Metabolism of Dichlobenil by Microorganisms in the Aquatic Environment

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The metabolism of [^{14}C]nitrile-labeled dichlobenil (2,6-dichlorobenzonitrile) by a microbial population in pond water and bottom sediment as well as by a pure culture of a bacterium isolated from the hydrosoil was investigated. More than 75% of dichlobenil added to water and sediment disappeared due primarily to volatilization of the herbicide. Dichlobenil was converted microbiologically to 2,6-dichlorobenzamide and certain unknown metabolites in the water and sediment. The radioactivity remaining in the sediment after methanol and HCl extractions varied from 3 to 5% of the originally applied ^{14}C. A cell suspension of an *Arthrobacter* sp. metabolized as much as 71% of dichlobenil to dichlorobenzamide during a 6-day incubation period, but produced very small amounts of other metabolites. When [^{14}C]amide-labeled dichlorobenzamide was incubated in water and sediment, about 6% of ^{14}C was converted to $^{14}CO_2$ 39 days after treatment. It appears that in the aquatic environment, dichlobenil can be metabolized to CO_2 via dichlorobenzamide primarily through microbiological action.

The herbicide dichlobenil (2,6-dichlorobenzonitrile) is useful in controlling a variety of aquatic weeds (Frank et al., 1963; Lawrence et al., 1963; Walker, 1964). It is important to have information on the fate of this herbicide in aquatic environments particularly from the standpoint of environmental pollution. Metabolism by microorganisms is one of the important factors which determine the fate of an herbicide in the aquatic environment.

Presently, very little is known about the metabolism of dichlobenil by aquatic microorganisms, although a few workers have investigated the persistence of this herbicide in the aquatic environment (Van Valin, 1966; Frank and Comes, 1967; Cope et al., 1969; Ogg, 1972). In terrestrial soil, dichlobenil has been shown to be converted to a persistent metabolite, 2,6-dichlorobenzamide (Benyon and Wright, 1968; Briggs and Dawson, 1970; Verloop and Nimmo, 1970). The present investigation was conducted to determine the metabolic transformation of dichlobenil by a mixed population of microorganisms present in the aquatic environment. In addition, the metabolism of the herbicide by a bacterial species isolated from hydrosoil was investigated.

MATERIALS AND METHODS

Chemicals. [^{14}C]Nitrile-labeled dichlobenil (2,6-dichlorobenzonitrile) with a specific activity of 28.8 μCi/mg was supplied by N. V. Philips-Duphar. Nonradioactive samples of dichlobenil, 3-hydroxy-2,6-dichlorobenzamide, 4-hydroxy-2,6-dichlorobenzamide, 3-hydroxy-2,6-dichlorobenzonitrile, and 4-hydroxy-2,6-dichlorobenzonitrile were supplied by Thompson-Hayward Chemical Co. and Philips-Duphar. 2,6-Dichlorobenzamide and 2,6-dichlorobenzoic acid were purchased from Aldrich Chemicals Co.

Metabolism of Dichlobenil in Pond Water and Sediment. Pond water and sediment were collected from a farm pond located 5 miles west of Syracuse, N.Y. The physical and chemical properties of the hydrosoil were: pH 7.8; cation exchange capacity, 5 mequiv/100 g; organic matter, 10.2%; sand, 24%; silt, 59%; and clay, 17%. The soil type was silt loam. The sediment was squeezed through cheesecloth prior to preparing a soil suspension. [^{14}C]Dichlobenil was added at a concentration of 5 ppm to 25 ml of soil suspension (5 g of sediment + 20 ml of pond water) in 50-ml erlenmeyer flasks. The soil suspension, sterilized by autoclaving for 30 min and by subsequent addition of 0.1% sodium azide, was maintained as a control. The flasks were stoppered with cotton plugs and incubated at 25° on a rotary shaker. Duplicate sterile and unsterile flasks were removed 7, 14, 21, and 28 days after treatment and analyzed for [^{14}C]dichlobenil and its possible metabolites. The sediment was separated from water by centrifugation and then refluxed with methanol for 2 hr. The methanol extract was separated from the sediment by centrifugation and the sediment was washed twice with 90% methanol. The water phase and the methanol extracts were combined; the amount of radioactivity in the combined extract was measured by adding 0.1-ml aliquots to 15 ml of liquid scintillation solution (2,5-diphenyloxazole, 15 g; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.75 g; naphthalene, 240 g; dioxane, 1200 ml; toluene, 1200 ml; and ethanol, 720 ml) and counting in a Nuclear-Chicago liquid scintillation counter. After methanol extraction, the sediment was refluxed with 2 *N* HCl in methanol for 1 hr and the extract was assayed for ^{14}C using the same scintillation solution. The amount of radioactivity remaining in the hydrosoil following extraction with methanol and methanolic HCl was determined by wet combustion (Smith et al., 1964). The $^{14}CO_2$ evolved from the combusted soil was trapped in a solution consisting of monoethanolamine and 2-methoxyethanol (1:2, v/v) (Metcalf et al., 1967). The amount of radioactivity in the trapping solution was determined by liquid scintillation counting. The observed radioactivity was corrected for any quenching by channels ratio method.

Dichlobenil was separated from its metabolites in the aqueous methanol phase by steam distillation at pH 9. The metabolites were then fractionated by extraction of the residual solution with ether at different pH values, as described by Verloop and Nimmo (1969). In this fractionation procedure, neutral compounds were extracted at pH 11; free phenols were extracted at pH 4; ether-extractable acids were separated by extraction at pH 0.5. The amount of radioactivity in each fraction was determined by liquid scintillation counting. The metabolites in the different fractions were separated by thin-layer chromatography on silica gel plates. The plates were developed over a distance of 15 cm in the following solvent systems: chloroform–ethanol–acetic acid (89:10:1) (system I) and benzene–methanol (9:1) (system II) (Verloop and Nimmo, 1969). The pH 4 fraction was also chromatographed on thin-layer plates (Merck Polyamide 11F-254 of aluminum) in a solvent mixture consisting of benzene–methanol (4:1) to obtain a better resolution of the metabolites present in this fraction. The polyamide chromatograms were allowed to develop for 3 hr after solvent had reached the edge of the chromatogram. Radioactive compounds were detected by scanning the chromatograms on a Nuclear-Chicago Acti-

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Table I. Distribution of Radioactivity in Soil Suspension Treated with [^{14}C]Dichlobenil

	% of initial ^{14}C							
	Unsterile soil suspension, days after treatment				Sterilized soil suspension, days after treatment			
Fraction	7	14	21	29	7	14	21	29
Water phase + methanol soluble	54.2	28.6	15.8	4.9	55.9	38.6	26.1	24.0
Methanolic HCl soluble	0.8	2.6	3.6	2.3	0.7	1.1	0.3	0.5
Residue after extraction	0.9	3.3	4.8	4.3	0.6	1.1	0.1	0.5
Total	55.9	34.5	24.2	11.5	57.2	40.8	26.5	25.0

Table II. Distribution of [^{14}C]Dichlobenil and Metabolites in Water Phase + Methanol Extract of the Sediment

		% of initial ^{14}C							
		Unsterile soil suspension, days after treatment				Sterilized soil suspension, days after treatment			
Fraction	^{14}C-Labeled compd	7	14	21	29	7	14	21	29
Steam distillate	Dichlobenil	44.8	13.5	3.5	3.5	55.2	37.2	25.5	22.9
Non-steam-distillable									
Ether extract, pH 11	2,6-Dichlorobenzamide	5.4	2.9	2.5	0.9	0.5	0.7	0.5	0.9
Ether extract, pH 4	Unknown, M_1	2.4	9.5	6.4	0.1				
	Unknown, M_2	0.3	0.9	2.1	0.1				
	Unknown, M_3	0.7	1.1	0.8	0.1				
	Total	3.5	11.5	9.3	0.3				
Non-ether-extractable (aqueous phase)		0.5	0.7	0.5	0.2				

graph; nonradioactive compounds were detected by ultraviolet light absorption. Authentic compounds were cochromatographed for comparison with unknown metabolites. The identity of the metabolites was determined by comparing their R_f values with those of known compounds. The activity of each resolved ^{14}C-labeled metabolite was determined by scraping the radioactive zone off the plate into the previously described liquid scintillation fluid containing an added Cab-o-Sil (thixotropic gel suspension powder) and counting it for ^{14}C.

Metabolism of 2,6-Dichlorobenzamide in Pond Water and Sediment. [^{14}C]-2,6-Dichlorobenzamide was added at a concentration of 0.5 ppm to a 100-ml suspension of water and hydrosoil (20 g of hydrosoil + 80 ml of pond water). The soil suspension was incubated for 40 days at 25° and $^{14}CO_2$ evolution was measured in a closed system through which CO_2-free air was circulated. The CO_2 present in the incoming air was removed by passing it through 1 *N* NaOH. The air was then passed through sterile distilled water before passing it through the soil suspension. The $^{14}CO_2$ evolved from the soil was collected in a CO_2-trapping solution described earlier, after passing the gas through a toluene trap. A sterilized soil suspension served as a control. The CO_2-trapping solution was removed and replaced every week and assayed for ^{14}C. At the completion of the incubation period, the sediment was separated from the water phase and extracted with methanol as described earlier. The water phase and the methanol extracts were combined and analyzed for dichlorobenzamide and metabolites by extraction with ether at different pH values as discussed previously.

Metabolism of Dichlobenil by an Aquatic Bacterium. A population of aquatic microorganisms capable of metabolizing dichlobenil was developed by enrichment culture technique using benzonitrile, an unsubstituted analog of dichlobenil, as the sole carbon source. The enrichment medium contained 1 ml of benzonitrile, 0.4 g of KH_2PO_4, 1.6 g of K_2HPO_4, 0.5 g of NH_4NO_3, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.25 g of $FeCl_3 \cdot 6H_2O$ per liter of distilled water, adjusted to pH 7.0. The procedure for enrichment and isolation of microorganisms has been discussed elsewhere (Sikka and Saxena, 1973).

An *Arthrobacter* sp. isolated by enrichment culture technique was grown in the mineral medium containing 0.1% benzonitrile at 28°. The cells were collected by centrifugation after 30 hr of incubation (log phase), washed twice with 0.1 *M* phosphate buffer, and resuspended in a fresh mineral medium to obtain an optical density of 0.5 at 600 nm. [^{14}C]Dichlobenil was added to the cell suspension at a concentration of 5 ppm and the culture was incubated at 28° on a rotary shaker. Aliquots of cell suspension were removed at various times after treatment, and the cells were separated from the medium. The pellet was extracted with methanol followed by another extraction with 80% methanol. The cell extract and the medium were combined and the combined solution was analyzed for [^{14}C]dichlobenil and metabolites as described previously.

RESULTS AND DISCUSSION

Metabolism of Dichlobenil in Pond Water and Sediment. Table I shows the distribution of ^{14}C recovered

from sterilized and unsterile soil suspensions which were treated with 5 ppm of [^{14}C]dichlobenil. The level of total ^{14}C in the unsterilized soil suspension declined rapidly during the first week following treatment. Subsequently, the amount of ^{14}C decreased more gradually. After 4 weeks, the amount of radioactivity had dropped to 11.5% of the initially added ^{14}C. The bulk of ^{14}C in the soil was associated with the water phase and the methanol extract. The amount of ^{14}C remaining in the hydrosoil following extraction with methanol did not exceed 5% of the initially added radioactivity. In the sterilized soil, more than 75% of the dichlobenil had disappeared within 4 weeks of treatment. On the basis of the radioactivity remaining in the sterile soil suspension, it is suggested that the loss of ^{14}C from the unsterilized soil was mainly nonbiological, probably resulting from evaporation of dichlobenil because of its volatile nature (Massini, 1961).

The extent of transformation of [^{14}C]dichlobenil in unsterilized and sterilized soil suspensions is shown in Table II. Dichlobenil seemed to disappear rapidly from the soil suspension. After 4 weeks, 22.9% of the added dichlobenil was recovered from the sterilized soil; after the same length of time, only 3.5% was present in the unsterilized soil. A relatively greater disappearance of [^{14}C]dichlobenil from the unsterile soil suspension as compared to the sterile soil seems to indicate that, in addition to nonbiological factor(s), microbial degradation was also contributing to the disappearance of the herbicide from the unsterile soil. The radioactivity in the non-steam-distillable fraction containing possible dichlobenil metabolites accounted for 9.4% of the initially added ^{14}C 7 days after treatment. After 14 days, ^{14}C in this fraction had increased to 15.1% and then decreased. The ^{14}C activity in a similar fraction from the sterilized suspension never exceeded 1.0%. Analysis of this fraction from unsterile soil revealed the presence of two major metabolites. In contrast, only traces of these metabolites were detected in the sterilized soil suggesting that transformation of dichlobenil in the unsterilized soil was largely a biological process. Our findings are not in agreement with those of Briggs and Dawson (1970) who reported that the degradation of dichlobenil in soil was primarily nonbiological, since they did not detect any measurable difference in the rate of conversion of the herbicide between sterile and nonsterile soils.

Most of the radioactivity attributed to dichlobenil metabolites in the unsterile soil was present in the pH 11 and pH 4 fractions. After 7 days, the pH 11 fraction accounted for 5.4% of the initial radioactivity. The level of ^{14}C in this fraction gradually decreased with a further increase in incubation time. Thin-layer chromatographic analysis of this fraction in two different solvent systems showed that all the ^{14}C was present in the form of a single compound which cochromatographed with authentic 2,6-dichlorobenzamide. A decrease in the amount of [^{14}C]dichlorobenzamide after 7 days suggested that this compound was further transformed in the hydrosoil as a result of microbial activity. In contrast to our results, the findings of Verloop and Nimmo (1969) and Benyon and Wright (1972) showed that dichlorobenzamide was resistant to further degradation in the terrestrial soil under the laboratory or field conditions.

The radioactivity in the pH 4 fraction increased to 11.5% of the initially added ^{14}C 14 days after treatment and decreased thereafter. Thin-layer chromatography of this fraction on polyamide plates showed the presence of three unknown ^{14}C-labeled metabolites (M_1, M_2, and M_3). The metabolite M_1 contained a major portion of the radioactivity associated with the pH 4 fraction. These metabolites were not detected in the extracts of sterile soil. No attempts were made to identify these metabolites on account of the low radioactivity in these compounds. It is speculated that these metabolites are weak acids, possibly phenolic derivatives of dichlobenil. It was observed that

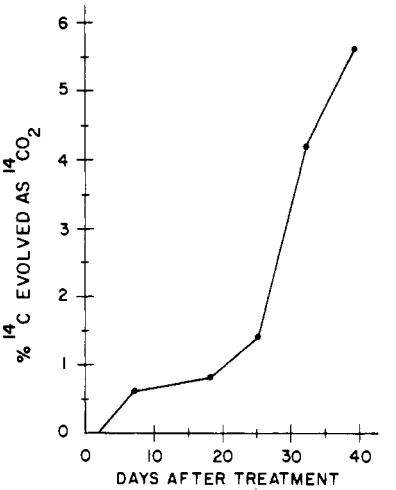

Figure 1. $^{14}CO_2$ evolution from pond water and hydrosoil treated with [^{14}C]-2,6-dichlorobenzamide.

Table III. Relative Distribution of ^{14}C in 2,6-Dichlorobenzamide and Metabolites

Fraction	^{14}C-Labeled compd	% initial ^{14}C
Ether extract, pH 11	2,6-Dichlorobenzamide	66.2
Ether extract, pH 4	Unknown, M_4	20.5
Ether extract, pH 0.5	Unknown, M_5	3.2
	Unknown, M_6	0.8
Non-ether-extractable (aqueous phase)	Unknown	3.7

the ^{14}C in metabolite M_1 increased from 2.5 to 9.5% of the initial radioactivity during the 7–14 day period. This increase was accompanied by a decrease in the amount of 2,6-dichlorobenzamide, suggesting that the latter was converted to M_1.

The aqueous phase following extraction with ether at pH 4 contained a very small amount of radioactivity and therefore further fractionation of this phase was not carried out.

The amount of radioactivity extracted from the sediment with methanolic HCl and that remaining in the sediment was higher in the unsterilized soil than in the sterilized soil. It is likely that higher residual radioactivity in the unsterile sediment resulted from the formation of metabolites which had higher affinity for the sediment as compared to dichlobenil. There also exists the possibility that autoclaving altered the physicochemical characteristics of the soil, causing a decrease in its capacity to adsorb dichlobenil.

It may be mentioned that on account of the high volatility of dichlobenil, it was not possible to measure $^{14}CO_2$ evolution from hydrosoil treated with [^{14}C]dichlobenil using the experimental procedure described for studying the metabolism of [^{14}C]dichlorobenzamide.

Metabolism of Dichlorobenzamide in Water and Sediment. The findings of Verloop and Nimmo (1970) showed that 2,6-dichlorobenzamide was persistent in terrestrial soil because no degradation products could be detected 6 months after the addition of dichlorobenzamide. Our results, however, seem to indicate that dichlorobenzamide was further transformed in pond water and sediment under the laboratory conditions. To test this possibility, the carbonyl carbon labeled [^{14}C]dichlorobenzamide was added to a sample of pond water and sediment

Table IV. Relative Distribution of Radioactivity in Dichlobenil and Metabolites in *Arthrobacter* Cell Suspension

Fraction	^{14}C-Labeled compd	% Initial ^{14}C at days after incubation			
		1	2	3	6
Steam distillate	Dichlobenil	79.9	50.7	28.9	8.7
Non-steam-distallable					
Ether extract, pH 11	2,6-Dichlorobenzamide	9.1	33.7	50.5	70.7
Ether extract, pH 4	Unknown, M_7	0.3	0.6	0.8	3.2
	Unknown, M_8	0.1	0.1	0.2	0.5
Ether extract, pH 0.5	Unknown, M_9	0.1	0.1	0.1	0.2

and its degradation was determined by trapping the evolved radioactive CO_2 and by analyzing the water and sediment for dichlorobenzamide and its possible metabolites.

The evolution of $^{14}CO_2$ from the soil suspension treated with [^{14}C]dichlorobenzamide is shown in Figure 1. Only 5.6% of the originally added [^{14}C]benzamide was converted to $^{14}CO_2$ within 40 days after treatment. Although CO_2 is not the main degradation product, the results confirmed our earlier findings that dichlorobenzamide was degraded in the aquatic environment. The distribution of radioactivity in compounds in soil suspension 40 days following incubation with [^{14}C]dichlorobenzamide is shown in Table III. About 28% of the originally added ^{14}C was present as the metabolites whereas the remaining ^{14}C was in the form of dichlorobenzamide. Only a trace amount of radioactivity was found in the hydrosoil after methanol extraction. The results indicate that dichlorobenzamide was metabolized, though not extensively, under the present experimental conditions. The pH 4 fraction contained the major metabolite as a result of dichlorobenzamide metabolism. Benyon and Wright (1972) suggested that decarboxylation of any benzoic acids formed in soil resulted in CO_2 evolution. Although we could not demonstrate the presence of [^{14}C]dichlorobenzoic acid, the evolution of the radioactive CO_2 suggests the possibility that [^{14}C]dichlorobenzamide was converted to an analog of [^{14}C]benzoic acid which was subsequently decarboxylated to give off $^{14}CO_2$. It was shown in our experiment that $^{14}CO_2$ was evolved through this process, since the initially added [^{14}C]dichlorobenzamide was labeled at the amide moiety. A small amount of the radioactivity in the pH 0.5 fraction suggests that conversion of the dichlorobenzamide to the benzoic acid(s) is a slow process. These findings indicate that aquatic microorganisms degrade dichlobenil to CO_2 possibly via the formation of dichlorobenzamide and dichlorobenzoic acid.

Metabolism of Dichlobenil by an Aquatic Bacterium. Since our studies with hydrosoil indicated that microorganisms were involved in degradation of dichlobenil, the metabolism of the herbicide by a bacterial species isolated from the aquatic environment was investigated. Using enrichment culture technique, an attempt was made to develop a population of microorganisms capable of metabolizing dichlobenil. We were not able to isolate an organism which was able to utilize dichlobenil as the sole source of carbon. In an attempt to induce the formation of enzymes capable of metabolizing the herbicide, analog enrichment technique was used. A bacterial species capable of utilizing benzonitrile (an unsubstituted analog of dichlobenil) as the sole source of carbon was isolated from hydrosoil by an enrichment culture technique. The species was identified as belonging to the genus *Arthrobacter*.

When a cell suspension of *Arthrobacter* was incubated with [^{14}C]dichlobenil, the herbicide was rapidly metabolized. The distribution of ^{14}C among the labeled products in the cell suspension at different times after incubation of the organisms with [^{14}C]dichlobenil is shown in Table IV. The major ^{14}C-labeled metabolite resulting from the conversion of dichlobenil was 2,6-dichlorobenzamide. More than 70% of the initially added ^{14}C was present in the form of this metabolite 6 days after incubation. Small amounts of radioactivity were present in the pH 4 and pH 0.5 fractions. No attempts were made to identify the metabolites in these fractions because of small amounts of these compounds. These results show that hydrolysis of dichlobenil is the major process in the transformation of the herbicide by *Arthrobacter* whereas hydroxylation of the herbicide is a minor metabolic process.

Our findings have clearly demonstrated that microorganisms are directly responsible for dichlobenil degradation. Verloop and Nimmo (1969) also reported that dichlobenil was degraded by soil microorganisms. However, their conclusion was an indirect one since it was based on the absence of degradation in sterilized soil. In the pure culture studies, *Arthrobacter* transformed dichlobenil mainly to 2,6-dichlorobenzamide, whereas in the water and sediment system, additional metabolites were also detected. This difference in the metabolic conversion can be explained by the fact that the water-sediment system is populated by diverse microorganisms and that the joint action of more than one species is responsible for the metabolism of dichlobenil under these conditions.

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