last application, and were bioassayed for diazinon-degrading ability. Water from diazinon-treated plots was also tested for its ability to degrade ethyl parathion and Dursban. Incubation of Dursban was carried out in darkness because it decomposes in light.

The metabolism of diazinon in paddy water from diazinon-treated and untreated rice fields was studied with the use of radioactively labeled diazinon supplied by Geigy Agricultural Chemicals, Basle, Switzerland. The insecticide was labeled at the fourth position on the pyrimidine ring with a specific activity of 2.6 μCi/mg. Before the labeled diazinon was used its purity was confirmed by thin-layer chromatography.

The incubation mixture contained in a test tube consisted of 5 ml of paddy water, 3 ml of unlabeled aqueous diazinon solution, and 2 ml of aqueous labeled diazinon. In another treatment, 30 mg of streptomycin sulfate was added to the incubation mixture to determine the metabolism of diazinon in the absence of the microbial factor. Each treatment was replicated three times. Five days after incubation at room temperature (23 ± 2°C) 20 ml of chloroform–diethyl ether (1:1) was added to the incubation mixture in each tube. The mixture was shaken for 10 min and allowed to stand for 30 min, after which the solvent layer was removed. The process was repeated once. The two samples of the solvent layer

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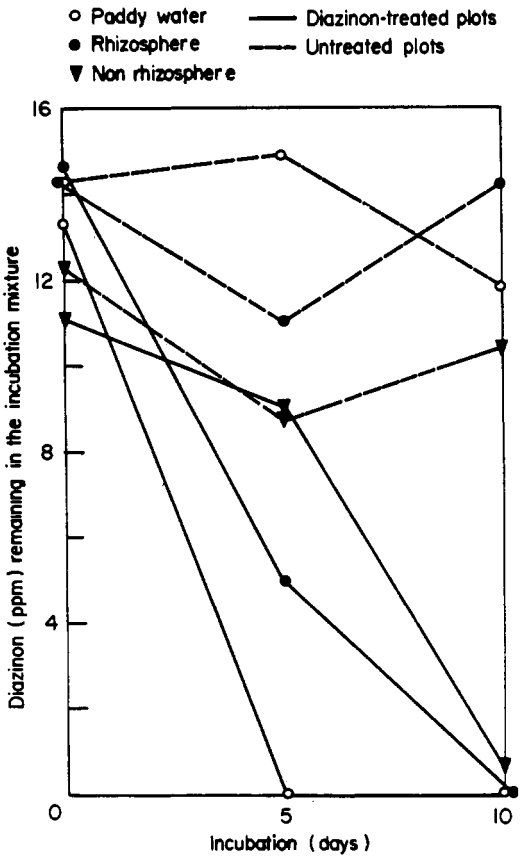

Figure 1. Degradation of diazinon incubated with paddy water, rice rhizosphere soil, and nonrhizosphere soil collected from diazinon-treated and untreated rice fields (IRRI, 1970)

thus removed were pooled together and evaporated to dryness at room temperature. The residues were then dissolved in 2 ml of methanol and separated by thin-layer chromatography, and the chromatograms were exposed to Kodak X-ray film for 5 days.

Thin-Layer Chromatography. The residues dissolved in methanol were spotted along with diazinon and its hydrolysis product, 2-isopropyl-6-methyl-4-hydroxypyrimidine, on chromatoplates coated with silica gel G 250 μ thick. The plates were developed with chloroform + acetone (7:1) for a distance of 15 cm and dried. The authentic compounds were located as described previously (Sethunathan and Yoshida, 1969). The areas in the parallel track opposite the authentic compounds were scraped and suspended in 10 ml of scintillation solution (5 g of PPO and 0.3 g of POPOP per liter of toluene, Packard Instrument Co., La Grange, Ill.). The radioactivity was determined on a Tri-Carb scintillation counter, Model 314 EX (Packard Instrument Co., La Grange, Ill.).

The radioactivity remaining in the water phase after solvent extraction was assayed by adding 1 ml of the water fraction to 10 ml of Bray's solution (Bray, 1960).

RESULTS AND DISCUSSION

Diazinon-Degrading Factor. Samples of paddy water, rice rhizosphere soil, and nonrhizosphere soil from untreated fields showed no diazinon-degrading ability, while samples from diazinon-treated plots readily decomposed the insecticide (Figure 1). The degrading activity of the samples from diazinon-treated plots was in the order of paddy water > rhizosphere soil > nonrhizosphere soil. Since diazinon was degraded more actively under aerobic than under anaerobic conditions (Sethunathan and Pathak, 1971), the insecticide was expected to break down rapidly in the paddy water, which contains dissolved oxygen released by algae (IRRI, 1968) and diffused from the atmosphere. The rice rhizosphere was also expected to harbor aerobic microflora because of the oxidizing capacity of rice roots under waterlogged conditions. The nonrhizosphere soil included a predominantly reduced layer with small segments of oxidized layer on the top.

Table I. Degradation of Diazinon on Incubation with Paddy Water Collected from Different Locations in the Philippines

Source of paddy water	Diazinon (ppm) recovered at indicated days after incubation			
	0	3	6	12
Muñoz, Nueva Ecija				
Diazinon-treated rice field[a]	23.0	0	0	0
Untreated rice field	22.8	21.8	22.4	20.3
Iloilo City				
Diazinon-treated rice field[a]	33.3	0	0	0
Untreated rice field	35.0	24.6	22.8	21.8
Camarines Sur, Bicol				
Diazinon-treated rice field[a]	40.3	3.3	0	0
Untreated rice field	44.3	39.3	39.8	37.5

[a] At the time of sampling each field had received a total of three diazinon treatments at 2 kg/ha a.i. every 20 days.

Table II. Persistence of Diazinon in Paddy Water Alone and in Paddy Water Soil Systems

Treatment	Diazinon (ppm) recovered at indicated days after incubation		
	0	5	10
Diazinon + paddy water	23.1	0	0
Diazinon + paddy water + soil	15.2	11.2	0

Table III. Effect of Tween 80 Detergent on the Persistence of Diazinon Incubated with Paddy Water from Diazinon-Treated Field

Treatment	Diazinon (ppm) at indicated days after incubation			
	0	5	10	20
Diazinon + paddy water	17.3	9.2	0	0
Diazinon + paddy water + Tween 80 (250 ppm)	16.9	15.8	5.8	0
Diazinon + paddy water + Tween 80 (500 ppm)	17.1	15.2	14.7	4.3

Diazinon was degraded when incubated with paddy water from diazinon-treated fields, but not when incubated with water from untreated plots from three different locations in the Philippines (Table I). This indicates that the development of a diazinon-degrading factor may be a common phenomenon in areas where diazinon has been used continuously for some years.

Diazinon was rapidly degraded in paddy water from diazinon-treated plots after an initial lag of 1 or 2 days. But it was expected to degrade slowly when incubated with water and soils because some insecticide might be adsorbed on soil particles. This expectation was confirmed. Diazinon in an almost soil-free paddy water system was decomposed in 5 days while in soil paddy water system it persisted up to 10 days after treatment (Table II). Addition of Tween 80, a detergent, to paddy water slowed down the rate of degradation (Table III). Similar results have been reported for

Table IV. Metabolism of ^{14}C-Diazinon Incubated with the Paddy Water from a Rice Field Previously Treated with Diazinon

Incubation period, hr	Diazinon recovered,[b] ppm	Radioactivity (cpm) recovered[a] Diazinon[c]	Hydrolysis product[c]	Water phase[d]
0	14.5	...	...	...
20	14.5	31000	1000	400
55	5.9	12300	10900	5000
75	0.3	600	12000	5800
100	0	200	200	4200

[a] All figures adjusted for background. Initial radioactivity added to each tube was about 50,000 cpm. [b] As determined by gas–liquid chromatography. [c] Determined after separation of chloroform–diethyl ether fraction by thin-layer chromatography. [d] Remaining in water phase after solvent extraction.

Table V. Persistence of Diazinon on Incubation with Paddy Water from Field Plots Treated with Different Insecticides at Maligaya Rice Research and Training Center, Philippines

Water from plots treated with[a]	Diazinon recovered, ppm Days after incubation 0	3	12
Dursban	27.8	27.5	24.0
Carbofuran	27.2	27.2	25.5
Sandoz-6626	27.7	26.8	24.5
Phosvel	27.8	28.2	26.2
γ-BHC	27.0	27.0	24.8
Diazinon	25.5	0	0
Untreated	27.2	26.5	25.7

[a] All insecticides were applied to the paddy water at 2 kg/ha a.i. every 20 days except for S-6626 that was used at the rate of 1 kg/ha.

Table VI. Persistence of Different Insecticides on Incubation with Paddy Water from Diazinon Treated and Untreated Control Plots

Insecticide	Days after incubation	Insecticide recovered, ppm Diazinon-treated field water + insecticide	Untreated field water insecticide
Diazinon[a]	0	38.2	43.8
	12	0	40.7
Dursban[b]	0	7.2	7.2
	12	3.3	4.8
Ethyl parathion[a]	0	9.8	9.8
	12	9.9	10.1

[a] In aqueous solutions. [b] In alcoholic solution.

parathion and diazinon in upland soils (Lichtenstein, 1966). Some detergents are known to inhibit microorganisms.

Metabolism of Diazinon in Paddy Water. The autoradiochromatogram of the incubation mixtures at 5 days after diazinon was incubated with paddy water from treated plots and with paddy water from untreated plots is shown in Figure 2. This figure confirms the results of diazinon degradation obtained by gas–liquid chromatography technique. No radioactive metabolite was recorded on the autoradiograph of the incubation mixture of diazinon and water from diazinon-treated plot at 5 days after incubation. This indicated that the pyrimidine ring carrying the radioactive carbon was completely mineralized, evidently to $^{14}CO_2$. Our previous results support this hypothesis. We found that more than 66% of the ^{14}C added to paddy water from diazinon-treated plots was released as $^{14}CO_2$ in 5 days (Sethunathan and Pathak, 1971). The presence of a prominent radioactive spot identical to diazinon in the autoradiograph showed that streptomycin added to the incubation

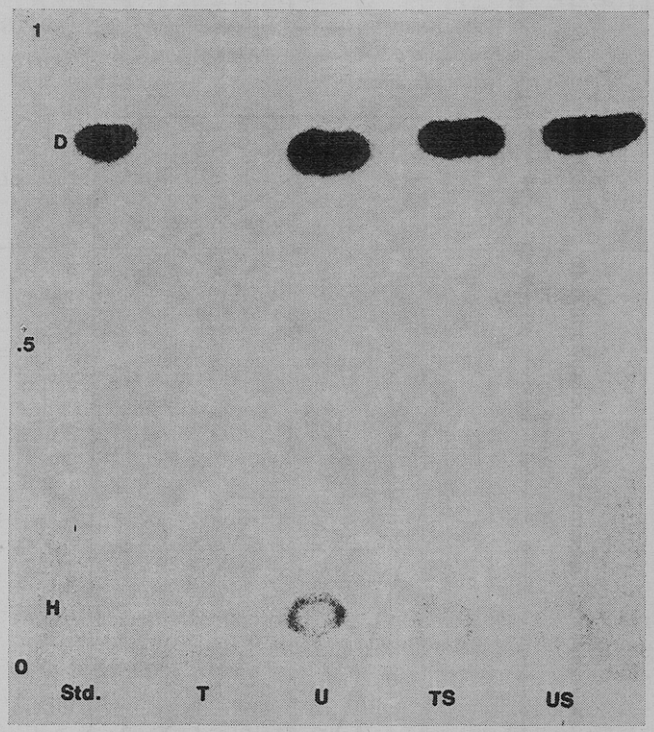

Figure 2. Autoradiochromatogram of ^{14}C-diazinon (D) after incubations for 5 days with paddy water from diazinon-treated (T), and untreated field plots (U). TS = T + streptomycin, US = U + streptomycin. H = 2-isopropyl-6-methyl-4-hydroxypyrimidine

mixture prevented the breakdown of diazinon (Figure 2). Thus microbes participated actively in the rapid degradation of the insecticide. When the labeled diazinon was incubated with water from untreated plots most of the radioactivity occurred at the position of diazinon, with faint activity at the position of hydrolysis product. The small amount of the hydrolysis product recorded was most likely formed by some chemical and biological action that commonly occurs in nonsterilized systems (Sethunathan and MacRae, 1969).

Earlier tests had shown that, when incubated with paddy water from diazinon-treated plots, about 90% of the added ^{14}C diazinon was degraded in 3 days, but only 10% of it was recovered as $^{14}CO_2$ (Sethunathan and Pathak, 1971). The insecticide was degraded completely in 5 days of incubation and more than 66% of the added ^{14}C was recovered as $^{14}CO_2$, which showed that at 3 days of incubation most of the insecticide was converted to some metabolite or metabolites that were mineralized to CO_2 within 2 days. Another experiment confirmed this conclusion. On incubation with water from a diazinon-treated field, the concentration of diazinon declined progressively with a simultaneous increase in its hydrolysis product (Table IV). No hydrolysis product was recovered at 100 hr after incubation and the autoradiographic analysis of the incubation mixtures revealed similar results. Evidently the hydrolysis product that formed was metabolized to $^{14}CO_2$ between 75 and 100 hr after incubation (Sethunathan and Pathak, 1971). No radioactive metabolite other than this hydrolysis product was recorded on the autoradiograph of the incubation mixture. About 10% of the added radioactivity remained in the water phase after solvent extraction.

Specificity of the Factor. Diazinon incubated with water from a diazinon-treated rice field was completely degraded (Table V). However, it was not significantly degraded even up to 12 days of incubation with water from plots treated

with Dursban, Sandoz-6626, Phosvel, lindane, or carbofuran. Furthermore, water from diazinon-treated plots did not affect the degradation of ethyl parathion or a solution of Dursban in alcohol. Only diazinon was appreciably degraded (Table VI). Since all three compounds possess a P–O–ester bond at which diazinon is hydrolyzed the diazinon-degrading factor appears to be highly specific.

LITERATURE CITED

Bray, G. A., *Anal. Biochem.* **1**, 279 (1960).
IRRI, International Rice Research Institute, Annual Report (1968).
IRRI, International Rice Research Institute, Annual Report (1969).
IRRI, International Rice Research Institute, Annual Report (1970).
Lichtenstein, E. P., *J. Econ. Entomol.* **59**, 985 (1966).
Pathak, M. D., *Intern. Pest Control* **10**(6), 12 (1968).
Sethunathan, N., Caballa, S., Pathak, M. D., *J. Econ. Entomol.* **64**, 571 (1971).
Sethunathan, N., MacRae, I. C., J. AGR. FOOD CHEM. **17**, 221 (1969).
Sethunathan, N., Pathak, M. D., *Canad. J. Microbiol.* **17**, 699 (1971).
Sethunathan, N., Yoshida, T., J. AGR. FOOD CHEM. **17**, 1192 (1969).
Yoshida, T., unpublished data (1971).

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Presence of Residues in Eggs Laid by Chickens Receiving Decoquinate

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Decoquinate-*3*-^{14}C was administered at a level of 0.003% in feed to two laying hens (White Leghorn–Babcock 300) over a 19-day period. All eggs laid by the birds during the medication period and during a 15-day withdrawal period were collected and assayed for radioactivity. A plateau of radioactivity corresponding to 0.88 ppm equivalents of decoquinate was obtained after 10 days of continuous medication. Most of the residue was located in the yolk. The residues in the whites and yolks of eggs obtained in the plateau period were examined by thin-layer chromatography. Decoquinate plus one nondecoquinate component were detected in egg yolks, while decoquinate was the only residue detected in egg white.

Decoquinate [ethyl 6-(decyloxy)-7-ethoxy-4-hydroxy-3-quinolinecarboxylate] has high anticoccidial activity when administered to chickens in the diet at a low concentration (Ball *et al.*, 1968; Johnson *et al.*, 1968). When Craine *et al.* (1971) administered single oral doses of decoquinate-^{14}C to chickens, less than 2% of the radioactivity was excreted in urine. Only minor amounts of the decoquinate dose were converted to nondecoquinate metabolites. Filer *et al.* (1969) obtained a plateau of radioactivity in tissues of broiler chickens after administration of decoquinate-^{14}C through the feed. Using a fluorometric method of measure, Button *et al.* (1969) found residues in tissues of chickens medicated with decoquinate. In both cases a rapid disappearance of residue occurred when medication ceased. Using a tlc analytical method, Ferrando *et al.* (1971) found residues of decoquinate mainly in fatty tissues.

When birds were medicated on successive days with decoquinate-*3*-^{14}C, Craine *et al.* (1971) obtained a plateau of tissue residues within 3 days. The extracted radioactive residues were examined by thin-layer chromatography. Two nondecoquinate components in addition to decoquinate were detected in kidney, liver, and bile. In contrast, only decoquinate was present in muscle, skin, and fat. In the present report decoquinate-^{14}C was fed to laying hens at a recommended use level to determine whether residues accumulated in eggs.

EXPERIMENTAL

Chemicals and Materials. Decoquinate-*3*-^{14}C (Filer *et al.*, 1969) was obtained from May & Baker, Ltd. (Dagenham, England). Particle size of the compound was reduced as described previously (Craine *et al.*, 1971). The compound had a specific activity of 0.61 μCi/mg and radiopurity of 99.9% based on thin-layer chromatographic analysis. The sources of other chemicals were those described by Craine *et al.* (1971).

Dosage Preparation. The decoquinate-*3*-^{14}C (107 mg) was placed in a plastic bag containing 3600 mg of oiled corn meal, mixed to a uniform consistency by kneading and transferred to a 10-l. screw-cap plastic bottle. Laying hen ration was mixed with the decoquinate–corn meal mixture at a level so that each laying hen would receive approximately 3 mg of labeled decoquinate per day. Portions (5 g) of the meal were extracted with chloroform and radioactivity was measured in two aliquots of each extract to determine homogeneity.

Radioactivity Measurement. Radioactivity was measured in a scintillation spectrometer in glass counting vials using a colloidal silica gel suspension system (Green, 1970). Counting efficiency for individual samples was determined with an external γ source. Portions (1.0 ml) of the ethanolic or chloroform–ethanolic extracts of eggs were placed in counting vials which were heated in a water bath to evaporate the solvent. Portions of the lyophilized egg yolk or white (100 mg) were weighed into counting vials. One milliliter of Soluene 100 (digestion fluid) was added and the vials were heated at 50°C for 8 hr. To reduce color a few drops of 50% hydrogen peroxide were added. After 15–20 hr, 1 ml of methanol and 15 ml of the counting system were added and the vials counted. The specific activity of the compound allowed detection of 0.1 ppm of decoquinate.

Birds. Laying hens 36 weeks of age (White Leghorn–Babcock 300—replacements) were housed in a laying battery. Six birds were given a laying hen mash *ad libitum* for a 10-day

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