Degradation of 4-Aminopyridine in Soil

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The rate of 4-aminopyridine (4-AP) degradation was studied in soil and in pure cultures of five soil microorganisms. In three different soils, between 6 and 24% of the 4-AP-2-^{14}C applied was recovered as $^{14}CO_2$ after 60 days. When chopped corn containing 3% 4-AP-2-^{14}C was incubated 70 days on moist soil, 16% of the radioactivity was recovered as carbon dioxide. On incubation of 4-AP-2-^{14}C in flooded soils, 21 and 24% of the radioactivity was lost after 60 days in two different soils. Pure cultures of *Pseudomonas fluorescens*, *Enterobacter aerogenes*, *Aspergillus niger*, *Streptomyces griseus*, and *Agrobacterium tumefaciens* were unable to metabolize 4-AP to a significant extent during 120 h of incubation. The compound did not retard growth of these organisms at 100 μg/ml.

The compound 4-aminopyridine (4-AP) has been found to be a successful chemical frightening agent of birds (Goodhue and Baumgartner, 1965). It has proved effective in protecting fields of corn and sunflowers from blackbirds (De Grazio et al., 1971, 1972; Stickley et al., 1972; Pfeifer, 1968–1972). The absorption and metabolism of the compound by plants and its phytotoxicity have been studied (Starr and Cunningham, 1974, 1975a). Data on the leaching and degradation of 4-AP in soil (Starr and Cunningham, 1975b) became available to us after completion of our studies. We report here data concerning the decomposition of 4-AP in soil and by pure cultures of soil microorganisms.

MATERIALS AND METHODS

The properties of the soils used in this study are reported in Table I. The soils were air-dried and passed through 20 mesh screen before use.

4-Aminopyridine-2-^{14}C. The ^{14}C-labeled 4-AP used had a specific activity of 5.1 μCi/mg. Radiochemical purity was checked by TLC and found to be greater than 98%.

Chromatography. Alumina TLC plates were developed with acetone–concentrated NH_3 (97:3). Silica gel G thin-layer plates were developed with methanol or ethyl acetate–methanol–concentrated NH_3 (100:25:1).

Preparation of 3% 4-AP-2-^{14}C on Chopped Corn. A method for preparing baits of the frightening agent is to mix chopped grain containing 3% 4-AP with untreated chopped grain (De Grazio et al., 1972). The bait is spread by aerial application over the cropland to be protected. Chopped corn containing 4-AP-2-^{14}C was prepared to test for decomposition of 4-AP on the bait.

Dried chopped corn was collected between sieve openings of 0.358 and 0.396 cm. Chopped corn (3.0 g) was placed in a 25-ml round-bottomed flask. To this were added 5.06 mg of 4-AP-2-^{14}C and 85.1 mg of 4-AP dissolved in 2.0 ml of 50% acetone. The flask was stoppered and shaken 2 h and then taken to dryness on a rotary evaporator at 40 °C. The corn was dried an additional 5 h at 70 °C. After transfer of the corn, the flask in which the corn was dried was rinsed with ethanol, and the ethanol was found to contain 2.6% of the radioactivity originally added. A portion of the treated corn was extracted by refluxing with 95% ethanol for 18 h. The extract contained 95% of the applied radioactivity. The recovered ^{14}C chromatographed as 4-AP on TLC plates.

Assay of Radioactivity. Radioactivity was measured with a scintillation counter. Aqueous samples (0.5 ml) were assayed in 5.0 ml of scintillation solvent. The scintillation solvent consisted of 1600 ml of toluene, 800 ml of Triton X-100, 12.0 g of 2,5-diphenyloxazole, and 1.2 g of dimethyl-1,4-bis[2-(5-phenyloxazolyl)]benzene. Counting efficiency was determined by the addition to the sample of 50 μl of toluene-^{14}C of known specific activity. TLC plates (with plastic backing) were assayed with a radiochromatogram scanner (Packard Instruments, Model 7201), or by cutting them into sections and placing the individual sections in scintillation vials for counting. $Ba^{14}CO_3$ was assayed by the method of Nathan et al. (1958). The counting efficiency was determined with $Ba^{14}CO_3$ of known specific activity.

Table I. Characteristics of the Soils Used in the Study

Soil	pH	Org matter, %	Mechanical anal., % Sand	Silt	Clay
Barnes sandy loam	7.4	4.2	64	18	18
Towner loamy fine sand	7.2	1.1	85	10	5
Fargo clay	7.7	3.4	1	33	66

Aerobic Decomposition of 4-AP-2-^{14}C in Soil. Air-dried soil (50 g) was placed in 250-ml biometer flasks (Bartha and Pramer, 1965), and 1.0 ml of 0.50 mg/ml of 4-AP-2-^{14}C in ethanol was added to each flask to give 10 ppm of 4-AP. After evaporation of the ethanol, the soil was stirred 5 min, then water was added to bring the soil to 80% of the soil moisture capacity, and the soil was stirred again 3 min. In the side arm was placed 10.0 ml of 0.5 N CO_2-free NaOH. The flasks were sealed and incubated at 20 °C. The NaOH was removed for $^{14}CO_2$ assay and replaced with fresh alkali every 24 h for the first 10 days and then every 24 to 120 h thereafter. Immediately after removal from the biometer flasks, the NaOH was diluted with 10 ml of 0.5 M $BaCl_2$ and centrifuged at 2000*g* in covered centrifuge tubes for 15 min. The $Ba^{14}CO_3$ precipitate was suspended in 15 ml of CO_2-free water and centrifuged as before. The precipitate was resuspended in 15 ml of ethanol, centrifuged, dried at 105 °C, weighed, and assayed for ^{14}C.

Degradation of 4-AP-^{14}C on Chopped Corn. Air-dried soil (50 g) was placed in a series of biometer flasks and water was added to 80% of the soil moisture holding capacity. In each of a set of two flasks 100 mg of chopped corn containing 3% 4-AP-2-^{14}C was scattered on the soil surface. In a second set of flasks, 100 mg of untreated chopped corn was added to each flask, and in a third set, no further additions were made. In the side arm of each flask was placed 10.0 ml of 0.5 N CO_2-free NaOH. The flasks were sealed and incubated at 20 °C. At 1- to 3-day

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intervals the alkali was removed from the side arms and assayed for ^{14}C as described above.

Flooded Soil Experiments. Air-dried soil (50 g) was placed in 250-ml flasks and 1.0 ml of 250 μg/ml of 4-AP-2-^{14}C in ethanol was added to each flask. After evaporation of the solvent, the soil was stirred 5 min, and water was added to cover the soil 1 cm. The flasks were loosely covered and incubated at 25 °C. Water was added to the flasks as needed to maintain the water level. The ^{14}C remaining in the soil after incubation was estimated by rinsing the contents of a flask into an empty chromatography column (2.2 cm × 60 cm) and allowing the water to drain. An aliquot of the effluent was assayed for radioactivity. The column was then washed with 600 ml of 1 N HCl. The flow rate was 0.2 to 1.0 ml/min. The combined effluents were assayed for ^{14}C. In control experiments recoveries were between 80 and 90% depending on the soil used.

Chromatography of Soil Extracts. Water and HCl eluates from the flooded soil experiments were neutralized with NaOH and concentrated to dryness at 40 °C under reduced pressure. No loss of radioactivity occurred during the concentration step. The residue was extracted with ethanol and the extract streaked on TLC plates and the plates developed. The soil, after the HCl wash, was extracted (Soxhlet) 18 h with 95% ethanol. The ethanolic extracts, after concentration, were chromatographed on TLC plates.

At the conclusion of the incubation of 4-AP-^{14}C treated soils in the biometer flasks, a portion of the soil was washed with 1 N HCl and then extracted with ethanol continuously for 18 h. The ethanol extracts and HCl washes were chromatographed in the same manner as the flooded soil extracts.

Studies with Soil Bacteria. All organisms were grown in nutrient broth except *Aspergillus niger* which was grown in trypticase soy broth. Cultures (5 ml), 48 h old, were used to inoculate 200 ml of media containing 0, 10, and 100 μg of 4-AP-2-^{14}C. The cultures were incubated at 24–26 °C on a rotary shaker (250 rpm) for 120 h. The cultures were then brought to volume and centrifuged at 6000*g* for 20 min, and aliquots of the supernatant were assayed for ^{14}C and chromatographed on TLC plates. The cells were extracted with hot 95% ethanol and the extract was assayed for ^{14}C and chromatographed on TLC plates.

Growth rates of the organisms were studied under similar conditions used to test for the metabolism of 4-AP, except that nonradioactive 4-AP was used. The growth of *Pseudomonas fluorescens*, *Enterobacter aerogenes*, and *Agrobacterium tumefaciens* was followed by a change in absorbance at 650 nm. Growth of *Streptomyces griseus* and *Aspergillus niger* was followed by dry weight increase of the cells.

RESULTS AND DISCUSSION

The collection of $^{14}CO_2$ was used in these experiments to estimate the rate of decomposition of 4-AP-2-^{14}C. However, not all positions of the pyridine ring contained ^{14}C (C-2 and -6 are equivalent assuming no significant isotope effect) and release of $^{14}CO_2$ should not be interpreted to mean complete oxidation of all the carbons of 4-AP. After incubation, the 4-AP-2-^{14}C treated soils were extracted with 1 N HCl and ethanol. No radioactive substances other than 4-AP-^{14}C were detected on chromatography of the extracts. This suggests no significant accumulation of degradation products other than CO_2. However, because of the position of the ^{14}C label in the compound, this interpretation must be accepted with caution. Starr and Cunningham (1975b) reported finding

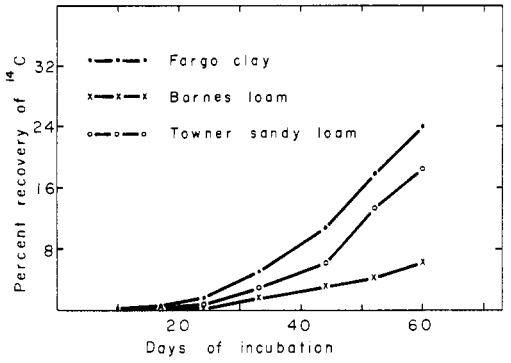

Figure 1. Percent recovery of ^{14}C as $^{14}CO_2$ from soils treated with 10 ppm of 4-aminopyridine-2-^{14}C. Each point represents the mean from two replicates.

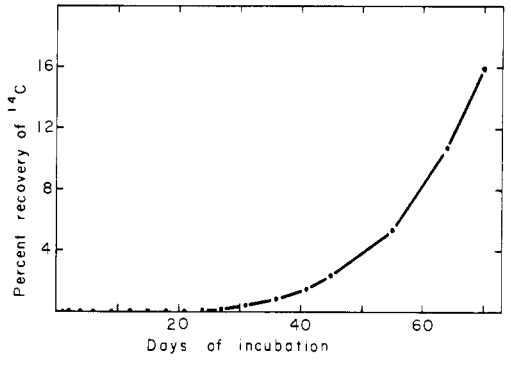

Figure 2. Percent recovery of ^{14}C as $^{14}CO_2$ from chopped corn, containing 3% 4-AP-2-^{14}C, placed on moist soil. Each point represents the mean from two replicates.

two apparent 4-AP metabolites in soil which had been incubated 3 months with ^{14}C-labeled 4-AP under aerobic conditions. The metabolites were no longer detectable on chromatograms after removal of contaminating pigments in the soil extracts.

Destruction of 4-AP in Soil under Aerobic Conditions. When 4-AP-2-^{14}C was incubated with moist soil, a lag period of approximately 20 days occurred before a significant rate of $^{14}CO_2$ release was observed (Figure 1). The lag phase suggests the increase of a population of organisms capable of degrading the pesticide (Kaufman, 1970). Although the rate of $^{14}CO_2$ formation was different with each soil used, the general pattern of release was similar. The rate of $^{14}CO_2$ formation did not show a relationship with the organic content of the soil. In this study, between 6 and 24% of the 4-AP-2-^{14}C applied to three soils was recovered as $^{14}CO_2$ over a 60-day period. Starr and Cunningham (1975b), with similar application rates and ^{14}C-labeled 4-AP, observed recovery of radioactive CO_2 between 0.35 and 54% over a 3-month period using six soils of contrasting physical properties.

Baits of 4-AP on chopped corn have been found to be effective in protecting ripening corn (De Grazio et al., 1971, 1972; Stickley et al., 1972) and sunflowers (Pfeifer, 1968–1972) from severe blackbird damage. We tested the stability of 4-AP on chopped corn to microbial degradation on moist soil. The results are shown in Figure 2. The lag period followed by a rapid release of $^{14}CO_2$ was similar to the results obtained when 4-AP-^{14}C was mixed with moist soil (Figure 1). Under the conditions employed here, the high local concentration of 4-AP on the cracked corn did not significantly retard degradation of the compound.

Both treated and untreated corn are used in baits of 4-AP (Stickley et al., 1972). The bait is often broadcast over the crop and therefore much of the corn rests on the

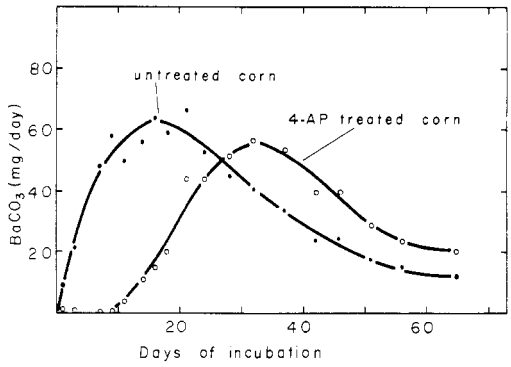

Figure 3. $BaCO_3$ collected from untreated chopped corn and chopped corn containing 3% 4-AP placed on moist soil. The data were corrected for blank determinations using soil but no corn. Each point represents the mean from two replicates.

Table II. Recovery of Radioactivity from Cultures of Soil Microorganisms Grown in the Presence of 4-AP-2-^{14}C

Organism and ATCC no.	Days of incubation	% recovery of ^{14}C[a] at concn of 4-AP-2-^{14}C 10 μg/ml	100 μg/ml
Pseudomonas fluorescens 13525	6	97.7	99.0
Enterobacter aerogenes 13048	5	102.0	99.2
Aspergillus niger 16888	5	98.3	101.1
Streptomyces griseus 10137	5	98.8	99.5
Agrobacterium tumefaciens 15955	5	96.2	98.4

[a] Each value represents the mean of two replicates.

soil surface. The results of an experiment to estimate the rate of microbial degradation of untreated and 4-AP treated corn are shown in Figure 3. The release of carbon dioxide in the biometer flasks was used to monitor the process. The data indicate a lag of about 10 days in microbial attack of the treated corn over that of the untreated corn. The lag may reflect a period of population increase for organisms tolerant to the high concentration of 4-AP on the bait.

Studies with Pure Cultures of Microorganisms. Five common soil microorganisms were used in an experiment to determine whether these species could utilize 4-AP as a carbon source or partially metabolize the compound. The number of cells of the microorganisms in the pure cultures employed in this study (100 ml) is usually much larger than would be expected for that species in the soil used for the aerobic experiments (50 g of air-dried soil). Therefore, the period of incubation used with the pure cultures was less than that used in the experiments with soil. The nutrient levels in the pure cultures do not accurately reflect those in soil. However, the organisms did pass through different phases of nutrient availability which may also occur in soil at one time or another, including optimum nutrient conditions early in the incubation, to starvation later in the incubation period. Under these conditions, the organisms were apparently unable to either utilize 4-AP as a carbon source or to carry out a single metabolic conversion of the compound. No significant loss of radioactivity was observed in the cultures of the five microbial species when exposed to 10 and 100 μg/ml of 4-AP-2-^{14}C (Table II). All the ^{14}C remaining in the culture media as well as that in the ethanol extracts of the cells chromatographed as 4-AP. During the incubation period, *P. fluorescens*, *A. tumefaciens*, and *E. aerogenes* reached the stationary phase within 24 h, while *A. niger* and *S. griseus* reached the stationary phase within 60 h. Although the compound did not inhibit the growth of these organisms up to 100 μg/ml, the concentration of 4-AP on the chopped corn baits is higher.

Table III. Recovery of Radioactivity from Flooded Soil Containing 5 μg/g of 4-AP-2-^{14}C[a]

Days of incubation	Mean % recovery of ^{14}C and std dev: Fargo clay	Barnes loam
15	90.1 ± 5.4	92.3 ± 4.5
30	86.7 ± 5.6	84.9 ± 5.3
45	81.8 ± 4.2	82.5 ± 6.2
60	76.4 ± 6.5	79.0 ± 4.7

[a] Recovery at time zero was set at 100%.

Loss of 4-AP in Flooded Soils. The recovery of radioactivity from flooded soils treated with 4-AP-2-^{14}C was determined over a 60-day period (Table III). As in the case of soil under aerobic conditions, essentially all the ^{14}C chromatographed as 4-AP. Extraction of the soil with 1 N HCl was done because of the poor recoveries of the avicide on extraction with ethanol, methanol, and acetone. The loss of ^{14}C in the flooded soils did not show the lag period observed under aerobic conditions. The loss of extractable ^{14}C ranged from 21 to 24% over a 60-day period. Starr and Cunningham (1975b) used the release of $^{14}CO_2$ from flooded soils treated with 4-AP-^{14}C to estimate the decomposition rate of the compound. After a 2-month incubation period they recovered less than 1% of the applied radioactivity as carbon dioxide. The apparently greater degradation observed in the studies reported here may be due to the binding of 4-AP, or a metabolite of 4-AP, to the soil in a form not extractable.

Studies on the degradation of a number of substituted pyridines in soil, and by specific microorganisms, have been reported by other investigators. Naik et al. (1972) attempted to correlate the structures of 38 pyridine derivatives with their resistance to attack by soil microorganisms. In their studies, 1 mM solutions of the pyridine derivative containing 0.5% garden soil, 20 μg/ml of yeast extract, and inorganic nutrients were incubated and the amount of derivative remaining estimated by absorbance of light between 210 and 330 nm. These investigators found that 4-AP persisted in the solutions for more than 170 days under both aerobic and anaerobic conditions, while the isomer, 2-aminopyridine, persisted less than 96 days. Although our studies did not extend beyond 70 days of incubation, the results also suggest 4-AP is not rapidly destroyed in soil under aerobic and flooded soil conditions.

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Metabolism of 2,4-Dichlorophenoxyacetic Acid. 8. Gas–Liquid Chromatography of Trimethylsilyl Derivatives of Amino Acid Conjugates

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A gas–liquid chromatographic technique was developed for the analysis of 19 metabolites of 2,4-D or potential metabolites, with particular emphasis on the amino acid conjugates of 2,4-D. This technique involves trimethylsilyl derivatization of the metabolites by hexamethyldisilazane, separation by GLC with OV-1 and OV-17 stationary phases, and quantification with a flame ionization detector. The structures of the Me_3Si derivatives were confirmed by mass spectrometry. Preliminary studies on the practical application of this technique for the analysis of plant extracts were conducted.

2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most widely used herbicides in agricultural production. Methods currently being used for the analysis of 2,4-D residues are on the basis of the conversion of the extracted materials to methyl or butyl esters, separation on gas–liquid chromatography, and detection by electron capture or microcoulometric detectors (Pesticide Analytical Manual, 1969; Schultz, 1973; Yip, 1962, 1971; Yip and Ney, 1966). Residue analysts usually only consider the quantification of free 2,4-D and possibly some hydroxylated metabolites; however, 2,4-D is metabolized by plants to a number of metabolites including the biologically active amino acid conjugates (Feung et al., 1971, 1972, 1973a,b, 1974, 1975). Information on the amount and identification of these metabolites in the natural ecosystems is necessary from both toxicological and ecological standpoints. The overall objective of this study was to develop an analytical method which utilizes GLC for the analysis of 2,4-D metabolites, with special emphasis on the biologically active amino acid conjugates of 2,4-D. Such a technique would provide a means to quantify the metabolites of 2,4-D as well as aid in the identification of structurally unknown metabolites.

EXPERIMENTAL PROCEDURE

Reagents and Materials. All solvents used were of highest purity. 2,4-D was purchased from Aldrich Chemical Co., Inc. *n*-Octacosane and all silylation reagents were purchased from Supelco, Inc.: bis(trimethylsilyl)-trifluoroacetamide (BSTFA), Sylon HTP (a 3:1:9 mixture of hexamethyldisilasane–trimethylchlorosilane–pyridine), Sylon-BTZ (a 3:2:3 mixture of *N,O*-bis(trimethylsilyl)-acetamide–trimethylchlorosilane–trimethylsilylimidazole), hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS).

Pesticide Research Laboratory and Graduate Study Center and Department of Entomology, The Pennsylvania State University, University Park, Pennsylvania 16802.

4-Hydroxy-2-chlorophenoxyacetic acid (4-OH-2-Cl) was obtained from Dr. J. Fleeker, North Dakota State University, while 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D) and 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) were previously synthesized (Hamilton et al., 1971). All amino acid conjugates of 2,4-D were supplied by Dr. C. S. Feung, Pesticide Research Laboratory, The Pennsylvania State University. The amino acid conjugates of 2,4-D were: 2,4-dichlorophenoxyacetylalanine (2,4-D-Ala); 2,4-dichlorophenoxyacetylarginine (2,4-D-Arg), 2,4-dichlorophenoxyacetylaspartic acid (2,4-D-Asp), 2,4-dichlorophenoxyacetylcysteine (2,4-D-Cys), 2,4-dichlorophenoxyacetylglutamic acid (2,4-D-Glu), 2,4-dichlorophenoxyacetylglycine (2,4-D-Gly), 2,4-dichlorophenoxyacetylhistidine (2,4-D-His), 2,4-dichlorophenoxyacetylhydroxyproline (2,4-D-Hyp), 2,4-dichlorophenoxyacetylisoleucine (2,4-D-Ile), 2,4-dichlorophenoxyacetylleucine (2,4-D-Leu), 2,4-dichlorophenoxyacetyllysine (2,4-D-Lys), 2,4-dichlorophenoxyacetylmethionine (2,4-D-Met), 2,4-dichlorophenoxyacetylproline (2,4-D-Pro), 2,4-dichlorophenoxyacetylphenylalanine (2,4-D-Phe) 2,4-dichlorophenoxyacetylserine (2,4-D-Ser), 2,4-dichlorophenoxyacetylthreonine (2,4-D-Thr), 2,4-dichlorophenoxyacetyltryptophan (2,4-D-Trp), 2,4-dichlorophenoxyacetyltyrosine (2,4-D-Tyr), and 2,4-dichlorophenoxyacetylvaline (2,4-D-Val).

Instruments. A MicroTek 220 gas chromatograph equipped with an Infotronics Model II digital integrator was used for this investigation. Silylanized 6 ft × 4 mm i.d. glass columns were employed with various column packings; 1, 2, and 3% OV-1 and 10% OV-7 on 100/120 mesh Supelcoport, and 1% OV-17 on 80/100 mesh Supelcoport. Dual flame ionization detectors were used. The instrument conditions were as follows: column temperature varied from 150 to 280 °C; detector, 280 °C; inlet, 245 °C; and flow of 50–60 ml of N_2/min.

Mass spectra were obtained on a LKB-9000 gas–liquid chromatograph interfaced mass spectrometer using a 6 ft