2,4,5-Trichlorophenoxyacetic Acid (2,4,5-T) Decomposition in Tropical Soil and Its Cometabolism by Bacteria in Vitro

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Little loss of unlabeled 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was evident in four tropical soils in the first 2 months after addition of the herbicide, but the rate of disappearance then increased with time. Little disappearance was evident in 4 months in γ-irradiated soil. The production of $^{14}CO_2$ from ^{14}C-ring-labeled 2,4,5-T was detected in 1 week in two tropical soils, but 2 months was required for significant $^{14}CO_2$ production in two other tropical soils. Of 52 bacteria isolated from soil and sewage, 41 metabolized 2,4-dichlorophenoxyacetic acid (2,4-D) and often 2,4,5-T, but none grew by using the herbicides as carbon sources. The cometabolism of 2,4,5-T by these isolates led to chloride release, formation of phenolic products, or cleavage of the aromatic ring.

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,b). 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 whether the cometabolism of 2,4,5-T and 2,4-D was a characteristic of a variety of soil bacteria. The pathway of 2,4,5-T cometabolism is the subject of a separate report (Rosenberg and Alexander, 1980).

MATERIALS AND METHODS

Materials. 2,4-D and 2,4,5-T were obtained from Dow Chemical Co., Midland, MI; phenoxyacetic acid, 2,4-dichlorophenol, 2,4,5-trichlorophenol, and catechol were purchased from Eastman Organic Chemicals, Rochester, NY; and phenol and sodium benzoate were obtained from Mallinckrodt Chemical Works, New York, NY. Uniformly ^{14}C-ring-labeled 2,4,5-T (sp act., 1.61 mCi/mmol) was purchased from California Bionuclear Corp., Sun Valley, CA. The purity of the ^{14}C-labeled compound was 98% as determined by thin-layer chromatography. Before use, unlabeled 2,4,5-T and 2,4-D were recrystallized twice in benzene. The purity of the compounds was greater than 99% as determined by thin-layer chromatography.

Glassware. Glassware was cleaned by a 24-h immersion in 20% (v/v) HNO_3. Nitric acid was removed by multiple washings in tap water, followed by distilled water.

Isolation of Microorganisms. Bacteria were isolated by the enrichment culture technique, using 1.0 mL of municipal sewage or 1.0 g of soil as the initial source of inoculum. The inorganic salts solution for the enrichment contained 0.50 g of $(NH_4)_2SO_4$, 0.20 g of KCl, 0.20 g of $MgSO_4 \cdot 7H_2O$, 0.10 g of NaCl, 50 mg of $CaCl_2 \cdot 2H_2O$, and 20 mg of $FeCl_3 \cdot 6H_2O$ per liter, and it was buffered with 12 mM potassium phosphate buffer, pH 7.2. When used as the source of carbon, phenoxyacetic acid, sodium benzoate, or phenol was added to a final concentration of 1.0 mg/mL. The medium was sterilized by filtration through sterile 0.2-μm membrane filters (Millipore Corp., Bedford, MA). The enrichment cultures (5.0-mL total volume) were incubated without shaking in screw-cap tubes at 29 °C. The disappearance of the organic compounds was determined by measuring the loss of UV absorbance. Once significant loss and visible turbidity occurred, 1.0 mL of the enrichment culture was transferred to fresh medium. After two successive transfers, the enrichments using the organic compounds as the sole carbon source were streaked on agar media containing the inorganic salts solution amended with 500 μg of the compound/mL.

Other bacteria were isolated by the same method, but the enrichment solution contained 0.03% glucose, glycerol, and sodium succinate (basal medium) plus 100 μg/mL of one of the following: 2,4-D, 2,4,5-T, phenoxyacetic acid, 2,4-dichlorophenol, 2,4,5-trichlorophenol, sodium benzoate, or phenol. The colonies that appeared on the agar media with the aromatic compounds as sole carbon sources and those that developed on agar containing glucose, glycerol, succinate as well as one of the aromatic compounds were transferred serially two more times on agar. Colonies that developed on these final plates were inoculated into liquid media containing either (i) the salts solution and 300 μg of 2,4-D or 2,4,5-T/mL or (ii) the inorganic salts solution supplemented with 0.3 g/L each of glucose, glycerol, and sodium succinate as well as 50 μg of 2,4-D or 2,4,5-T/mL.

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The ability of the isolates to attack 2,4-D and 2,4,5-T was subsequently tested by measuring loss of UV absorbancy in the liquid.

Growth curves and measurements of 2,4-D and 2,4,5-T disappearance were performed at 29 °C by growing the organisms in basal medium alone, in basal medium amended with 50 μg of phenoxy compound/mL, or in inorganic salts solution with 300 μg of the phenoxy compound/mL. The cultures were incubated on a rotary shaker, portions were removed periodically, the optical density was determined at 420 nm, and the samples were centrifuged at 10000*g* 4 °C for 15 min prior to assay of the supernatant fluid for 2,4-D or 2,4,5-T disappearance. Nonbiological disappearance of 2,4,5-T was assessed, using sterile medium.

Resting Cell Preparations. For the preparation of resting cells, cultures were grown on a rotary shaker for 36 h at 29 °C in 1-L Erlenmeyer flasks containing 500 mL of basal medium with and without 25 μg of 2,4,5-T/mL. The cells were collected by centrifugation at 10000*g* at 4 °C, and they were washed three times with and resuspended in 10 mL of 10 mM phosphate buffer, pH 7.2, to an optical density of 1.5 at 420 nm. To 10 mL of the resting cell suspension was added 25 μg of 2,4,5-T/mL, and the suspensions were incubated on a rotary shaker for 48 h at 29 °C. The reaction mixtures were centrifuged, and the supernatant fluid was used for analysis. Nonbiological disappearance of 2,4,5-T was assessed with sterile medium.

Manometry. Standard manometric procedures were used (Umbreit et al., 1964). Each flask received 2.67 mL of cell suspension (optical density of 0.70–0.75) in the phosphate buffer and 0.33 mL of either a solution with 1.1 μmol of 2,4,5-T (sodium salt) or the inorganic salts solution with glucose or sodium benzoate as the carbon source.

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.

For further determination of the persistence and degradation of 2,4,5-T in soil, 25 g of 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, NJ) 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 γ 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, OH) 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 addition of 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. Before use, these soils either were stored at room temperature as a slurry in water (Maahas clay) or air-dried.

Analytical Methods. Turbidity was measured at 420 nm in a Bausch and Lomb spectrophotometer, Model Spectronic 20. The disappearance of 2,4,5-T, 2,4-D, and phenoxyacetic acid was monitored by absorbance measurements at 292, 280, and 268 nm, respectively, in 1-cm quartz cuvettes in a Beckman grating spectrophotometer, Model DB-G. Because the samples contained interfering ultraviolet-absorbing material, the reference cuvette was the corresponding but unamended sample.

Chloride release was determined by the technique of Bergmann and Sanik (1957), using the enrichment medium as the blank. Phenol production was estimated by the method of Chrastil (1975) with 2,4,5-trichlorophenol as standard, and catechol production was determined by the procedure of Arnow (1937) with catechol as the standard. Radioactivity in the scintillation vials was determined by counting in a Beckman liquid scintillation counter, Model LS-100C, and 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-1 or 100/120 mesh Gas-Chrom W (HP) in a 1.83 m × 2 mm (i.d.) glass column (Supelco, Inc., Bellafonte, PA). The operating temperatures were 225 °C for the injector and 250 °C for the interface (detector). The column was operated isothermally at 210 °C. The flow rate of the helium carrier gas was 30 mL/min. The retention time of 2,4,5-T was 4.1 min.

RESULTS

The breakdown of 2,4-D, 2,4,5-T, and phenoxyacetate was determined by using 15 mL of the primary effluent of municipal sewage added to 135 mL of basal medium. The degradation was followed by measurements of absorbancy of portions of the mixture that were clarified by centrifugation. Nearly all of the 2,4-D and phenoxyacetate had disappeared after 7 and 12 days, respectively; however, 2,4,5-T was not attacked after 60 days. Subsequent additions of 2,4-D and phenoxyacetic acid were destroyed without a lag period, suggesting the selection for organisms capable of attacking these compounds. In sterile samples of the dilute sewage, more than 95% of the initial absorbancy was still present after 16 days.

Similar results were obtained when Maahas clay (15 g) was added to 135 mL of medium supplemented with the phenoxy compounds, except that the time needed to observe 90% disappearance of 2,4-D and phenoxyacetate was 14 and 16 days, respectively, while 3 and 4 days were required for the disappearance of 75% of the subsequently added 2,4-D and phenoxyacetic acid, respectively (Figure 1). The patterns of degradation in suspensions containing the Trinidad, Nigerian, and Puerto Rican soils were similar to those in suspensions of the Philippine soil.

Bacteria were isolated from enrichment cultures inoculated with sewage or soil and containing phenoxyacetate, benzoate, or phenol as sole carbon source or containing 2,4-D, 2,4,5-T, 2,4-dichlorophenol, 2,4,5-trichlorophenol, phenoxyacetate, benzoate, or phenol in the basal medium

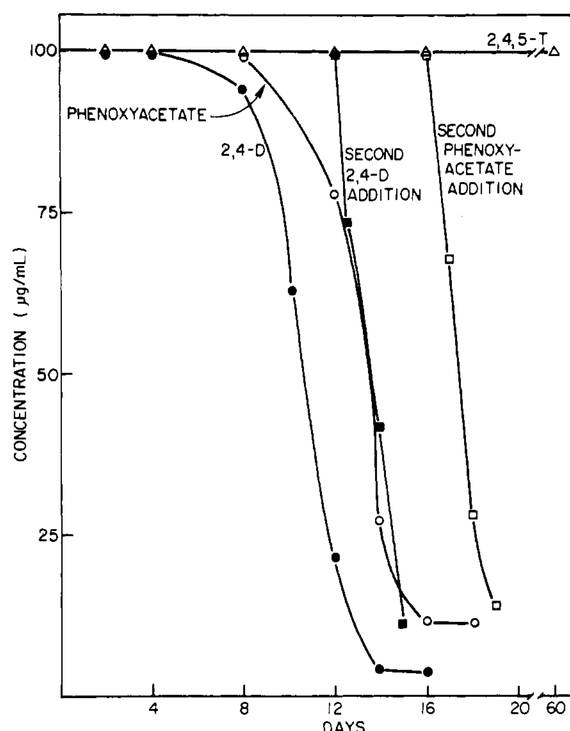

Figure 1. Disappearance of 2,4-D, 2,4,5-T, and phenoxyacetate from suspensions of Maahas clay amended with 100 μg/mL of each compound.

(the basal medium itself contained readily available carbon sources). When a significant loss of UV absorbancy and visible turbidity were evident in the medium, the enrichments were transferred from the liquid to the corresponding agar medium. The resulting colonies were then inoculated into either (i) fresh liquid medium containing the salts solution plus 300 μg of 2,4-D or 2,4,5-T/mL or (ii) the basal medium supplemented with 50 μg of 2,4-D or 2,4,5-T/mL or with no phenoxy compound. From these enrichments, 52 bacterial isolates were obtained.

For determination of the number of isolates that metabolized 2,4-D and 2,4,5-T, the bacteria obtained by enrichment in the basal medium were grown in the basal medium with and without the phenoxy compounds. Loss of the phenoxy compounds was assessed by measuring the decline in UV absorbancy. Growth of these bacteria, as measured turbidimetrically, was the same with or without the phenoxy compounds. The isolates that were able to grow by using phenoxyacetate, benzoate, or phenol as a sole carbon source were inoculated into an inorganic salts liquid medium with 300 μg of 2,4-D or 2,4,5-T/mL as sole carbon source or into the basal medium containing 50 μg of 2,4-D or 2,4,5-T/mL. No isolate used 2,4-D or 2,4,5-T as sole source of carbon.

A summary of the study to show the number of isolates capable of degrading 2,4-D and 2,4,5-T is given in Table I. The data from the soils were pooled. A total of 41 of the 52 bacteria thus originally obtained from sewage or soil metabolized 2,4-D only or 2,4,5-T and 2,4-D. These data show that some bacteria able to use phenoxyacetate, benzoate, and phenol as carbon sources could metabolize 2,4-D or 2,4,5-T and that some isolated because they could metabolize 2,4-dichlorophenol and 2,4,5-trichlorophenol also could metabolize the two herbicides.

The isolates were studied further to ascertain their morphological and biochemical characteristics. Of the 41 isolates, 88% were Gram-negative, 78% were rod-shaped, 58% were motile, 32% were pigmented and/or fluorescent, 68% were oxidase-positive, and 94% were catalase-positive.

Table I. Number of 2,4-D- and 2,4,5-T-Metabolizing Bacteria Isolated from Sewage and Soil

enrichment medium	no. of sewage isolates metabolizing		no. of soil isolates metabolizing	
	2,4-D only	2,4,5-T and 2,4-D	2,4-D only	2,4,5-T and 2,4-D
2,4-D[a]	2	1	2	2
2,4,5-T[a]	2	2	2	2
phenoxyacetate[a]	1	1	1	1
phenoxyacetate[b]	0	0	1	0
benzoate[a]	2	1	2	2
benzoate[b]	1	1	1	1
phenol[a]	1	1	1	1
phenol[b]	0	0	1	1
2,4-dichlorophenol[a]	0	0	1	1
2,4,5-trichlorophenol[a]	0	0	1	1

[a] In basal medium. [b] In inorganic salts medium.

Table II. Metabolism of 2,4,5-T, Release of Chloride, and Production of Phenol by Resting-Cell Suspensions of 19 Bacteria

bacterium	2,4,5-T lost,[a] %	chloride released,[b] %	phenol/2,4,5-T[c] (as %)
1A1	40	24	39
2A1	8	0	60
2A2	34	40	32
2A3	88	94	0
4A3	35	0	35
4A5	44	24	0
5A5	56	0	0
1B7	44	40	0
6B2	24	0	12
2C1	52	0	0
2C2	23	0	23
2C4	64	30	0
4C1	44	50	0
4C3	88	90	0
4C5	28	0	27
4D3	24	24	23
5D3	92	90	0
3E3	72	40	0
5F4	68	50	0

[a] Initial concentration: 25 μg/mL. [b] Percent of the organic chlorine released as chloride. [c] Percent of the theoretical yield of phenol, assuming all the 2,4,5-T was converted to a phenol.

The 19 bacterial isolates capable of cometabolizing 2,4,5-T as well as 2,4-D were grown in the basal medium supplemented with 25 μg of 2,4,5-T/mL. Resting-cell suspensions of these bacteria were prepared, and 2,4,5-T disappearance, phenol and catechol production, and chloride release were determined. In sterile controls, no chloride was released, no phenol was produced, and less than 5% of the 2,4,5-T disappeared. In contrast, extensive metabolism occurred with several of the isolates in a 48-h incubation period (Table II). Twelve isolates released chloride, and eight produced a phenolic compound from 2,4,5-T. No catechol was detected in the medium after a 48-h incubation period. The loss of UV absorbancy effected by some of the isolates, the release of chloride, and the absence of phenol and catechol indicated that certain isolates destroyed the aromatic ring. The production of phenol with and without chloride suggested that some of the isolates converted 2,4,5-T to 2,4,5-trichlorophenol and to mono- and dichlorophenols.

When grown in the basal medium amended with 50 μg of 2,4,5-T/mL, bacterium 4C3 multiplied as indicated by increases in optical density; at the same time, 2,4,5-T disappeared as measured by loss of UV absorbancy. The

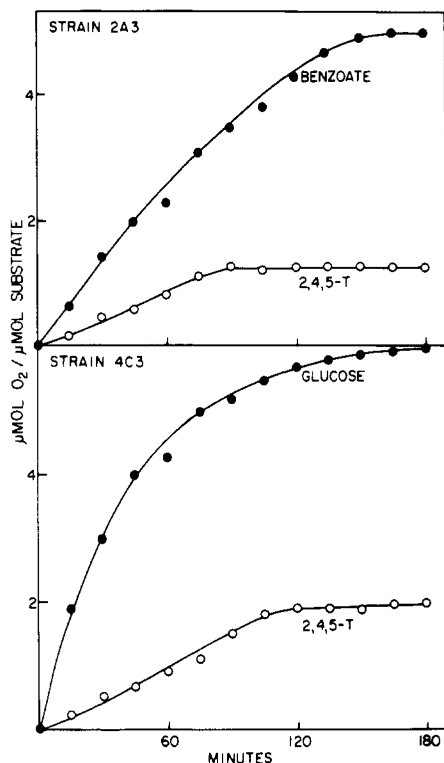

Figure 2. Metabolism of glucose, sodium benzoate, and 2,4,5-T by resting cells of bacteria grown in media with 0.5% glucose or 0.5% sodium benzoate.

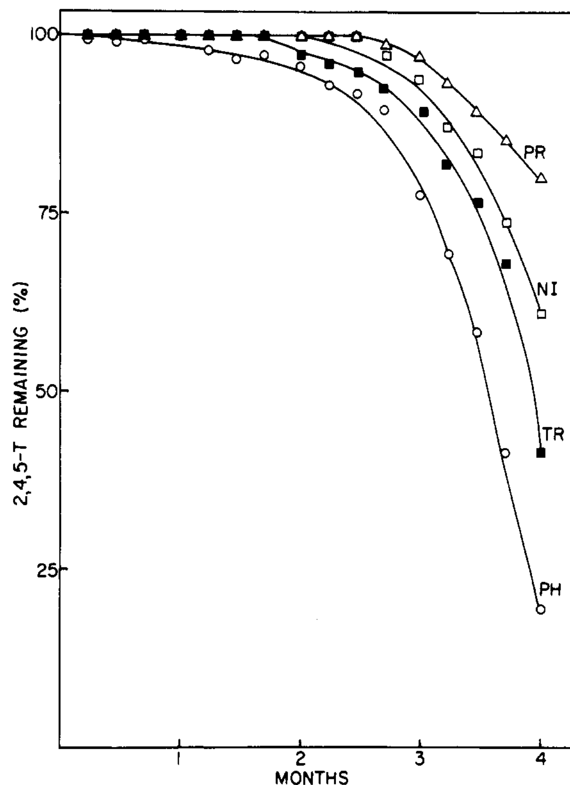

Figure 3. Disappearance of 2,4,5-T from Philippine (PH), Trinidad (TR), Nigerian (NI), and Puerto Rican (PR) soils.

loss of 2,4,5-T paralleled growth, and when growth reached the maximum optical density (at 30 h), about 80% of the absorbancy resulting from the herbicide had disappeared. Less than 2% of the pesticide disappeared from sterile 2,4,5-T-amended medium in 90 h, and no growth occurred in 90 h in the inorganic salts solution supplemented with 300 μg of 2,4,5-T/mL. The inability of 2,4,5-T to serve as sole carbon source and the disappearance of the compound in the presence of a second carbon source suggest that the chemical was cometabolized. Further support for cometabolism of 2,4,5-T was obtained in a study in which isolate 4C3 was grown in the inorganic salts medium with limiting glucose concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 5.5 mM) and amended with 0, 1.0, or 3.0 mM 2,4,5-T; under these conditions, the growth as measured turbidimetrically was the same whether 2,4,5-T was present or not, although 10–88% of the 2,4,5-T had disappeared. Similar data indicated that isolates 2A3 and 5D3 also cometabolized 2,4,5-T.

The ability of resting cell suspensions of two of these isolates to consume O_2 in the presence of 2,4,5-T was then investigated. Strain 2A3 was grown in a medium with benzoate as sole carbon source, and strain 4C3 was grown in a medium with glucose as the only carbon source. The values presented are means of duplicate determinations and are corrected for endogenous respiration. As shown in Figure 2, the two bacteria oxidized the herbicide. The extent of oxidation was equivalent to about 20 and 25% of the theoretical amount to completely oxidize the 2,4,5-T for strains 2A3 and 4C3, respectively. In each instance, greater activity was noted on the organic compound used for growth. Cells of glucose-grown strain 2A3 consumed almost no O_2 in 3 h in the presence of 2,4,5-T. The activity of strain 5D3 was essentially the same as that of bacterium 4C3.

For further determination of the persistence and degradation of 2,4,5-T in soil, 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 3). 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 γ-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 4. It is evident that the soils differed appreciably in their activities. In the Philippine soil, $^{14}CO_2$ evolution was evident in 1 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 4). The loss was evident after 2 weeks in the Philippine soil, but more than 2 months was needed for a detectable loss in the Puerto Rico soil. After 4 months, approximately 5–35% of the ^{14}C in the soils had disappeared, but less than 1% of the chemical had been lost from sterile soil.

DISCUSSION

The present data suggest that many bacteria are capable of cometabolizing 2,4-D and 2,4,5-T. As the latter compound is cometabolized, chloride and a phenol are frequently formed. The fact that some bacteria brought about loss of UV absorbancy indicates they are capable

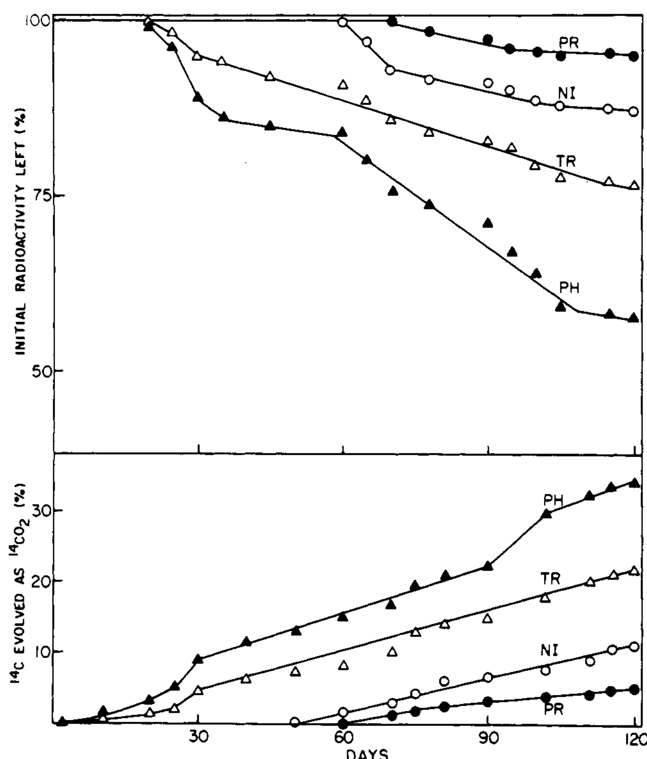

Figure 4. Disappearance of 2,4,5-T (^{14}C-ring-UL) (top) and evolution of $^{14}CO_2$ (bottom) from four tropical soils amended with 10 μg of labeled 2,4,5-T/g of soil (see Figure 3 for abbreviations).

of cleaving the ring. These results are in agreement with the findings of Ou and Sikka (1977), who found extensive degradation of the aromatic ring of a structurally similar molecule, 2-(2,4,5-trichlorophenoxy)propionate, by aquatic bacteria. Phenoxy herbicides with a chlorine in the meta position are generally resistant to microbial degradation (Burger et al., 1962), but it is clear from the data presented herein that high cell densities can bring about significant metabolism in short periods and that microorganisms transform, albeit slowly, the chemical in soil too. Earlier reports have shown a microbial involvement in the slow 2,4,5-T breakdown in soil (Sharpee, 1973; Yoshida and Castro, 1975).

Some of the bacteria that produced a phenolic compound from the herbicide also release chloride, which may indicate the conversion of 2,4,5-T to mono- or dichlorophenols. Other bacteria generated a phenol without chloride release, suggesting the formation of 2,4,5-trichlorophenol. Direct evidence for the production of 2,4,5-trichlorophenol from 2,4,5-T in soil was obtained by Sharpee (1973). In view of the appearance of chlorinated phenols, 2,4,5-T degradation may involve the cleavage of the acetate moiety as described for 2,4-D degradation (Tiedje and Alexander, 1969). The trichlorophenol that is then formed may be converted to 3,5-dichlorocatechol as described by Horvath (1970); such a catechol may be decomposed as described for 2,4-D since this catechol is also produced during the bacterial degradation of 2,4-D (Bollag et al., 1968a,b). Identification of products generated during microbial metabolism in vitro is the subject of a separate paper (Rosenberg and Alexander, 1980).

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 has 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.

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