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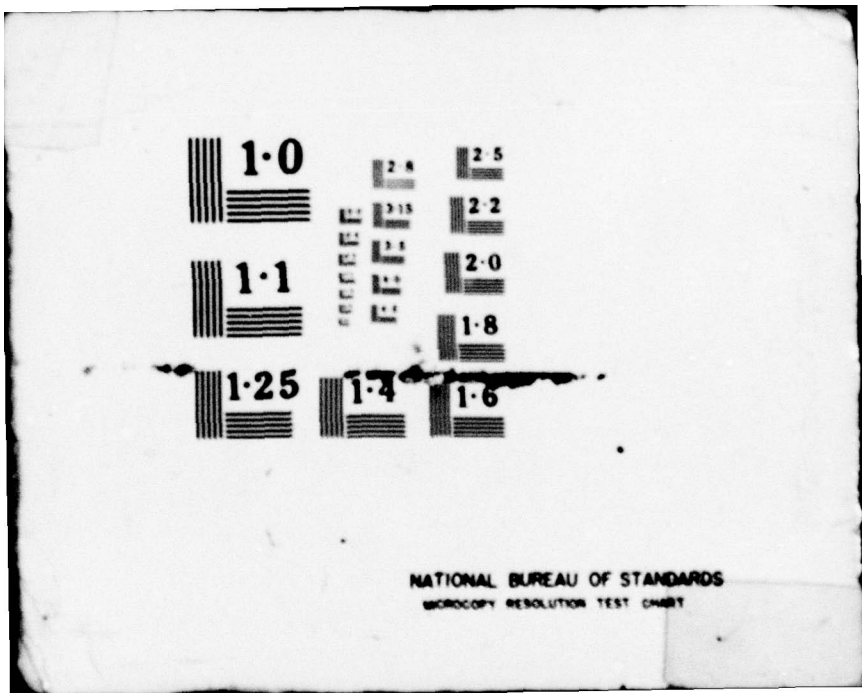
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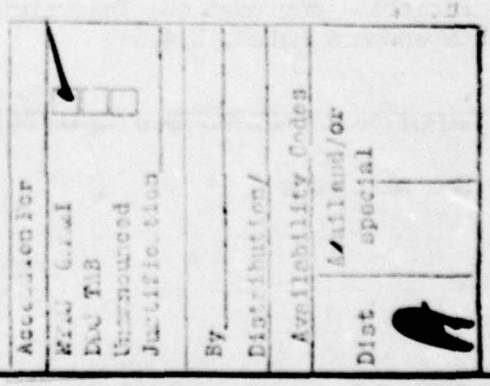
ABSTRACT

The toxicity and degradation of pesticides introduced into sanitary sewer facilities has yet to be extensively studied. For this reason, the effect and fate of eight pesticides were investigated using the BOD method with a sewage inoculum. Six of the eight chemicals (Carbaryl, Malathion, Diazinon, Dursban, Dichlorvos, and 2,4,-dichlorophenoxyacetic acid) did not retard oxygen consumption by a 0.33% sewage inoculum in a one-week test period at concentrations ranging from 0.1 to 100 μg/ml of active ingredient. Baygon at 10 and 100 μg/ml was toxic in the test period. Ficam at 1 and 10 μg/ml also slowed oxygen consumption during the decomposition of sewage as compared to a control with no pesticide. The BOD method was used to test the biodegradation of the pesticides with each chemical being used as a sole carbon source for an adapted or unadapted inoculum. Carbaryl was readily degraded, and Malathion and Diazinon also appeared to undergo oxidation, although the reaction was incomplete. Dichlorvos, Baygon, and 2,4-D were resistant to breakdown when tested by the BOD method.

Little loss of unlabeled 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was evident in four tropical soils in the first two months after addition of the herbicide, but the rate of disappearance then increased with time. Little disappearance was evident in four months in gamma-irradiated soil. The production of $^{14}CO_2$ from ^{14}C-ring-labeled 2,4,5-T was detected in one week in two tropical soils, but two months was required for significant $^{14}CO_2$ production in two other tropical soils.

In the decomposition of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in soil and soil suspension, 2,4,5-trichlorophenol appeared and then disappeared. No 2,4,5-T decomposition was evident in soil or soil suspension sterilized by gamma irradiation. A bacterial culture destroyed about 70% of the 2,4,5-T added to a glucose-inorganic salts medium in 80 h, and nearly 60% of the herbicide that was metabolized was recovered as 2,4,5-trichlorophenol. The bacterium did not use the trichlorophenol as a carbon source, and it did not release $^{14}CO_2$ from ^{14}C-2,4,5-trichlorophenol or ^{14}C-2,4,5-T. Soil suspensions converted 8% of labeled 2,4,5-T and 40% of labeled 2,4,5-trichlorophenol to $^{14}CO_2$ in 25 days. 2,4,5-Trichlorophenol was converted by microorganisms in the soil suspensions to products that were identified as 3,5-dichlorocatechol, 4-chlorocatechol, and succinate by gas chromatography and mass spectrometry and to products that were tentatively identified as cis,cis-2,4-dichloromuconate, 2-chloro-4-carboxymethylene-but-2-enolide, and chlorosuccinate by gas and thin-layer chromatography. Based on these results, a pathway of 2,4,5-T decomposition is proposed.



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ANNUAL REPORT

MICROBIAL DEGRADATION OF PESTICIDES

by

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12 November 1979

This report is in two sections. One section deals with studies of the degradation and effects of insecticides. The second part represents the final phase of studies on the degradation of 2,4,5-T.

I. MICROBIAL DEGRADATION AND EFFECTS OF INSECTICIDES

The literature on the effect and fate of organophosphorus, carbamate, and organochlorine pesticides in the environment continues to expand as new information is sought in order to minimize environmental pollution with these toxicants. Much of the information which already exists relates to the toxic effects of these pesticides on microorganisms growing in laboratory media and on microbial populations and communities in soils. Reviews by Martin (1972) and Powlson (1975) summarize much of this work, and these reviews are augmented by studies by Ballington et al. (1978) on Dichlorvos, Lejczak (1977) on Carbaryl and Baygon, Kuseske et al. (1974) on Baygon, and Hart and Larson (1966) on 2,4-dichlorophenoxyacetic acid. Although much recent work is concerned with persistence and degradation in soil of Dursban and Diazinon (Miles et al., 1979; Tu, 1978), Diazinon (Gunner, 1970), Baygon (Gupta et al., 1975), and Carbaryl (Rodriguez, 1977), not nearly as much work has been directed to the fate and effect in aquatic environments. Bourquin (1977) studied the degradation of Malathion in salt marshes, and Butcher et al. (1977), Sethunathan and Pathak (1972), Aly and El-Dib (1973), and Lichtenstein et al. (1966) studied the fate of Dursban, Diazinon, Baygon, and Carbaryl, respectively, in various freshwater environments. However, little work has been carried out on the effects of pesticides on microbial communites of sewage (Hashinaga et al., 1977).

The introduction of pesticides into sanitary sewer facilities may pose a significant problem, especially where disposal of shop wastes occurs on military installations (Meier et al., 1976). Although the average daily quantities of active pesticide ingredients discharged are usually below 2 ppm (Gebhart, personal communication, 1979), discharges occur at variable

intervals, causing locally higher concentrations. The toxicity of such an influx to the sewage microflora is, therefore, of concern. Further, the potential for degradation of these compounds by sewage microorganisms is also important inasmuch as the treated effluent is usually discharged into natural waterways. Hence, the present study was undertaken to determine the toxicity and potential for degradation of eight selected pesticides in the sewage environment.

MATERIALS AND METHODS

Stock solutions of all insecticides were prepared in anhydrous ethyl ether (absolute, Mallinckrodt, Inc., St. Louis, Mo.) and added to 300 ml standard BOD (biochemical oxygen demand) bottles (Wheaton Scientific, Millville, N.J.). The ether was allowed to evaporate and, upon the addition of 300 ml of the test solution, final concentrations of 0.1, 1.0, 10, and 100 µg/ml were obtained. A list of and some characteristics of the pesticides used are given in Table 1. A solution of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was prepared by dissolving 1.0 g in 100 ml of 0.2 M K_2HPO_4, from which appropriate volumes were drawn to yield final concentrations of 1.0, 10 or 100 µg/ml after addition of 300 ml of diluent. All stock pesticide solutions were prepared fresh for each experiment, and all glassware was washed by soaking at least 24 hours in a nitric acid bath (approx. 15% HNO_3, vol/vol) before use.

BOD diluent was prepared as outlined in Standard Methods for the Examination of Water and Wastewater (pp. 544-546). The final pH was approximately 7.2. The solution was aerated for 4-6 hours at room temperature. A fresh inoculum of municipal sewage was added at the rate of 0.33% (vol/vol) and allowed to mix. BOD bottles containing pesticide were filled with inocu-

TABLE 1

Pesticide characteristics

Compound class	Pesticide name[a]	Other name(s)	Water solubility (µg/ml)
Carbamate	Carbaryl	Sevin	40 at 30°C
Carbamate	Baygon	Propoxur	2000 at 20°C
Carbamate	Ficam	Bendiocarb	10
Thioate	Diazinon	-	40 at 25°C
Thioate	Dursban	Chlorpyrifos, Lorsban	2
Dithioate	Malathion	-	145
-	Dichlorvos	Vapona	ca 10,000
Phenoxy acid	2,4-Dichloro-phenoxyacetic acid	2,4-D	Soluble as K salt

[a]Chemical structure:

Carbaryl: (1-naphthyl N-methylcarbamate)

Baygon: 2-(1-methylethoxy)phenol methylcarbamate

Ficam: 2,2-dimethyl-1,3-benzodioxyl-4-ol-N-methylcarbamate

Diazinon: 0,0-diethyl-0-(2-isopropyl-6-methyl-5-pyrimidinyl)phosphorothioate

Dursban: 0,0-diethyl-0-(3,5,6-trichloro-2-pyridyl)phosphorothioate

Malathion: 0,0-dimethyl-S-(1,2-dicarbethoxyethyl)phosphorodithioate

Dichlorvos: 2,2-dichlorovinyl-0,0-dimethylphosphate

2,4-D: 2,4-dichlorophenoxyacetic acid

lated, aerated diluent and stoppered. The incubation was at 25°C. Initial dissolved oxygen (D.O.) was determined using the azide modified titration method outlined in Standard Methods (pp. 443-444), and the values are the averages of duplicate or triplicate bottles. Subsequent oxygen determinations were made using a polarographic oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Colo.) based on a 100% saturation control preserved by adding 1.0 g of KCN to one BOD bottle at zero time. Five replicates of the no-pesticide treatment as well as of each pesticide concentration were used. The average percentage of the initial D.O. of the five replicates was recorded. The measure of toxicity was made by comparing the resulting percent D.O. at the end of the test period (180-200 hours) for each concentration of each pesticide versus a control with no pesticide.

The assay for degradability of individual pesticides was performed as above except that the inoculum was either "old," carbon-depleted liquid from the toxicity phase of the experiments or one-week old, refrigerated sewage. Whereas the oxidation of carbonaceous material present in the sewage inoculum in combination with the introduced pesticide was measured in the toxicity experiments, in these experiments only pesticide carbon was available for degradation. The measure of degradation was made by comparing the final percent D.O. at the end of the 180-200 hour test period with the theoretically expected amount based on the stoichiometry of each reaction.

RESULTS AND DISCUSSION

The presence of Carbaryl, Malathion, Diazinon, Dursban, Dichlorvos, or 2,4-D in concentrations ranging from 0.1 to 100 µg/ml in 10-fold increments caused little or no apparent toxicity to the BOD of a 0.33% fresh sewage inoculum (Table 2). In all instances, 10 µg/ml of pesticide, in combination

TABLE 2

Toxicity of eight selected pesticides in sewage

Pesticide name	Pesticide concentration (µg/ml)			
	0.1	1.0	10	100
	% D.O. remaining after test period[a]			
Carbaryl	7[b]	13	12	5
Baygon	3	11	51	53
Ficam	ND[c]	35	57	ND
Diazinon	ND	ND	13	ND
Dursban	3	3	3	2
Malathion	2	2	2	ND
Dichlorvos	2	3	15	ND
2,4-D	ND	2	2	2

[a] Test period, 180-200 hours.

[b] Percentage given is highest value obtained in 1, 2, or 3 experiments done using this concentration.

[c] Not done.

with the carbonaceous materials in the sewage inoculum, provided more than adequate oxidizable material to assure 100% removal of oxygen from the saturated system. By the end of the 180-200 hour test period, the highest remaining O_2 concentration in BOD bottles to which any of these six pesticides was introduced was 15%. All values in Table 2 represent the highest percent D.O. remaining at the conclusion of all similar experiments with that particular pesticide. In separate trials with 100 µg of Dursban and 10 µg of Carbaryl/ml, a slight delay occurred before the onset of oxygen utilization (Figure 1) as compared to the no-pesticide control rate. However, complete oxygen consumption was witnessed in the test period, despite the initial lag. Alternatively, in other experiments with Dursban and Malathion, at 100 µg/ml, the initiation of oxidation was seemingly enhanced (Figure 2) as compared to the control. The effects of delay and enhancement evidenced in Figures 1 and 2 may be a result of variations in microbial populations in the sewage inocula, which were collected at different times for different experiments. The delay may result from some initial toxicity which was soon overcome or a binding to organic particulates of the molecules followed by slow release (Miles et al., 1979); the enhancement may result from chemical hydrolysis of the molecule followed by a rapid microbial oxidation (Konrad et al., 1969; Bourne, 1978).

Although six of the eight chemicals did not significantly alter the BOD, two carbamates (Baygon and Ficam) showed potentially toxic effects. At 10 and 100 µg/ml of Baygon and 1 and 10 µg/ml of Ficam, a decrease in the percent of oxygen consumed was seen (Table 2). Greater than 50% of the initial D.O. remained when Baygon was present, and more than 35% when Ficam was present. At the lower Baygon concentrations, the final D.O. was also less than 15%, the highest value remaining in experiments with the other six pesticides.

Figure 1. Possible toxicity of Carbaryl and Dursban to BOD of sewage.

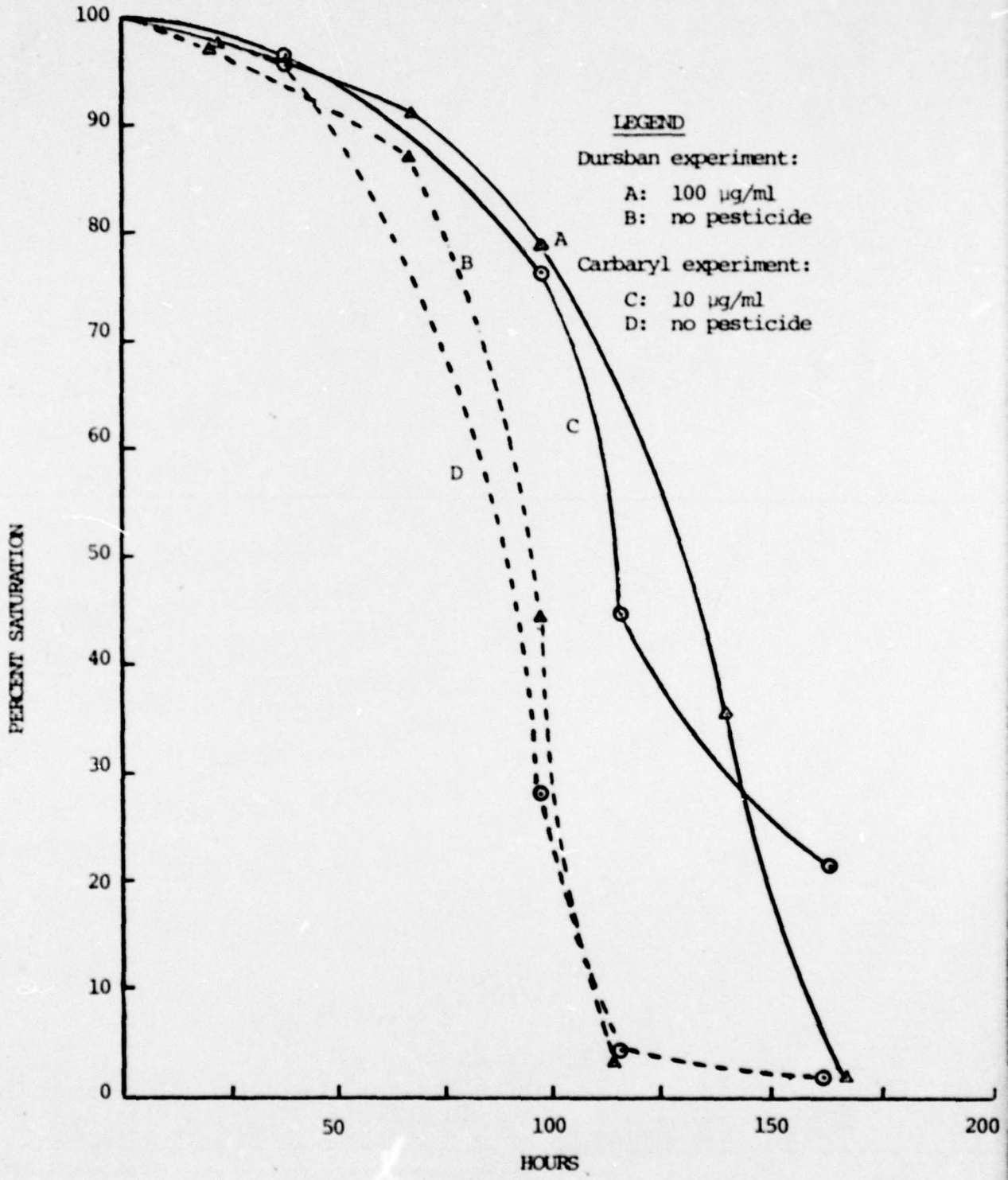
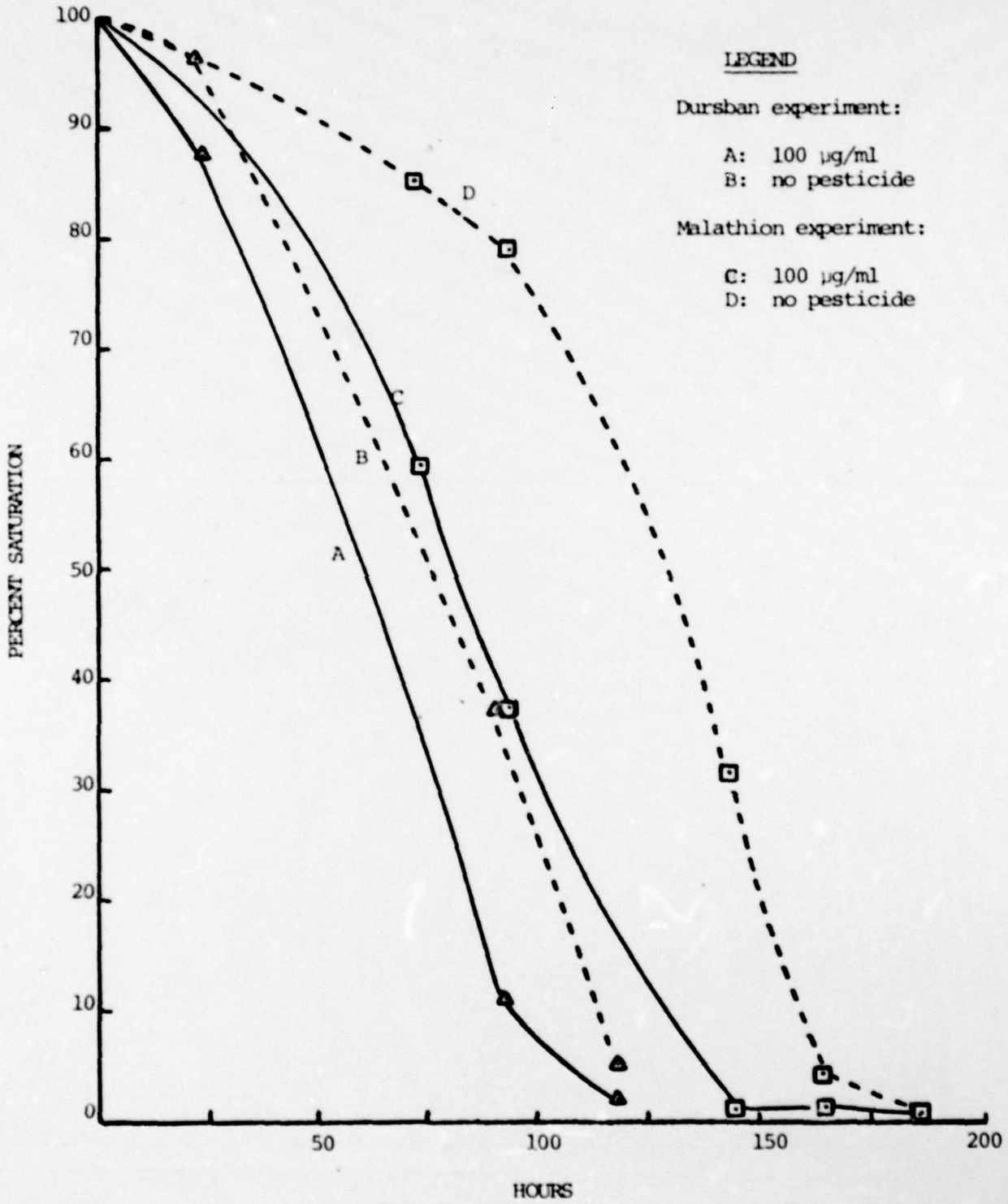


Figure 2. Possible enhancement of BOD of sewage by presence of Dursban and Malathion.



Experiments to determine the susceptibility of the eight pesticides when used as sole carbon sources by microorganisms (and thus be biodegraded) followed directly from the toxicity experiments just described. "Old," carbon-depleted inocula were obtained either from BOD bottles after completion of the toxicity phase of the experiment, or from sewage stored one week at 4°C. In the former, the inoculum should contain organisms tolerant of, if not adapted to, the particular pesticide to which it was exposed; stored sewage inoculum should not have these organisms. The solubilities of all the pesticides except Dursban exceeds the highest concentration, 20 µg/ml, used (Table 1). The water solubility of Dursban is 2 µg/ml.

Based on the theoretical value of 8 µg/ml D.O. in saturated BOD water, the introduction of 10 µg/ml of each of the pesticides except Dursban (23%) should provide sufficient pesticide carbon (111-294%) for total O_2 consumption. However, it is unlikely that the total molecule is immediately available, rather that portions are initially oxidized (Gupta et al., 1975; Liu and Bollag, 1971). Assuming this to be true, the application of 10 µg/ml of pesticide would provide enough immediately oxidizable carbon to account for only 28-79% of the possible O_2 consumption for five of the eight molecules. Malathion (11%) and Diazinon (127%) may still allow for complete O_2 depletion; Dursban (2%) is limited by its water solubility (Table 3).

The experimental results of oxygen consumption recorded in experiments in which 1, 10, or 20 µg/ml of pesticide were added are shown in Table 3. The endogenous oxidation in the treatments with no pesticide has been subtracted from the results presented. Little degradation of Baygon, Dichlorvos, and 2,4-D was evident at the concentrations employed. In all attempts, the amount of oxidation was a less than theoretical. When Diazinon and Malathion were used at 10 µg/ml, O_2 utilization occurred, but the extent was less than

TABLE 3

Degradability of eight selected pesticides in sewage

Pesticide name	Proposed oxidizable leaving group	Theoretical % D.O. consumable[a]		Experimental % D.O. consumed[b]		
		1 µg/ml[c]	10 µg/ml[c]	1 µg/ml[c]	10 µg/ml[c]	20 µg/ml[c]
Baygon	[B]-C(O)NHCH$_3$	8%	79%	5.8%[d]	4.2%	N.D.
Carbaryl	[C]-C(O)NHCH$_3$	8%	79%	9.8%	67.4%	N.D.
Ficam	[F]-C(O)NHCH$_3$	8%	79%	1.5%	(85.4/1/5.2)[e]	40.2%
Diazinon	[D]-P(S)(OC$_2$H$_5$)$_2$	13%	127%	N.D.[f]	20.2%	55.2%
Dursban	[DB]-P(S)(OC$_2$H$_5$)$_2$	2%	2%	N.D.	(2/85.8)	-1.4%
Malathion	[M]-CH(CH$_2$COOC$_2$H$_5$)-COOC$_2$H$_5$	11%	111%	N.D.	26.2%	47.2%
Dichlorvos	[V]-P(O)(OCH$_3$)$_2$	7%	73%	1.0%	5.7%	3.6%
2,4-D	[ph]-CH$_2$COOH	3%	28%	N.D.	2.9%	0%

[a] Based on 8 µg/ml O_2 at saturation.

[b] Value of no pesticide control has been subtracted.

[c] Initial concentration.

[d] Percentage given is average value obtained in 1, 2, or 3 experiments done using this concentration.

[e] Actual values obtained in repeat experiments.

[f] Not done.

theoretical. However, the percentage was doubled at the 20 µg/ml concentration, indicating that probably an accessible portion of the molecule was now available at twice the concentration, but the breakdown of the remainder of the molecules had yet to occur (Paris et al., 1975; Sethunathan and Yoshida, 1973). At the 1 and 10 µg/ml concentrations of Carbaryl, the percentage oxygen consumed approached the theoretical amount, the only pesticide of the eight to do so. Conflicting results were obtained in repeat experiments with 10 µg/ml Ficam and Dursban; in one experiment, complete oxidation was found, whereas no oxidation was seen in a second experiment. However, when 20 µg/ml of Ficam was used, O_2 consumption did reach 40%, whereas there was no increase with 20 µg/ml Dursban, although an increase may not be measurable because of the low solubility of Dursban and the relatively short incubation period. Differences in the activity of microorganisms in the inoculum also may be responsible for the conflicting results as fungal growth was visible in the Dursban experiment in which 85.8% of the O_2 was removed, while no fungus growth was noted in the other.

The degradation of Baygon and 2,4-D, although not noted here, has been reported in aquatic systems (Aly and El-Dib, 1973). Dichlorvos, normally applied as a vapor, and Ficam, a relatively new insecticide (Story, 1977), have been studied infrequently in aquatic environments. Malathion, Diazinon, and Dursban were all susceptible to microbial attack, but at a rate possibly dependent on the substituents present. Carbaryl was shown in this work to be readily degraded, thus confirming the report of Lichtenstein et al. (1966). The differences in the degradability of these compounds may be a result of the short incubation period used, the poor solubility of the molecule, or the absence of growth factors or other essential nutrients (Bourquin, 1977).

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II. 2,4,5-T DECOMPOSITION IN SOIL AND BY MICROORGANISMS IN CULTURE

Many pesticides are transformed in soil by agents that are destroyed by heat, and it is generally believed that the agents responsible for many of these reactions are microorganisms. Nevertheless, microorganisms able to use several of these pesticides as carbon or energy sources have yet to be isolated. On the other hand, microorganisms can often be obtained which cometabolize the molecules; that is, they metabolize the chemicals without using them as nutrients (Alexander, 1979). Evidence exists that 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are destroyed by microbiological means (Yoshida and Castro, 1975), and it is usually assumed that 2,4-D is solely metabolized by organisms growing on the herbicide because bacteria capable of using the compound as a carbon source have been isolated (Bollag et al., 1968a; 1968b). With 2,4,5-T, on the other hand, the only microorganisms found as yet to metabolize this herbicide do not use it as a carbon source (Horvath, 1970; Dimock, 1975).

A likely consequence of cometabolism, at least when the numbers of cometabolizing organisms in the soil are low, is the persistence of the substrate. The reason for the persistence under these conditions is that the small population cannot increase in size because the organisms get no carbon or energy, and hence the population does not increase in size in soils receiving the herbicide with no supplemental carbon (Alexander, 1979). Thus, 2,4,5-T often is found in soil several months after its first addition (Loos, 1975).

The present study was designed to establish the persistence of 2,4,5-T in tropical soils in which, contrary to expectations from studies in temperate soils and current interpretations of cometabolism, it sometimes is destroyed reasonably rapidly (Yoshida and Castro, 1975). The investigation was also

designed to determine whether its conversion might give rise to CO_2, as is characteristic of aerobic processes in soil brought about by microorganisms growing on many synthetic chemicals. Another purpose of the investigation was to ascertain the pathway of 2,4,5-T cometabolism.

MATERIALS AND METHODS

Degradation of 2,4,5-T and 2,4,5-T (^{14}C-ring-UL). Samples (100 g) of soil that had been dried in air and passed through a 2-mm sieve were placed in 1-L Erlenmeyer flasks and amended with 10 µg of 2,4,5-T/g of soil. Identical samples were irradiated with a ^{60}Co source with a dosage of 6 Mrad to sterilize the soil. Periodically, 10.0 g of soil was removed from the flasks and added to 250-mL Erlenmeyer flasks containing 10 mL of 0.1 N HCl and 100 mL of ethyl acetate, diethyl ether, and acetone (5:5:1). The latter flasks were shaken for 60 min, and after the soil settled, the solvent was decanted, dried with anhydrous sodium sulfate, and evaporated to approximately 2 mL with a stream of high purity dry N_2 in a hot water bath. The extract was derivatized with diazomethane (Daughton et al., 1976) for gas chromatography. The extraction was 85-90% efficient compared to 2,4,5-T standards. In experiments in which labeled 2,4,5-T was incubated in soil, thin-layer chromatography of the soil extracts showed that the only labeled compound that was present in the extract cochromatographed with authentic 2,4,5-T.

To determine further the persistence and degradation of 2,4,5-T in soil, 25 g of a silty clay from Trinidad (pH 6.1, 2.8% organic matter), Maahas clay from the Philippines (pH 6.8, 3.0% organic matter), a silty clay from Nigeria (pH 5.7, 2.4% organic matter), or Nipe clay from Puerto Rico (pH 5.8, 2.2% organic matter) in 250-ml biometer flasks (Bellco Glass, Vineland, N.J.) was amended with 10 µg of labeled 2,4,5-T/g of soil. The side arm contained 10 mL of 0.1 N KOH as the trapping solution for the $^{14}CO_2$ evolved. Some soil

samples were sterilized by gamma irradiation, and all samples were incubated at 29 °C. Periodically, duplicate 1.0-mL portions of KOH were removed and placed in disposable scintillation vials (Kimble, Toledo, Ohio) containing 15 ml of aqueous scintillant (ACS, Amersham/Searle Corp) and counted for radioactivity. The radioactivity collected in the KOH trap was verified as $^{14}CO_2$ by acidifying with concentrated HCl. No radioactivity remained in the acidified solution. Before adding 10 mL of fresh KOH, 0.1 g of soil was removed from each biometer flask and treated as described for unlabeled 2,4,5-T. The 50-µL portions were placed in scintillation vials with 15 mL of scintillant and counted.

Materials. 2,4,5-T was obtained from Dow Chemical Co., Midland, Mich., and 2,4,5-, 2,3,4-, 2,3,6-, 2,3,5-, 3,4,5-, and 2,4,6-trichlorophenols and catechol were from Eastman Organic Chemicals, Rochester, N.Y. Uniformly ring-labeled [^{14}C] 2,4,5-T (specific activity 1.61 mCi/mmol) and 2,4,5-TCP (specific activity 4.0 mCi/mmol) were purchased from California Bionuclear Corp., Sun Valley, Calif. The purities of the ^{14}C-labeled compounds were 98.0% and 98.5%, respectively, as determined by thin-layer chromatography. Unlabeled 2,4,5-T was recrystallized twice in benzene before use, and the purity of the compound was greater than 99% as determined by thin-layer chromatography. 3,5-Dichlorocatechol, cis,cis-2,4-dichloromuconate, 2-chloro-4-carboxymethylene-but-2-enolide, chlorosuccinate, succinate, 6-hydroxy-2,4-dichlorophenoxyacetic acid, and 4-chlorocatechol were obtained from Dr. J. M. Duxbury, Cornell University. Thin-layer and gas-liquid chromatography indicated that these chemicals were 85-95% pure. All compounds were dissolved in 95% ethanol prior to addition to media. Standards were prepared with pesticide-grade ethyl acetate (Fisher Scientific Co., Rochester, N.Y). All other chemicals were of the highest purity available commercially.

Glassware. Glassware was cleaned by rinsing in distilled water followed by a 24-h immersion in 20% (v/v) HNO_3. The nitric acid was removed by thorough washing in tap water followed by distilled water.

Incubation Conditions. The basal medium consisted of the inorganic salts solution supplemented with 5.glucose. Because the greatest rate and extent of transformation of phenoxy herbicides in the four soils studied occurred in a Philippine soil (Maahas clay, pH 6.8, 3.0% organic matter, 0.27% total nitrogen), it was used to investigate the metabolite formed in the transformation of 2,4,5-T and 2,4,5-TCP. The soil was amended with 10 µg of 2,4,5-T/g of soil and incubated for 4 months at 29 °C in 250-mL Erlenmeyer flasks. The soil samples were then flooded with 150 mL of basal medium, the flasks were mixed for 2 h at 29 °C and 150 rpm, the contents of the flasks were allowed to settle, and then 20-mL portions were transferred to either 125-ml baffled Erlenmeyer flasks or 250-mL biometer flasks (Bellco Glass, Vineland, N.J.). These suspensions were amended to final concentrations of 10 µg of unlabeled 2,4,5-T or 2,4,5-TCP/mL in the baffled Erlenmeyer flasks or 31 nCi (5.0 µg) of 2,4,5-T (^{14}C-ring-U)/mL or 0.22 µCi (12 µg) of 2,4,5-TCP (^{14}C-ring-U)/mL in the biometer flasks.

The flasks were incubated on a rotary shaker (150 rpm) at 29 °C. The $^{14}CO_2$ evolved from the biometer flasks was trapped in 0.1 N KOH contained in the sidearm, the trapping solution being replaced at appropriate time intervals. One-milliliter portions of the trapping solution were added to 15 mL of Aqueous Counting Scintillant (ACS, Amersham/Searle Corp., Arlington Heights, Ill.) in scintillation vials (Kimble, Toledo, Ohio), and the radioactivity was counted. At the same time, 50-µL portions of the inoculated suspensions were placed in scintillation vials with 15 ml of ACS, and the radioactivity was counted.

The transformation of unlabeled 2,4,5-T was determined in Maahas clay. For this purpose, 100-g portions of soil were air dried and passed through a 2-mm sieve. The herbicide (2.5 mg) in ethanol was added to the reaction flasks, the solution was evaporated to about 0.5 mL, and 1.0 mL of distilled water was added. The soil was then introduced into the flask, and distilled water was finally added to bring the soil to 70% of field capacity. The soil was aerated at a rate of 100 mL/min with water-saturated air that was first freed of CO_2 by passage through Ascarite. The temperature was 23 to 25 °C. In an attempt to enhance 2,4,5-T degradation, one set of flasks was amended with 1.0 mg each of sodium benzoate and glucose/g of soil. For all experiments, possible nonbiological transformation of 2,4,5-T and 2,4,5-TCP was assessed using soil inoculum, cell suspensions, or soil treated with 6 megarads of gamma-irradiation. Controls without added herbicide or 2,4,5-TCP were also prepared, incubated, and analyzed as described.

To determine the products of 2,4,5-T degradation, a bacterium obtained from Maahas clay was grown in the basal medium amended with 20 µg of unlabeled 2,4,5-T or 2,4,5-TCP/mL or with either 31 nCi of ^{14}C-ring-U 2,4,5-T/mL or 0.22 µCi of 2,4,5-TCP/mL. The organism was grown in either 125-mL baffled Erlenmeyer flasks or 250-ml biometer flasks at 29 °C on a rotary shaker operating at 150 rpm.

Analysis of unlabeled metabolites. The compounds were extracted from the mixtures inoculated with soil or the axenic bacterial culture with equal volumes of pesticide-grade ethyl acetate after acidifying to pH 2 with 1N HCl. The extraction was repeated three times, and the solvent phases were dried with anhydrous Na_2SO_4. Based on tests with known concentrations of 2,4,5-T and 2,4,5-TCP, the extraction procedure removed more than 90% of the chemicals from the sample. The solvent was concentrated to about 0.5 ml with dry

N_2 and treated with diazomethane (Daughton et al., 1976).

2,4,5-T and possible metabolites were extracted periodically from the soil by adding 100 mL of a solvent system containing ethyl acetate, diethyl ether, and acetone (5:5:1) to 25 g of soil. The soil was shaken for 60 min, the particles were allowed to settle, and the solvent phase was removed. The solvent was dried with anhydrous Na_2SO_4, concentrated under a stream of dry N_2 to about 5 mL, and treated with diazomethane. The efficiency of recovery of 2,4,5-T from the soil was 85% when tested with known concentrations of the herbicides. For gas chromatography and combined gas chromatography-mass spectrometry, 2,4,5-TCP and possible metabolites formed from 2,4,5-T were prepared in ethyl acetate and derivatized with diazomethane. Analysis was by gas-liquid chromatography or gas chromatography-mass spectrometry.

Analysis of labeled metabolites. The samples were extracted, dried, and concentrated (to 2 mL) as described above, and 50-µL portions were withdrawn for the determination of radioactivity. For chromatographic analysis, 50 µL of extract was spotted on thin-layer silica gel (GF) Redi/plates (Fisher Scientific Co., Pittsburgh, Penna.). Each spot was overlaid with 50 µL containing 150 µg of a possible microbial product generated from the parent chemical. The spots were allowed to dry, and the plates were developed in a solvent system of isopropyl alcohol, ethyl acetate, and ammonium hydroxide (7:9:4). The chromatograms were examined under UV light, the spots containing the metabolites were scraped off the plate, and the resulting material was placed in scintillation vials containing 15 mL of ACS. The radioactivity was then counted.

A second set of thin-layer chromatography plates was developed, dried in air, and placed against Kodak no-screen SB-5 X-ray films (Eastman Kodak Co., Rochester, N.Y.) for 3 to 4 weeks to detect radioactive areas. The

films were developed as described by Wang and Willis (1965) with Kodak X-ray developer and rapid fixer.

Analytical Methods. The disappearance of 2,4,5-T and 2,4,5-TCP was determined by measurement of the UV absorbance of the solution at 292 and 310 nm, respectively, using 1.0-cm quartz cuvettes and a Beckman DU-2 spectrophotometer. All readings were corrected for material absorbing at the same wavelengths. Phenol was determined by the method of Chrastil (1975) using a Bausch and Lomb Spectronic-20 spectrophotometer. Chloride was estimated by the technique of Bergmann and Sanik (1957), and the values obtained were corrected for the chloride in the basal medium. Radioactivity was determined by counting in a Beckman liquid scintillation counter, model LS-100C. All counts were corrected for quenching and background.

Gas chromatographic analysis was performed with a Perkin-Elmer gas-liquid chromatograph, model 3920B, equipped with a flame ionization detector. The packing was 3% OV-17 on 100/120 mesh Gas Chrom W (HP) in a 1.83-m by 2-mm (i.d.) glass column (Supelco, Inc., Bellefonte, Penna.). The operating temperatures were 215 °C for the injector and 250 °C for the interface (detector). The column was maintained for 2 min at 60 °C and programmed at 8°/min to 250 °C, or it was operated isothermally at 135 °C. The flow rate of the helium carrier gas was 30 mL/min. The quantities of 2,4,5-T and its possible metabolites were determined by comparison with standard curves prepared from the authentic chemicals.

Mass spectra were obtained with a Finnigan 3300 mass spectrophotometer, electron impact 70 eV, coupled with a Finnigan 3300 gas chromatograph via a heated single-stage jet separator and using a glass column identical to the one described previously except that it was U-shaped and 1.53-m long. The spectra were compared with those of authentic compounds.

RESULTS

The tropical soils were amended with 10 μg of unlabeled 2,4,5-T. The data show that 2,4,5-T was destroyed in the four tropical soils (Figure 1). After an initial period of up to 2.5 months, during which there was little loss, 2,4,5-T disappearance (as determined by loss of UV absorbance and gas chromatographic analysis) became marked and ranged from 20 to 80% by 4 months. Only 2% loss was detected in gamma-irradiated soil in 16 weeks.

The same soils were amended with 10 μg of labeled 2,4,5-T/g of soil and incubated at 29 °C in biometer flasks. The evolution of $^{14}CO_2$ from these soils is depicted in Figure 2. It is evident that the soils differed appreciably in their activities. In the Philippine soil, $^{14}CO_2$ evolution was evident in one week, but more than 2 months elapsed before $^{14}CO_2$ in appreciable amounts was released from the Puerto Rico soil; 34% of the ^{14}C was released from the former and only 5.2% from the latter soil after 4 months. These data confirm the ring cleavage of 2,4,5-T because the herbicide contained the label in the ring. Less than 1% of the ^{14}C was released as $^{14}CO_2$ in irradiated soil in 4 months.

The disappearance of the ^{14}C-labeled 2,4,5-T from the soil was measured at the same time as $^{14}CO_2$ evolution was monitored. The data show that the appearance of labeled CO_2 occurred concomitantly with the loss of labeled 2,4,5-T (Figure 2). The loss was evident after two weeks in the Philippine soil, but more than two months was needed for a detectable loss in the Puerto Rico soil. After 4 months, approximately 5 to 35% of the ^{14}C in the soils had disappeared, but less than 1% of the chemical had been lost from sterile soil.

The metabolism of 2,4,5-T and 2,4,5-TCP by the mixed populations in a soil suspension was determined by loss of UV absorbance at 292 and 310 nm, respectively, and by gas chromatographic analysis of 2,4,5-T. This mixture

Figure 1. Disappearance of 2,4,5-T from Philippine (PH), Trinidad (TR), Nigerian (NI), and Puerto Rican (PR) soils.

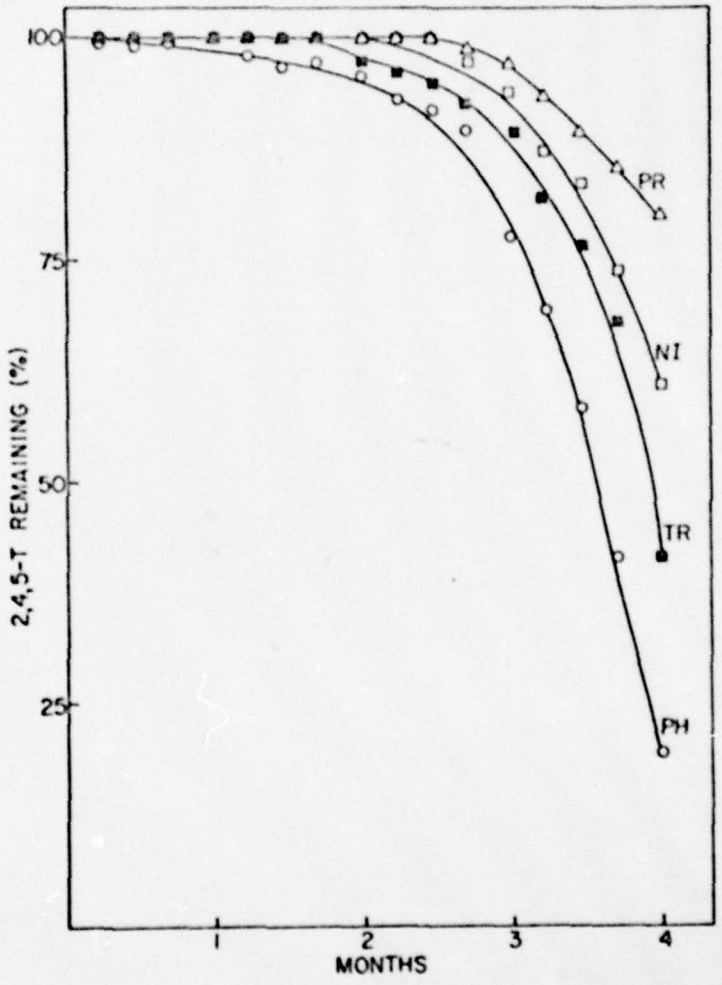
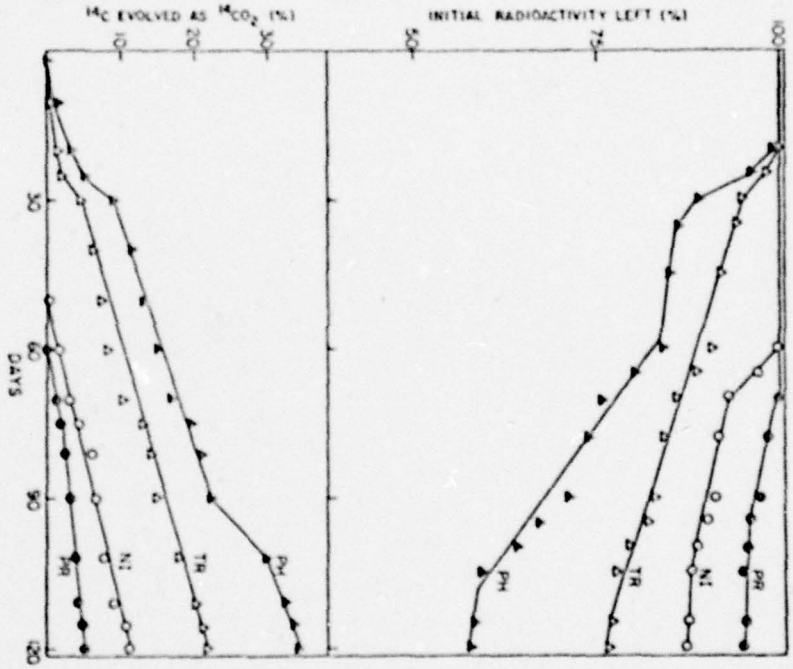


Figure 2. Disappearance of 2,4,5-T (^{14}C-ring-UL) (top) and evolution of $^{14}CO_2$ (bottom) from four tropical soils amended with 10 µg of labeled 2,4,5-T/g of soil (see Figure 1 for abbreviations).



of organisms from soil rather than soil itself was used to facilitate the demonstration of microbial transformations in the preliminary studies. The data suggested that 2,4,5-T disappeared slowly, and gas chromatographic analysis showed that only about 10% was lost after 25 days (Figure 3). The solution gave a reaction in the Chrastil (1975) test suggesting a phenol, and it also contained a product absorbing light at 310 nm, which is the wavelength at which 2,4,5-TCP absorbs maximally. The absorption maxima for 2,4,5-, 2,3,4-, 2,3,6-, 2,3,5-, 3,4,5-, and 2,4,6-trichlorophenols were 310, 283, 289, 280, 305, and 317 nm, respectively. Authentic 2,4,5-TCP added to the soil suspension disappeared rapidly, and more than 80% of the compound was lost at 25 days as judged by decline in UV absorbancy. About 75% of the chlorine in the herbicide was liberated as free chloride. After 25 days, the soil suspension receiving 2,4,5-T was extracted with ethyl acetate and saved for gas chromatography-mass spectrometry. In soil suspensions sterilized with gamma irradiation, no 2,4,5-T decomposition was evident.

The breakdown of 2,4,5-T (^{14}C-ring-U) and 2,4,5-TCP (^{14}C-ring-U) was also determined by measuring the evolution of $^{14}CO_2$ and the decrease of ^{14}C in the soil suspension. The amount of ^{14}C in the soil suspension containing labeled 2,4,5-T did not decrease until day 16, and after 25 days about 12% of the ^{14}C had disappeared from the liquid (Figure 4). Concurrent with the decrease in radioactivity in the suspension was the evolution of $^{14}CO_2$, and about 8% of the initial radioactivity had been evolved as $^{14}CO_2$ by day 25. In contrast, when the soil suspension was incubated with labeled 2,4,5-TCP, the radioactivity in the liquid started to decrease at 4 days, and nearly 50% of the ^{14}C had disappeared from the suspension by day 25. As the ^{14}C derived from 2,4,5-TCP was lost from the suspension, $^{14}CO_2$ was evolved until more than 40% of the initial radioactivity was recovered as $^{14}CO_2$ by day 25. The suspen-

Figure 3. Changes in the concentrations of 2,4,5-T and 2,4,5-TCP in a soil suspension containing 10 μg of 2,4,5-T or 2,4,5-TCP/mL. The product formed from 2,4,5-T absorbed maximally at 310 nm.

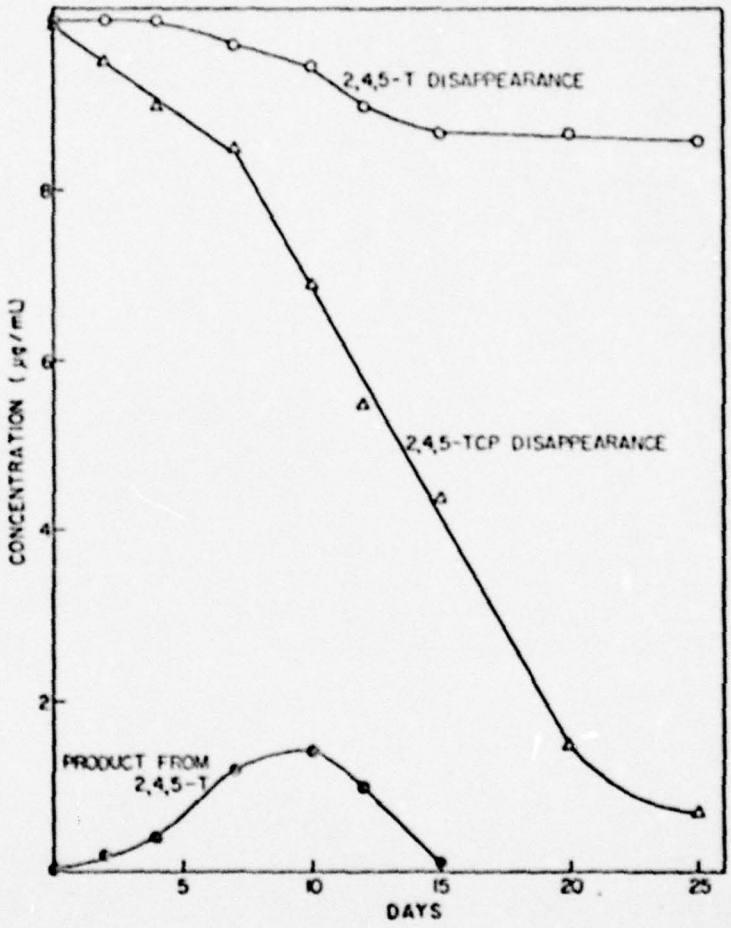
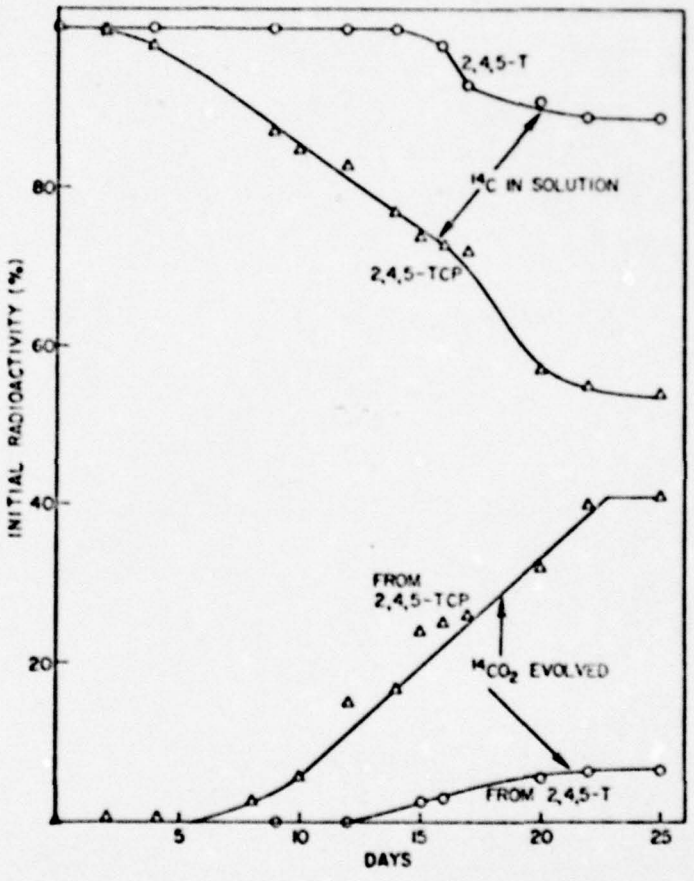


Figure 4. Metabolism of labeled 2,4,5-T or 2,4,5-TCP and evolution of $^{14}CO_2$ by soil suspensions.

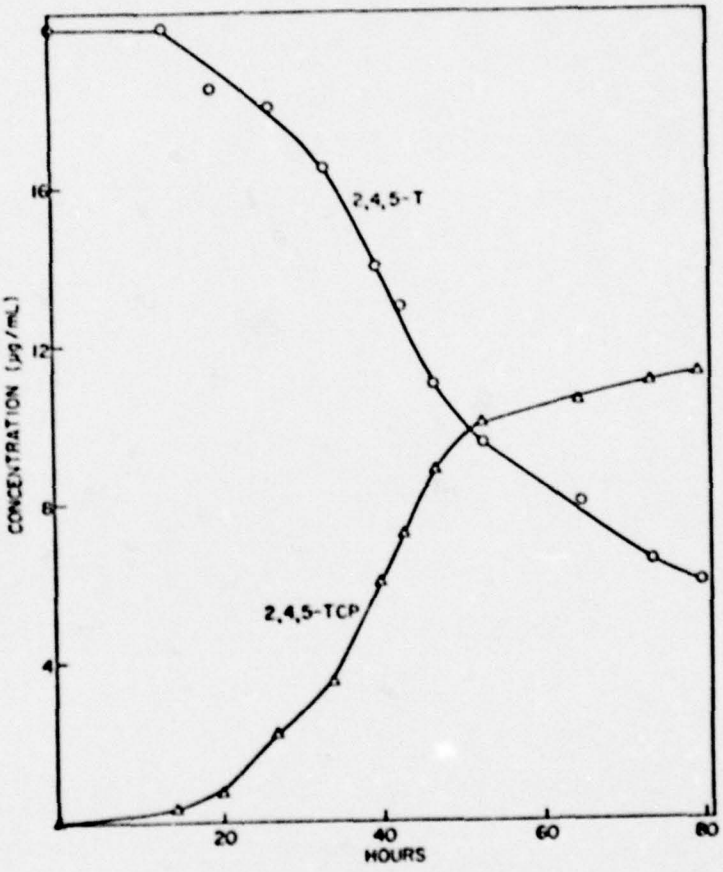


sion was saved for thin-layer chromatography and autoradiography.

A bacterium was isolated from Maahas clay by inoculating soil into a medium containing 100 mg of 2,4,5-T, 0.30 g of glucose, 0.30 g of glycerol, 0.30 g of sodium succinate, 0.50 g of $(NH_4)_2SO_4$, 0.20 g of KCl, 0.20 g of $MgSO_4.7H_2O$, 0.10 g of NaCl, 50 mg of $CaCl_2.2H_2O$, and 20 mg of $FeCl_3.6H_2O$ per liter and 12 mM potassium phosphate buffer. When measurements of the UV absorption indicated that the 2,4,5-T was destroyed and a change in visible turbidity was evident, the enrichment culture was transferred to fresh medium. After two successive transfers, the enrichment was streaked on a solid medium of the same composition. The isolate was identified as Pseudomonas fluorescens based on its physiological and morphological characteristics. The bacterium was grown in the basal medium amended with 20 µg of unlabeled 2,4,5-T and 0.5 µCi of ^{14}C-ring-U 2,4,5-T/mL. When compared to sterile controls, a decrease in 2,4,5-T concentration was evident at 20 h, and about 70% of the chemical had disappeared at 80 h (Figure 5). The analysis of 2,4,5-T was by measuring absorbancy at 292 nm and by gas chromatography of samples of the solution that had been extracted with ethyl acetate and derivatized. At about 20 h, the solution contained a substance absorbing maximally at 310 nm, giving a positive phenol reaction, and having the same retention time as 2,4,5-TCP by gas chromatographic analysis. Assuming that the unknown was 2,4,5-TCP and using 2,4,5-TCP as a standard for quantification, the yield of the presumed 2,4,5-TCP reached a value of nearly 60% of the maximum that could be produced from 2,4,5-T. No free chloride was released into the medium.

The isolate of P. fluorescens could not use 2,4,5-TCP as a carbon source for growth. Moreover, it did not metabolize 2,4,5-TCP in the basal medium supplemented with 20 µg of unlabeled 2,4,5-TCP/mL or with 2.0 µCi (7.7 µg) of labeled 2,4,5-TCP/mL. The bacterium did not liberate $^{14}CO_2$ from labeled

Figure 5. Changes in the concentrations of 2,4,5-T and 2,4,5-TCP effected by a bacterium grown in basal medium amended with 20 µg of 2,4,5-T.

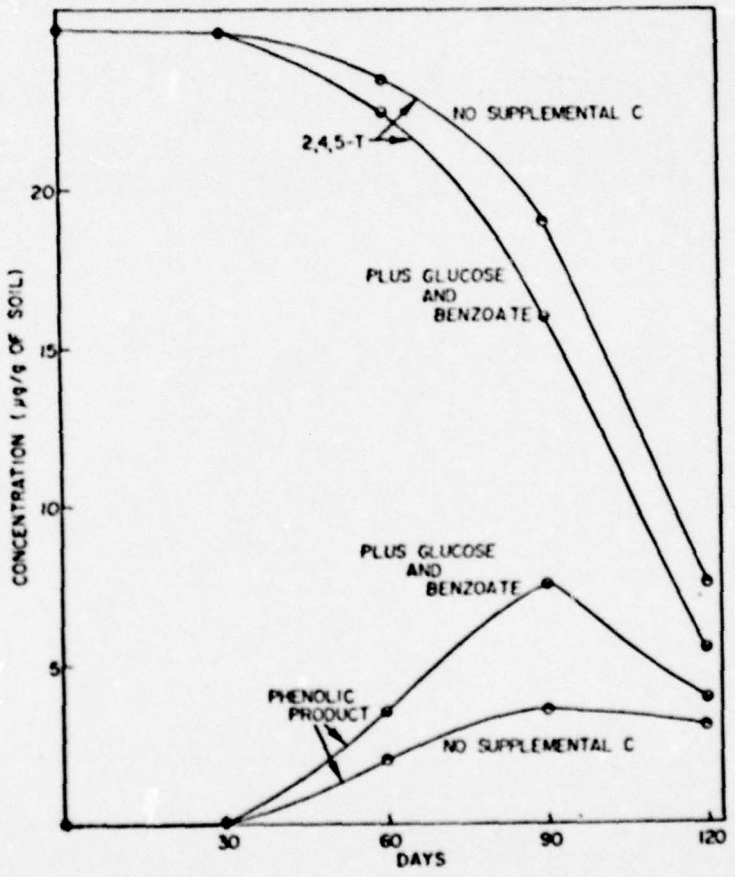


2,4,5-T and 2,4,5-TCP when growing in the basal medium. Thus, the isolate cometabolized 2,4,5-T and converted it to a product that might be 2,4,5-TCP, but it could not degrade the phenol further. The cultures grown in the basal media amended with unlabeled 2,4,5-T were extracted with ethyl acetate, derivatized, and saved for further analysis.

The degradation of 2,4,5-T was determined in Maahas clay amended with 25 µg of the herbicide/g of soil. Some of the soil samples were supplemented with 1.0 mg each of glucose and sodium benzoate/g of soil. Portions (25 g) of the soil samples were removed at 30, 60, 90, and 120 days and extracted, and the extracts were analyzed by gas chromatography. The disappearance of 2,4,5-T was not evident until day 60 regardless of whether the soil had received the glucose-benzoate mixture (Figure 6). This supplement thus did not enhance 2,4,5-T metabolism in the first few days, when the two available carbon sources were likely being utilized. The rate of 2,4,5-T loss appeared to be greater in the carbon-supplemented soil than in the soil receiving 2,4,5-T alone, but the difference was slight and because only two soil replicates were examined, it is not known whether the difference was statistically significant. No 2,4,5-T decomposition was evident in soil by gamma-irradation.

A product was found in the extracts of soil that had been incubated with 2,4,5-T for 60 days. This compound gave a positive test for phenol, and it had the same retention time (270 s) by gas chromatography as authentic 2,4,5-TCP. The concentration of the compound rose with time and then decreased somewhat after 90 days. No other products were found by gas chromatography of the extracts of the 2,4,5-T-amended soil as compared with soil not receiving the herbicide. The extract was saved for further analysis.

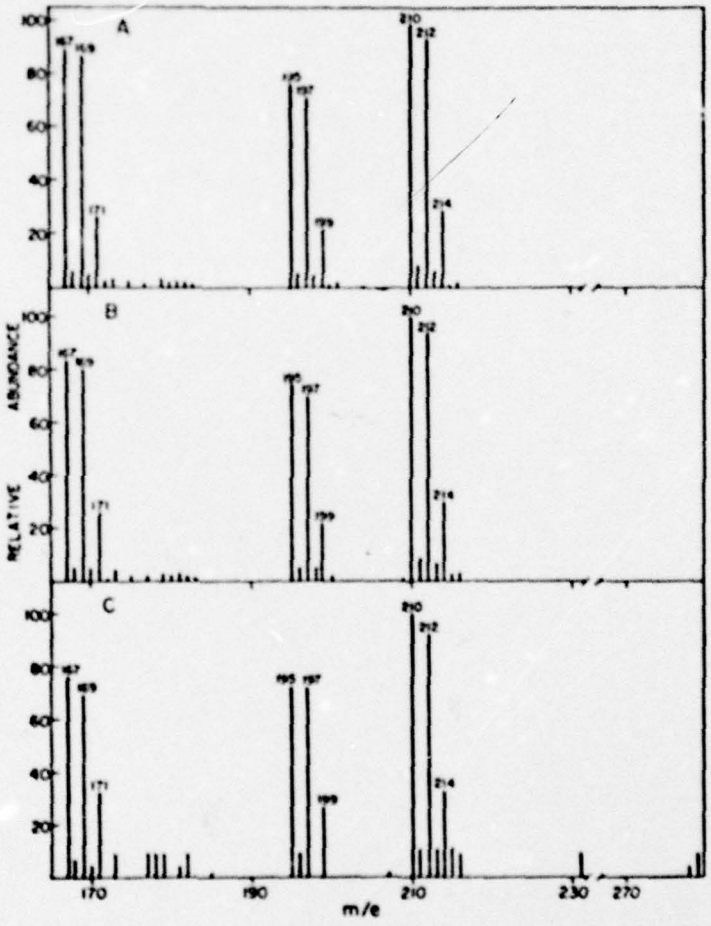
Figure 6. Changes in the concentrations of 2,4,5-T in Maahas clay amended with 25 µg of 2,4,5-T/g of soil and formation of a phenolic product. The soil was either treated with 1.0 mg of glucose and sodium benzoate/g or not so amended.



Derivatized extracts of the soil suspension, P. fluorescens culture, and soil that had been incubated with 2,4,5-T were analyzed by gas chromatography. The methyl derivative of a product in each of these extracts had the same retention time as the methylated derivative of authentic 2,4,5-TCP. The retention times for 2,4,5-, 2,3,4-, 2,3,6-, 2,4,6-, 3,4,5-, and 2,3,5-trichlorophenols were 270, 180, 350, 240, 265, and 105 s, respectively, on the OV-17 column operated isothermally at 135 °C. Although 2,4,5- and 3,4,5-trichlorophenols had similar retention times, their mass spectra differed significantly. Figure 7 shows the spectra for authentic 2,4,5-TCP (A), the metabolite from the P. fluorescens culture (B), and the product obtained from soil (C). The product from the soil suspension incubated with 2,4,5-T had an identical spectrum to that of the other two metabolites. The compounds had molecular ions with m/e of 210 and fragmentation patterns identical to that of authentic 2,4,5-TCP. Thus, microorganisms in axenic culture, soil suspension, and Maahas clay converted 2,4,5-T to 2,4,5-TCP.

Because the soil suspension liberated $^{14}CO_2$ from labeled 2,4,5-TCP, the phenol was further metabolized; hence, the extracts from these suspensions were analyzed for possible products formed from 2,4,5-TCP. The derivatized extract from the inoculum amended with unlabeled 2,4,5-TCP was analyzed by gas chromatography, and the retention times were compared with retention times of authentic 3,5-dichlorocatechol, cis-cis-2,4-dichloromuconate, 2-chloro-4-carboxymethylene-but-2-enolide, chlorosuccinate, succinate, and 4-chlorocatechol. These compounds have been previously identified as products generated in the metabolism of 2,4-dichlorophenoxyacetate (Bollag et al., 1968a, 1968b, Duxbury et al., 1970; Sharpee et al., 1973; Tiedje et al., 1969). Based on comparisons with retention times for these derivatized standards, all of the products were found in the extract of the soil suspension incubated

Figure 7. Mass spectra of the methyl derivatives of authentic 2,4,5-TCP (A) and of a product extracted from a bacterial culture (B) and Maahas clay soil (C) incubated with 2,4,5-T.



with 2,4,5-TCP. The retention times were 16.3, 13.1, 16.7, 10.0, 8.6, and 13.4 min, respectively, on the temperature-programmed OV-17 column.

Mass spectra were obtained for three of these products. One was identical to the mass spectrum of authentic 3,5-dichlorocatechol (Figure 8). The compound showed a molecular ion with m/e of 206, although the major peak occurred at 191, which probably represents the loss of a methyl group and the formation of $-O-CH_2-O-$ from the oxygens of the catechol. The spectrum of the second product was identical to that of 4-chlorocatechol (Figure 9), and the product had a molecular ion with m/e of 172 and a fragmentation pattern identical to that of authentic 4-chlorocatechol. The third product did not show the expected molecular ion at m/e 146, but it did have a base peak at m/e 115 and a fragmentation pattern identical to that of authentic dimethyl succinate (Figure 10).

The other three metabolites that were tentatively identified by gas chromatography were further analyzed by thin-layer chromatography and autoradiography. Extracts from the soil inoculum amended with labeled 2,4,5-TCP were spotted on silica gel plates, and these spots were overlain with 50 µL of a solution containing each of the authentic chemicals. A second set of plates was used for autoradiography. The authentic chemicals were located on the chromatograms by their UV absorption or, in the case of chlorosuccinate, by the use of a spray for halogenated compounds. The plates were first sprayed with a solution of 1.0 g of $AgNO_3$ in 100 mL of 0.5 N NH_4OH solution, they were dried briefly, and then the plates were sprayed with 0.1% ethanolic fluorescein solution (Fisher Scientific Co.). These spots were then scraped off and transferred to scintillation vials containing 15 mL of ACS solution. The radioactivity would have been generated by 2,4,5-TCP metabolism. On the basis of radioactivity on the X-ray film and spot formation, the products appeared to

Figure 8. Mass spectra of the methyl derivatives of authentic 3,5-dichlorocatechol (A) and of a product formed in soil suspension incubated with 2,4,5-TCP (B).

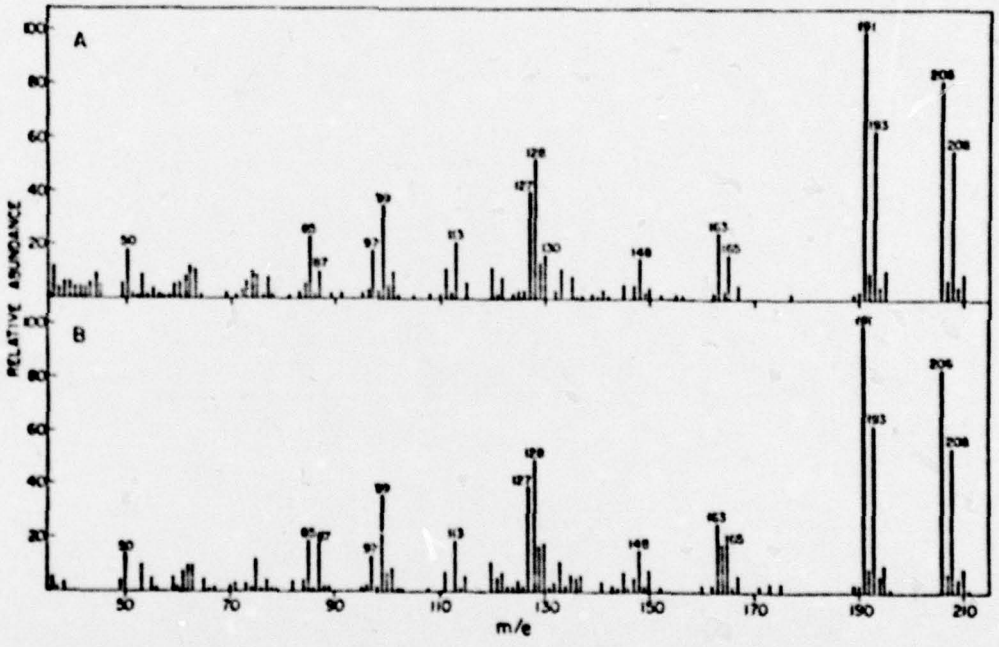


Figure 9. Mass spectra of the methyl derivative of authentic 4-chlorocatechol (A) and of a product formed in soil suspension incubated with 2,4,5-TCP (B).

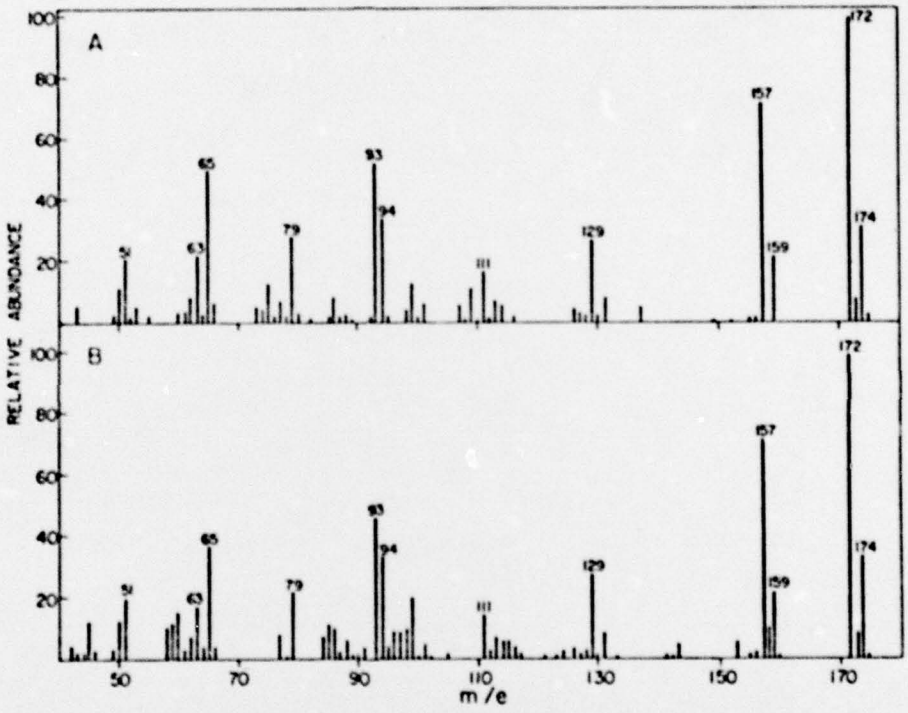
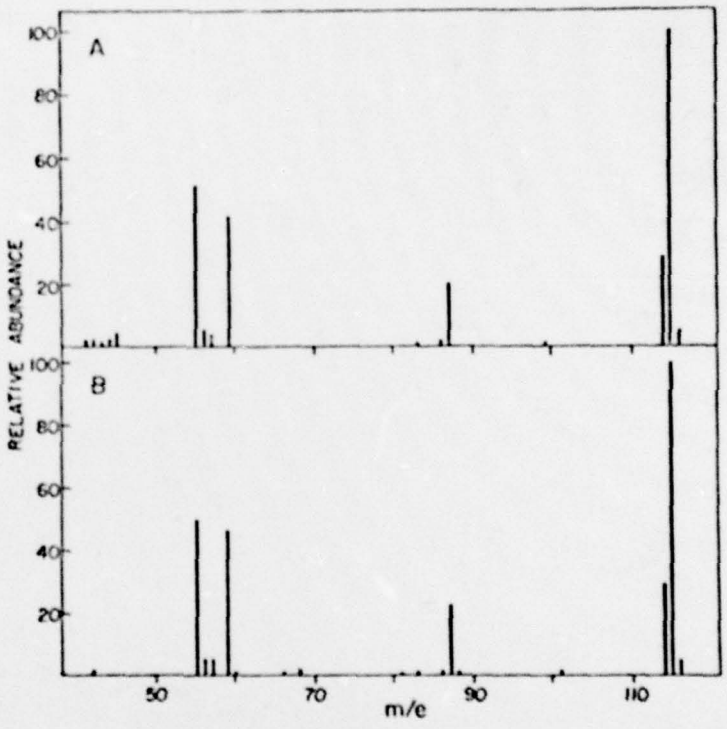


Figure 10. Mass spectra of the methyl derivative of succinate (A) and of a product formed in soil suspension incubated with 2,4,5-TCP (B).



be cis,cis-2,4-dichloromuconate, the chlorobutenolide, and chlorosuccinate. No such products were found in the sterile soil suspensions incubated with 2,4,5-TCP; hence, the products are derived from microbial metabolism.

DISCUSSION

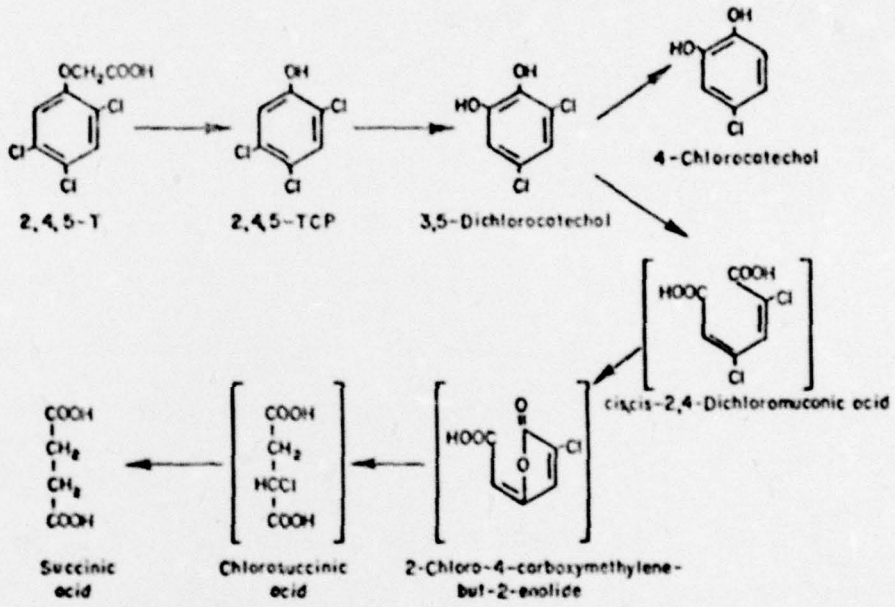
In view of the present findings and the report by Koch (1975) that 2,4,5-T is converted to CO_2, one might expect that microorganisms could be obtained which use it as a source of carbon and energy. However, such an organism had yet to be found, and all of the active bacteria attack 2,4,5-T by cometabolism. Because cometabolizing species do not replicate at the expense of the compound on which they act, the rate of decomposition will remain low should the initial cell number be small. This long persistence coinciding with an apparent microbial transformation is typical of the behavior of 2,4,5-T in soil (Audus, 1951). The fact that some soils more readily destroy the herbicide than others may reflect the content in these soils of nutrients that support replication of the cometabolizing populations, the larger microbial numbers or biomass then being more active in the cometabolic transformation.

The breakdown of 2,4,5-T in soil has been observed by a number of investigators. For example, Yoshida and Castro (1975) detected the microbial destruction of 2,4,5-T in two Philippine soils 12 weeks after addition of the herbicide. Koch (1975) reported the evolution of $^{14}CO_2$ from ring-labeled 2,4,5-T applied to Johnson Island coral, and Sharpee (1973) showed microbial activity on 2,4,5-T in a temperate soil as well as in model aquatic ecosystems. The most extensive microbial destruction of a trichlorophenoxy herbicide was reported by Ou and Sikka (1977), who showed that 2-(2,4,5-trichlorophenoxy) propionate was converted to CO_2. In the present study, 2,4,5-TCP, 3,5-di-

chlorocatechol, 4-chlorocatechol, and succinic acid were identified as products by mass spectrometry. In prior work, it has been found that 2,4,5-TCP is produced from 2,4,5-T in soil and water (Sharpee, 1973) and during the utilization of 2-(2,4,5-trichlorophenoxy)propionate by a mixed microbial culture (Ou and Sikka, 1977). Based on a positive Arnow-catechol test and thin-layer chromatography, Horvath (1970) proposed that Brevibacterium sp. generated 3,5-dichlorocatechol from 2,4,5-T. However, the present inquiry provides definite evidence for the microbial formation of 3,5-dichlorocatechol, and several other intermediates formed in the metabolism of 2,4,5-TCP also have been tentatively identified. Based on these findings, 2,4,5-T appears to be acted on by an initial cleavage of the ether linkage to yield 2,4,5-TCP, which is then converted to 3,5-dichlorocatechol. The benzene ring is then apparently opened to yield products tentatively identified as cis,cis-2,4-dichloromuconate, 2-chloro-4-carboxymethylene-but-2-enolide, and chlorosuccinate, and succinate is the final product of dehalogenation (Figure 11). The pathway of metabolism beyond 3,5-dichlorocatechol thus is analogous to the sequence described for 2,4-D degradation (Loos, 1975). The precursor of 4-chlorocatechol is likely the dichlorocatechol, but it is not presently clear how the dichlorocatechol is generated from 2,4,5-TCP because both a dehalogenation and a hydroxylation are required.

The data presented here demonstrate that 2,4,5-T was acted on by cometabolism because the isolate could not use 2,4,5-T as a carbon and energy source, although it could metabolize the herbicide. An isolate able to use the herbicide as a carbon source for growth has yet to be obtained. However, a soil suspension contained organisms able to transform 2,4,5-TCP to succinic acid, indicating that the 2,4,5-T is ultimately converted to compounds that undoubtedly serve as carbon and energy sources for microorganisms. The usually long

Figure 11. Proposed pathway for the microbial metabolism of 2,4,5-T.



persistence of 2,4,5-T in soil is thus likely a result of the inability of the small cometabolizing population to replicate by using the pesticide as a carbon source, but a product of the cometabolism that can be used as a carbon source would not be found in nature in appreciable concentrations because that product would serve as a substrate for microbial growth.

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