Fate of Diazinon in Submerged Soil

Accumulation of Hydrolysis Product

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In a study of the persistence of diazinon (C^{14}-labeled at the 4 position on the pyrimidine ring) in submerged soils, soil microflora appeared to assist in its degradation into a less toxic hydrolysis product, (2-isopropyl-6-methyl-4-hydroxy pyrimidine). This hydrolysis product, was, however, resistant to further degradation under submerged conditions.

Diazinon [*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate] has been used increasingly in agriculture to control insect pests of various crops. Earlier literature on diazinon residues in soils has been mainly confined to nonflooded conditions (Getzin and Rosefield, 1966; Getzin, 1967; Gunner *et al.*, 1966). Getzin (1967) reported that greater amounts of the hydrolysis product were recovered from soil fumigated with propylene oxide than from nonfumigated soil. Conversely, little $C^{14}O_2$ was released from the fumigated soil treated with C^{14}-labeled diazinon, while large amounts were released from nonfumigated soil. He also suggested that the initial step in the degradation of diazinon in nonflooded soils is hydrolysis at the heterocyclic phosphate bond (phosphorus-oxygen-pyrimidine bond), followed by disruption of the pyrimidine ring and the subsequent release of $C^{14}O_2$. Soil microflora appeared to play a major role in the degradation of the breakdown products of diazinon, but not in the degradation of the parent molecule. Trela *et al.* (1968) recently observed that the degradation of diazinon into pyrimidine and phosphorothioate derivatives was greatly stimulated in the presence of microorganisms isolated from diazinon-treated soil.

Diazinon has been found to effectively control rice stemborers and some leaf hoppers when a granular form of this insecticide, Basudin 10-G, is applied to the standing water (IRRI, 1967; Pathak, 1966). Reports on diazinon residues in submerged soils are rather scanty. However, previous studies (Sethunathan and MacRae, 1969a) on the persistence of diazinon in submerged neutral or alkaline soil showed that diazinon disappeared at a faster rate from nonsterilized soils than from sterilized soils, thus indicating the participation of soil microflora in its degradation. Surprisingly, only a small amount of $C^{14}O_2$ was released from nonsterilized soils treated with C^{14}-labeled diazinon (labeled at the 2-position on the pyrimidine ring). This result is not in agreement with the results on $C^{14}O_2$ evolution from ring-labeled diazinon reported for nonflooded soils (Getzin, 1967). This difference suggested that the fate of diazinon under submerged conditions might be different from that in nonflooded conditions. The present paper reports the results obtained in the study of the fate of diazinon in submerged soils using C^{14}-labeled diazinon and following the radioactivity.

MATERIALS AND METHODS

C^{14}-Labeled Diazinon and Related Analogs. Unlabeled and labeled diazinon and its related analogs, *viz.*, diazinon and 2-isopropyl-6-methyl-4-hydroxy pyrimidine, were kindly supplied by Geigy Agricultural Chemicals, Basel, Switzerland. Labeled diazinon (labeled at the 4 position of the pyrimidine ring) had a specific activity of 2.6 μc. per mg. The purity of the labeled diazinon was confirmed before use with thin-layer chromatography.

$C^{14}O_2$ Evolution. C^{14}-labeled diazinon was added to 20 grams of air-dried, screened (2 mm.), nonsterilized Maahas clay (pH 6.6, organic matter 2.0%) in glass tubes (25 × 200 mm.). Sterilized soil samples were also prepared by autoclaving thin layers of soil at 121° C. for 1 hour each for 3 days consecutively, and then transferring 20-gram amounts to sterile tubes (Sethunathan and MacRae, 1969a), to confirm the significance of the microbial role in the degradation of diazinon. The soils were then flooded with 20 ml. of 40 p.p.m. of unlabeled aqueous diazinon solution which was previously sterilized by passing it through a Millipore filter (pore size 0.45 ± 0.02 micron). For 30 days, the $C^{14}O_2$ evolution was followed as described earlier (Sethunathan and MacRae, 1969a). Three replicates for each treatment were run. Bulk of the nonsterile submerged soil was reduced within a few days after submergence because of the activity of soil microflora leaving the upper layer of submerged soil, a few millimeters thick, in the oxidized state. This oxidized zone was brownish in view of the presence of ferric oxide. Such a characteristic brownish oxidized layer was absent in sterilized soil, denoting the absence of microbial activity. However, these soil samples were not tested for the microbial counts.

Degradation Studies in Nonflooded Condition. To compare the results obtained under flooded conditions with those of nonflooded conditions, 0.5 ml. of C^{14}-labeled diazinon solution was added to 20-gram samples of sterilized and nonsterilized Maahas clay. Each tube also received 5 ml. of unlabeled diazinon solution (40 p.p.m.), which had been previously sterilized by filtering, to provide 50% water-holding capacity. The soils were then incubated at room temperature and the levels of diazinon and the hydrolysis product on the 30th day were determined after extraction using thin-layer chromatography (TLC) and the isotopic technique.

Identification of Degradation Compound. The method of extraction of diazinon and its metabolites from nonsterilized and sterilized soil samples was based on that of Lichtenstein *et al.* (1967) with some modifications. The soil sample from each tube was shaken first with 60 ml., then with two 40-ml. portions of hexane-acetone (1 to 1) for 5 minutes each, and was centrifuged at 9000 r.p. m. after the addition of 2% Na_2SO_4 solution. The supernatant was transferred to a separatory funnel and the hexane fraction pooled. The water-acetone phase of the soil extract was re-extracted, first with 60 ml. and twice with 40-ml. portions of chloroform–diethyl ether

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Table I. R_f Values for Diazinon, Diazoxon, and 2-Isopropyl-6-Methyl-4-Hydroxy Pyrimidine

Compound	Chloroform: Acetone (7:1)	Acetone
Diazinon	0.87	0.92
Diazoxon	0.64	...
2-Isopropyl-6-methyl-6-hydroxy pyrimidine	0.16	0.68

(1 to 1). Both hexane and chloroform–diethyl ether fractions were evaporated to dryness at room temperature (about 28° C.). The residue was dissolved in 2 ml. of methanol, transferred to a glass vial, again evaporated to dryness, and finally redissolved in 1 ml. of methanol for detection in TLC. The remaining aqueous fraction was used for the determination of C^{14} activity. Analysis by TLC showed that most of the diazinon appeared in the hexane fraction while the chloroform–diethyl ether fraction mainly yielded the hydrolysis product, 2-isopropyl-6-methyl-4-hydroxy pyrimidine.

Thin-Layer Chromatography (TLC). Unlabeled compounds of diazinon and two of its potential analogs, diazoxon and 2-isopropyl-6-methyl-4-hydroxy pyrimidine, were spotted on 250-micron silica gel G plates alongside the hexane and chloroform–diethyl ether fractions of diazinon residues. Chromatograms were then developed in the chloroform-acetone (7 to 1) for a distance of 15 cm. After masking the side of the plate containing samples, the standards were located by their characteristic fluorescence on exposure to short-wave ultraviolet light after spraying the chromatoplate with 0.1% Rhodamine B in 95% ethanol.

The regions corresponding to the developed standards were then carefully scraped from the parallel track on the TLC plate containing radioactive samples. These scrapings from each region were added to 10 ml. of scintillation solution (5 grams of PPO and 0.3 gram of POPOP per liter of toluene, Packard Instrument Co., La Grange, Ill.), allowed to settle down for 3 hours, and the radioactivity was determined in a Tri-Carb scintillation counter, Model 314 EX (Packard Instrument Co., La Grange, Ill.). Counts of TLC scrapings of the standards were also taken.

The identification of 2-isopropyl-6-methyl-4-hydroxy pyrimidine was further confirmed by using another mobile phase, acetone. The R_f values for diazinon and its two analogs are given in Table I.

Gas Chromatography. The gas chromatograph, employed for detection of diazinon and diazoxon, was an Aerograph Model 200, fitted with a phosphorus detector. The column was spiral borosilicate glass (5 feet in length × 1/8-inch I.D.) containing acid-washed Chromosorb G (30 to 60-mesh) coated with 5% DC 200 silicone. The operating temperatures were 195° C. for the column and detector and 225° C. for the injector. The flow rates of nitrogen (carrier gas), hydrogen, and air were 15 to 20 ml., 15, and 170 ml. per minute, respectively.

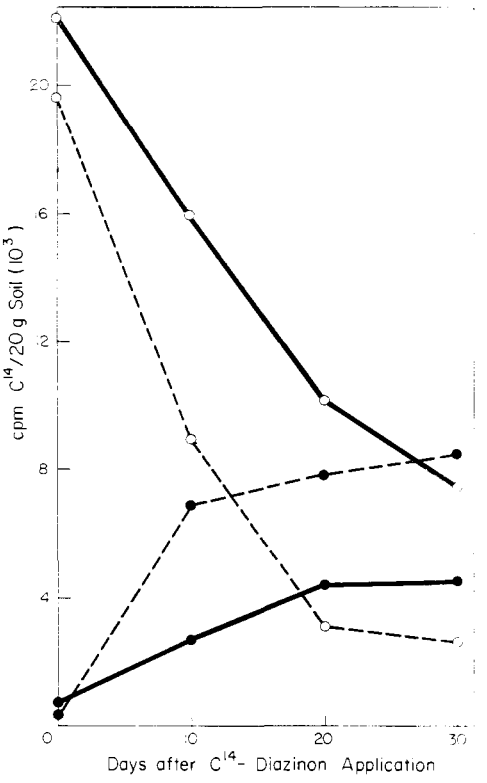

Figure 1. Formation of 2-isopropyl-6-methyl-4-hydroxy pyrimidine from C^{14}-diazinon in submerged Maahas clay

○ Diazinon
● 2-Isopropyl-6-methyl-4-hydroxy pyrimidine
— Sterilized
- - - - - Nonsterilized

Radioactivity in Soil. To determine the radioactivity in the soil or water phase, samples were mixed after solvent extraction with a small amount of CuO and then combusted in the combustion tube of a Coleman Carbon-Hydrogen Analyzer at 800 to 850° C. for 10 minutes. The resulting $C^{14}O_2$ was swept with oxygen (flow rate 120 ml. per minute) and trapped in 5 ml. of hydroxide of hyamine—10X (Packard Instrument Co., La Grange, Ill.). An aliquot (3 ml.) of the hyamine solution was added to 10 ml. of scintillation solution and the radioactivity determined.

RESULTS AND DISCUSSION

Formation of 2-Isopropyl-6-Methyl-4-Hydroxy Pyrimidine. Studies on the identification of degradation compounds of C^{14}-labeled diazinon in submerged soils showed that a radioactive hydrolysis product of diazinon, 2-isopropyl-6-methyl-4-hydroxy pyrimidine, accumulated to a greater extent in nonsterilized soil than in sterilized soil (Figure 1). This difference

Table II. Distribution of Radioactivity Recovered from Different Fractions 30 Days after C^{14}-Diazinon Application to Submerged Maahas Clay (C.P.M./20 G. Soil)[a]

Treatment	$C^{14}O_2$ (C.P.M.)	Hexane[b]	Chloroform–Diethyl Ether[c]	Water[d]	Soil Bound	Initial Radioactivity Recovered	Total Radioactivity Recovered
Nonsterilized	60	28,000	85,000	4,000	81,000	197,000	198,060
Sterilized	0	75,000	45,000	2,000	68,000	221,000	190,000

[a] Fractionation method is described in Materials and Methods.
[b,c] Counts from T.L.C. scrapings.
[d] Remaining fraction after extracting chloroform–diethyl ether layer from acetone-water fraction.

was particularly marked during the first 10 days. A reversible relationship between decrease in diazinon levels and accumulation of hydrolysis product was observed. Evidently, the hydrolysis of diazinon occurs at a faster rate in nonsterilized flooded soil than in sterilized flooded soil, especially during the first 10-day period. This difference would suggest the active participation of soil microflora in the degradation of diazinon to its hydrolysis product. In this process, soil microflora may be involved in a direct attack on the heterocyclic phosphate bond. However, following submergence of soil, the physical and chemical changes which are due to microbial activities might contribute to the diazinon hydrolysis, thus suggesting an indirect role of soil microflora. Furthermore, preliminary experiments showed that the pH of nonsterilized diazinon-treated soil increased from 6.6 to 7.2 following submergence while that of sterilized soil remained at initial 6.6. Diazinon is known to be stable at this range of pH (Margot and Gysin, 1957).

Under upland conditions, Getzin (1967), using C^{14}-labeled diazinon, observed that at 3 weeks after application about 8 and 2% of the total radioactivity were recovered as 2-isopropyl-6-methyl-4-hydroxy pyrimidine from fumigated and nonfumigated soils, respectively. This finding was attributed to a more rapid decomposition of the hydrolysis product (to CO_2) in nonfumigated soil than in fumigated soil. On the other hand, in submerged soils, of the C^{14}-labeled diazinon applied, about 43 and 20% radioactivity accumulated in nonsterilized and sterilized soils, respectively, as hydrolysis product 30 days after application (Table II).

Fate of C^{14}-Diazinon. The distribution of C^{14} added as diazinon after 30 days of incubation in submerged soil is shown in Table II. As previously stated, TLC analysis indicated that only diazinon was identified in hexane fraction. With chloroform–diethyl ether fraction, most of the radioactivity was located at the R_f position of 2-isopropyl-6-methyl-4-hydroxy pyrimidine with faint radioactivity at the R_f position of diazinon. No radioactivity was observed in the R_f position of diazoxon or in any position other than that of parent diazinon or its hydrolysis product on the TLC plate spotted with hexane and chloroform–diethyl ether fractions. Thus the only metabolite detected from the soil was 2-isopropyl-6-methyl-4-hydroxy pyrimidine which carried the radioactive pyrimidine ring. The absence of diazoxon in the extracts was further confirmed using gas chromatograph fitted with a phosphorus detector. The radioactivity included under the hexane fraction in Table II represents cumulative counts at the R_f position of diazinon from both the hexane and chloroform–diethyl ether fractions. Diazinon degraded faster in nonsterilized flooded soils than in sterilized flooded soils leading to a greater accumulation of the hydrolysis product in the former. A good percentage (36 to 40%) of radioactivity was recovered from the soil as soil-bound in both sterilized and nonsterilized soils. This radioactivity would indicate a possible binding of diazinon or its degradation product or products to soil particles during incubation. Although volatilization of diazinon was not significant under the experimental conditions, in actual field condition it may be significant. However, in sterilized soil, 14% of the radioactivity could not be accounted for, perhaps because of an experimental error. A preliminary recovery test for C^{14} immediately after the application of C^{14}-diazinon to submerged soils indicated recovery of only 83.7 to 93.8%. Apparently, the major course of diazinon degradation in neutral soils such as Maahas clay is through hydrolysis, both chemical and microbial, although in acid soil, chemical degradation of diazinon could be more significant (Sethunathan and MacRae, 1969a).

Table III. Recovery of C^{14}-Diazinon and C^{14}-2-Isopropyl-6-Methyl-4-Hydroxy Pyrimidine 30 Days after C^{14}-Diazinon Application to Nonflooded Maahas Clay (C.P.M./20 G. Soil)

	Nonsterilized	Sterilized
Diazinon	14,000	24,000
2-Isopropyl-6-methyl-4-hydroxy pyrimidine	19,000	29,000

Note: 0.5 ml. of aqueous C^{14}-diazinon solution (about 50,000 c.p.m.) was added to each tube.

Mineralization of Pyrimidine Ring. The mineralization of the pyrimidine ring of the diazinon molecule in submerged soil was investigated by measuring the release of $C^{14}O_2$ from sterilized and nonsterilized Maahas clay treated with C^{14}-labeled diazinon (Table II). Nonsterilized soil released more $C^{14}O_2$ than sterilized soil in which liberation of $C^{14}O_2$ was minor, suggesting that cleavage of the pyrimidine ring was caused by microbial action. Nevertheless, the metabolism of the pyrimidine ring appeared to be rather negligible in oxygen-depleted submerged soils since only 0.3% of the originally applied C^{14}-labeled diazinon (labeled at the 4 position in the pyrimidine ring) was accounted for as $C^{14}O_2$. This information is not surprising since only a few anaerobic bacteria are known to destroy common pyrimidines and attempts to isolate them from soil by means of enrichment culture techniques generally have been unsuccessful (Barker, 1961).

Metabolism of the pyrimidine ring would be expected to be more effective using ring-labeled diazinon in nonflooded 'aerobic' soil as reported by Getzin (1967) than in flooded soil if an enzymatic action involved in ring cleavage requires molecular oxygen. Thus, under nonflooded conditions, more hydrolysis product was recovered from sterilized soils than from nonsterilized soils (Table III). In submerged soil which is predominantly anaerobic, the surface layer of soil, a few millimeters thick, is considered in oxidized state. The $C^{14}O_2$ evolution from nonsterilized flooded soil may result from the rupture of a portion of the pyrimidine ring at this oxidized zone. However, the bulk of the pyrimidine ring with labeled carbon remains intact. In this cleavage, actinomycetes may play a major role in view of the earlier findings that applications of diazinon to submerged Maahas clay have a stimulatory effect on this group causing a pigmented zone at the surface layer (Sethunathan and MacRae, 1969b). The importance of synergistic action of mixed microflora in the degradation of diazinon has been reported recently (Gunner and Zuckerman, 1968).

From the foregoing, it appears that the major step in the degradation of diazinon in flooded soil is hydrolysis resulting in the formation of 2-isopropyl-6-methyl-4-hydroxy pyrimidine as one of the degradation products. Under submerged conditions, where the bulk of soil microflora is anaerobic, oxidation is negligible and the hydrolysis product tends to accumulate and persist in large quantities without being oxidized. The more rapid degradation of diazinon and the greater recovery of hydrolysis product from nonsterilized soils suggest that in flooded soils, soil microflora play an important role—direct or indirect—in the degradation of diazinon to its hydrolysis product, 2-isopropyl-6-methyl-4-hydroxy pyrimidine, but not thereafter. The accumulation of 2-isopropyl-6-methyl-4-hydroxy pyrimidine in submerged soils should not, however, pose a serious residue problem

since based on the anticholine esterase activity of the two compounds (Margot and Gysin, 1957), the hydrolysis product of diazinon is far less toxic than the parent molecule. In addition, drying the soil or increasing aeration during land preparation for the succeeding rice crop may completely eliminate this degradation product by oxidation.

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Received for review November 18, 1968. Accepted May 16, 1969. Study supported in part by a grant from the National Science Development Board of the Philippines.