The Fate of 4-Amino-3, 5, 6-Trichloropicolinic Acid in Spring Wheat and Soil

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Because of the great importance of measuring the effects of pesticides on environmental contamination, a study was carried out to determine the fate of 4-amino-3,5,6-trichloropicolinic acid, TORDON(R) herbicide, in wheat and soil. The results of this study are reported here.

Experimental

Chemicals and Equipment. 4-Amino-3,5,6-trichloropyridine-2,3,4,5,6-C^{14}_5-2-carboxylic-C^{14} acid was prepared as follows: Acetylene-1,2-C^{14}_2, prepared from barium carbonate-C^{14}(1), was converted to acetaldehyde-1,2-C^{14}_2 (2). This, in turn, was converted to paraldehyde by contacting it with ferric chloride. The trimer was changed to a mixture of 5-ethyl-2-methylpyridine and 2-picoline (3). To convert 5-ethyl-2-methylpyridine to 2-picoline, the desired intermediate, the compound was oxidized to 6-methylnicotinic acid (4, 5) and then decarboxylated through the intermediacy of the calcium salt (5).

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2-Picoline hydrochloride was chlorinated in a sealed tube using liquid chlorine and a trace of benzoyl peroxide, with no solvent, at 120°C. for 48 hrs. The desired product, totally labeled 2,3,4,5-tetrachloro-6-trichloromethylpyridine, about 10% of the total chlorinated material, was isolated by means of column chromatography using Dicalite impregnated with paraffin oil as the stationary phase and 95% methanol saturated with paraffin oil as the developer. At this stage, the product was contaminated with paraffin oil. The perchlorinated 2-picoline was converted to 4-amino-2,3,5-trichloro-6-trichloromethylpyridine by heating with isopropyl alcohol saturated with anhydrous ammonia in a sealed tube at 100°C. for 6 hrs. The product was hydrolyzed using 80% sulfuric acid (6) to give 4-amino-3,5,6-trichloropicolinic acid. The product was cleaned up by means of thin layer chromatography using Silica Gel F-254 2 mm., 20 x 20 cm. (Brinkman Industries, New York) and toluene/acetic acid (19/6) as developer. The R_f value for 4-amino-3,5,6-trichloropicolinic acid was 0.17; 3 successive developments were carried out, the plate being allowed to dry between developments. The yield, based on acetyldehyde, was 1%. This procedure gave totally labeled herbicide of 100% radioactive purity. The infra-red and ultraviolet spectra and chromatographic mobility were identical in all respects with known herbicide.

4-Amino-3,5-dichloro-6-hydroxypicolinic acid was prepared by heating together for 16 hrs. under reflux 4-amino-3,5,6-trichloropicolinic acid and excess of 10% sodium hydroxide solution. The crude product was collected by adjusting the pH to 1 with hydrochloric acid and filtering. The product was triturated with dimethyl formamide,

filtered, redissolved in excess of 2% sodium hydroxide solution, filtered, and reprecipitated by adjustment of the pH to 1 with hydrochloric acid. The product was collected by filtration: yield, 52%; m.p., 250-251°C. (d.). Anal. Calcd. for $C_6H_4Cl_2N_2O_3$: N, 12.56. Found: N, 12.72.

4-Amino-2,3,5-trichloropyridine was prepared from 4-amino-2,3,5-trichloropicolinic acid by a decarboxylation reaction. The product was purified by sublimation at atmospheric pressure, m.p. 155°C.; literature, 153-153.5°C. (7).

The gas chromatograph was an Aerograph Model A-100 (Varian Aerograph Co.). The chromatographic column was an 18" x 1/4" stainless steel tube packed with 10% Dow 710 Silicone on Fluoropack. The helium carrier gas flow rate was 40 ml./minute. Radioactivity assays were carried out using a Model 4322 scintillation counter (Packard Instrument Company) and an Autoscaler for G-M counting (Tracerlab, Inc.).

Planting Logistics. Soil in glazed, 1-gallon crocks treated with totally labeled herbicide at rates on an acid equivalent basis of 0.1 and 0.91 pound/acre of soil surface and with non-radioactive herbicide at the rate of 0.91 pound/acre was sown to spring wheat, Selkirk variety. The high rate is equivalent to 0.90 ppm. in a 3" acre. The pots were maintained out of doors. The regimen of care, watering, fertilization, etc., was normal for this crop.

Extraction Techniques. The plant material was subjected to 3 successive extractions in hot 80% methanol containing sodium

bicarbonate, 50 mg./25 ml. Each extraction was carried out for 15 min. This procedure removed at least 90% of the radioactive residue from the plant material. The combined extracts were evaporated in vacuo to a small volume such that they could be utilized directly for chromatographic study.

Soil samples were extracted in a high speed homogenizer at room temperature with acetone, 80% ethanol, and 1.5N ammonia, in that order. Each extraction was carried out for 15 min. and the soil was allowed to dry at room temperature between extractions. Each of these extracts was concentrated to a small volume in vacuo at 50°C.

The soil was next heated under reflux with 6N hydrochloric acid for 24 hrs. and the cooled hydrolysate, after removal of the soil, was evaporated to dryness as above. It was necessary to resort to 2 triturations with ethanol followed by evaporations of the ethanol extracts in order to get a syrup completely soluble in water. The insoluble solid residues remaining from the ethanol triturations did not contain any detectable radioactivity.

Following the acid hydrolysis, the dried soil was heated at 80-90°C. for 3 hrs. with 0.1N sodium hydroxide solution.

After acidification of the aqueous solutions resulting from the acid hydrolysis and alkaline extraction, the solutions were extracted 5 times with ethyl acetate. The resulting ethyl acetate and water phases were each assayed for radioactivity and submitted to paper chromatography after appropriate concentration.

Paper Chromatography. Paper chromatography of all extracts was carried out on 1"-wide strips of Whatman's No. 1 or No. 3 filter paper. Radioactivity was located by counting segments of the paper strips. The position of pyridine reference compounds was determined by means of ultraviolet contact printing, as described by Markham and Smith (8), or by observation of the fluorescence quenching on chromatograms sprayed with an 0.05% solution of fluorescene in 95% ethanol followed by illumination with a 2537 Angstrom light source. The position of oxalic acid was determined with the aid of a 1% bromcresol green spray (9).

Results and Discussion

A radioautograph of a 5-week-old wheat seedling grown on soil treated with the high level application of the herbicide clearly showed the presence of residues of some sort in all of the organs. The residue levels seemed somewhat higher in the characteristically deformed leaves than in normal appearing ones.

Plant growth, total uptake of radioactivity, and specific activity in terms of ppm. of 4-amino-3,5,6-trichloropicolinic acid were measured from zero to 98 days. It was found that, in each of the 3 cases, the data could be represented by zero-order rate curves as readily as by more complex functions. Accordingly, the expressions shown in Table 1 represent these rates. The effect of the herbicide on plant growth can readily be seen from the expressions in Table 1: There is a regular decrease in the regression coefficient as the treatment rate increases. There is a 57-fold increase in the rate

of total radioactivity uptake corresponding to a 9-fold increase in the treatment rate as determined from regression coefficients in Table 1. There is no significant rate of increase in the specific activity for the low treatment rate, but there is for the high treatment rate, again from the appropriate regression coefficients in Table 1. Clearly, the plant growth rate is not keeping pace with the uptake of total radioactivity in the case of the high treatment level.

Identification of radioactive compounds found in the wheat was based exclusively on those plants treated at the rate of 0.91 pound/acre. The residue level was so low in the plants treated at the smaller, more practical rate that detection of anything except the chief constituent would not have been possible.

Extracts from 98-day-old plants were not only submitted to paper chromatography; they were also separated into acidic and neutral fractions. These, in turn, were submitted to paper chromatography and the data are shown in Table 2. This fractionation indicated that all the radioactive material in the 80% methanol extract with an R_f value greater than 0.5 in solvent system No. 1 appeared to be neutral material and that at 0.5 and 0.05, acidic.

The predominant radioactive entity observed was the major acidic metabolite in Table 2. Clearly, the R_f data suggest that this compound is 4-amino-3,5,6-trichloropicolinic acid. The identity of this compound was confirmed by working up the acid metabolite from 830 g. of mature

TABLE 1 - REGRESSION EQUATIONS DESCRIBING PLANT GROWTH AND RADIOACTIVITY UPTAKE FROM SOIL TREATED WITH 4-AMINO-3,5,6-TRICHLOROPICOLINIC ACID

Measurement	Treatment, Lbs./Acre[a/] of 4-Amino-3,5,6-trichloropicolinic Acid		
	0	0.10	0.91
Plant growth as g. of plant material	g. =-2.73 + 0.19 days $S_{g.}$ = 1.92, S_b = 0.02	g. =-0.79 + 0.14 days $S_{g.}$ = 1.96, S_b = 0.02	g. = -1.16 + 0.08 days $S_{g.}$ = 1.75, S_b = 0.02
Total radioactivity uptake as ug. of TORDON herbicide	--	ug. = 0.18 + 0.01 days $S_{ug.}$ = 0.84, S_b = 0.01	ug. = -10.46 + 0.57 days $S_{ug.}$ = 3.54, S_b = 0.05
Specific activity, as ppm. of 4-amino-3,5,6-trichloro-picolinic acid	--	ppm. = 0.13 - 1.4×10^{-4} days $S_{ppm.}$ = 0.08, S_b = 7.1×10^{-4}	ppm. = 2.33 + 0.037 days $S_{ppm.}$ = 1.28, S_b = 0.008

a/ $S_{g.}$, $S_{ug.}$, $S_{ppm.}$, = Standard error of the estimate; S_b = Standard error of the regression coefficient.

wheat plants which had been planted in soil treated with non-radioactive TORDON herbicide. These plants, containing the untagged material, were enriched with 28 g. of plants grown on the radioactive material. In this way, the recovery process could be followed radiochemically. Large-scale paper chromatography using Whatman's No. 3-mm. paper, development in solvent system No. 1, elution, and then development with solvent system No. 2 gave a syrup which was converted to methyl esters by means of diazomethane in ether. From this crude product, the methyl ester of 4-amino-3,5,6-trichloropicolinic acid was isolated in pure form by means of vapor phase chromatography at 205°C. The infra-red absorption spectrum of this compound was identical in all respects with that of a known sample of this compound. In addition, the material isolated from the vapor phase chromatography column was radioactive.

Prediction that the first minor acidic metabolite was oxalic acid was strongly supported by the following experiment: The radioactive acid metabolite fraction from 1.9 g. of 98-day-old wheat plants was converted into methyl esters with diazomethane. This, along with known dimethyloxalate, was submitted to vapor phase chromatography at 75°C. and the issuing dimethyloxalate was collected and found to be radioactive.

The second minor acidic metabolite was provisionally identified as 4-amino-3,5-dichloro-6-hydroxypicolinic acid by the similarity of its R_f values relative to those of the known compound.

Examination of the R_f values of the neutral metabolite in Table 2

TABLE 2 - PAPER CHROMATOGRAPHY OF PLANT EXTRACTS

Compound	R_f Values in a/						
	1	2	3	4	5	6	7
Known Compounds							
TORDON herbicide	.54	.47-.51	.48-.57				
4-Amino-2,3,5-trichloropyridine	.94-.96	.94	.94-.96	.58-.61	.84	.26	
Oxalic acid	.01-.04	0-.03	0-.02				0
4-Amino-3,5-dichloro-6-hydroxypicolinic acid	.14-.18	.20-.21	.25-.26				
Extracts of 90-Day-Old Wheat Plants							
80% methanol extract	.05						
	.5						
	.9						
Major acidic metabolite	.54	.45	.57				
First minor acidic metabolite	.07		.07				0
Second minor acidic metabolite	.18	.21	.25				
Neutral metabolites	.94	.85	.95	.58	.84	.12	
Major hydrolysis product	.54	.51	.48				
First minor hydrolysis product	.01	0	0				0
Second minor hydrolysis product	.18	.21	.29				

a/ Solvent systems: (1) n-butanol saturated with 1.5N ammonia; (2) benzene/propionic acid/H_2O (2/2/1); (3) n-butanol/triethylamine/water (5/1/2); (4) cyclohexane/acetic acid/water (2/2/1); (5) benzene saturated with ethyleneglycol; (6) n-heptane saturated with ethyleneglycol; and (7) ethanol/1.5N ammonia (100/1).

shows a close correspondence with those for the known 4-amino-2,3,5-trichloropyridine, a possible metabolite of 4-amino-3,5,6-trichloropicolinic acid. However, chromatography of extracts of wheat plants grown on soil treated with carboxyl-tagged 4-amino-3,5,6-trichloropicolinic acid (10) also revealed the presence of neutral radioactive metabolites with high R_f values in solvent system No. 1. As a consequence of this experiment, it was concluded that the neutral metabolites must consist largely of compounds not directly resulting from decarboxylation of 4-amino-3,5,6-trichloropicolinic acid. It was eventually found that hydrolysis either with hydrochloric acid or with pancreatin would split at least 80% of the radioactivity in the neutral metabolite fraction into radioactive acids extractable with ethyl acetate. The R_f values of these hydrolysis products are recorded in Table 2, and they agree well with those found for reference samples of oxalic acid, 4-amino-3,5-dichloro-6-hydroxypicolinic acid, and TORDON herbicide. The proportion of the 3 acidic hydrolysis products was the same as that in the extracts.

When the hydrolysis-resistant portion from pancreatin treatment of the neutral metabolite fraction, along with 4-amino-2,3,5-trichloropyridine, was passed through the vapor phase chromatography column at 180°C., the issuing pyridine derivative was found to be radioactive. The magnitude of the radioactivity agreed well with the value representing the hydrolysis-resistant portion of the neutral metabolite fraction. Thus, the evidence seems to be fairly convincing that 4-amino-2,3,5-trichloropyridine is present in the extracts, but only to a very minor extent.

It seems likely that the saponifiable portion of the neutral metabolite fraction consists of 4-amino-3,5,6-trichloropicolinic acid and, at least, oxalic acid and 4-amino-3,5-dichloro-6-hydroxypicolinic acid conjugated as lipids. No attempt has been made at further characterization of these materials. The hydrolysis to simpler compounds by pancreatin occurs readily and permits the quantitative estimation of these lipids.

The rate at which the 3 principal radioactive entities found in the wheat extracts changed with time was determined from the radioactive assay data of paper chromatograms at 4 different time periods, from 11 through 84 days, using No. 1 solvent system. These data were reduced to expressions of linear regression and are shown in Table 3. 4-Amino-3,5,6-trichloropicolinic acid, starting at a level of about 80% of the total radioactivity, appears to be decreasing slowly with time. Only 80-90% confidence can be attached to the disappearance rate as estimated from Student's t-value. The neutral metabolites start at a level of about 10% of the total radioactivity and increase at a rate not greatly different from that of TORDON herbicide disappearance. A somewhat higher degree of confidence, 95-98%, can be placed on this rate of increase. The amounts of oxalic acid and 4-amino-3,5-dichloro-6-hydroxypicolinic acid, as a percent of the total radioactivity, appeared to be constant with time.

An examination of ripe wheat grain taken from 98-day-old wheat plants which had been grown in the plot treated with the high level of the herbicide clearly showed no differences, either qualitatively or quantitatively, in the nature of the residual radioactive entities

relative to the pattern described for the foliage.

Reconciliation of all the data at 84 days, including 4-amino-3,5,6-trichloropicolinic acid, oxalic acid, and 4-amino-3,5-dichloro-6-hydroxypicolinic acid formed after hydrolysis of the neutral metabolites, indicates the following distribution: 4-amino-3,5,6-trichloropicolinic acid, 83%; oxalic acid, 8%; 4-amino-2,3,5-trichloropyridine, 4%; and 4-amino-3,5-dichloro-6-hydroxypicolinic acid, 5%.

The soil from this experiment, after the wheat plant was harvested, was allowed to stand fallow without watering for the next 2 years. It was essentially air-dried for the last 18 months of this 2-year period. It was found to contain radioactivity representing 0.15 ppm. of the herbicide, equivalent to a loss of 83% of the amount initially applied.

As a result of the extractions and acid hydrolysis, there remained only 4% of the initial burden of radioactivity as non-extractable, unknown radioactive material in the soil. The efficiency of radioactivity removal from soil as a result of these treatments is shown in Table 4. Essentially none of the radioactivity was removed from the soil by acetone extraction and, thus, the amount remaining after solvent extraction represents radioactivity not removed by ethanol followed by ammonia. Each of the extracts and the hydrolysis mixture were examined as to their content of discrete radioactive compounds by methods already described, and these results are shown in Table 5.

The small discrepancy in the total burden of radioactivity in

TABLE 3 - RATES OF CHANGE OF RESIDUAL 4-AMINO-3,5,6-TRICHLOROPICOLINIC ACID AND ITS METABOLITES IN WHEAT PLANTS

Compound	Linear Regression Equation [a]	t [b]
4-amino-3,5,6-trichloro-picolinic acid	% = 78.9 - 0.14 days $S_{\%}$ = 4.1, S_b = 0.07	1.91
Neutral metabolites	% = 9.7 + 0.16 days $S_{\%}$ = 1.6, S_b = 0.03	5.65
Oxalic acid	% = 6.6 $S_{\%}$ = 1.3	---
4-amino-3,5-dichloro-6-hydroxypicolinic acid	% = 4.1 $S_{\%}$ = 2.0	---

a/ Assays were carried out at 11, 24, 52, and 84 days.

% = Percent of total radioactivity.

$S_{\%}$ = Standard error of the estimate

S_b = Standard error of the regression coefficient

b/ $t = \frac{b}{S_b}$, where b is the regression coefficient.

the soil as recorded in Table 4 and that value which can be calculated from Table 5 arises because the values in Table 5 were calculated directly from the extracts and final extracted soil. In other words, they were determined independently of data recorded in Table 4. In any event, the difference is not large.

4-Amino-3,5,6-trichloropicolinic acid was found to be the largest single radioactive entity present in the extracts and, in fact, represented 54% of the total radioactive burden in the soil.

The radioactive material listed as "unknown" in Table 5 consists of at least 6 discrete entities, and none of these materials bear any resemblance to possible simple metabolites of 4-amino-3,5,6-trichloropicolinic acid. These compounds are felt to represent extensive breakdown products of the herbicide molecule, products of bacterial degradation far removed from the parent compound. These conclusions are based on information gleaned from paper chromatography and solvent partition studies, ethyl acetate/water.

A significant feature of this metabolism study is the almost total lack of simple, or first-generation, breakdown products of the herbicide. Still, it is clear that a large amount of the herbicide has disappeared in the period of time covered by the experiment. Other work with this herbicide (10) reports the same lack of closely related degradation products. We conclude that first-generation degradation products of the herbicide are much more readily disposed of by living systems than is the herbicide itself; in other words, the rate-determining step in the disappearance of radioactivity is that operating on the herbicide, i.e., the first step in the total reaction sequence.

TABLE 4 - EFFICIENCY OF RADIOACTIVITY REMOVED FROM SOIL

Extractions and Treatments	Radioactivity Remaining As 4-Amino-3,5,6-trichloropicolinic acid equivalent	
	µg.	% of Total
None	122.0 ± 2.4	100
After solvent extrn.	51.3 ± 1.7	42
After acid hydr.	11.7 ± 0.3	10
After NaOH extrn.	4.7 ± 0.9	4

a/ ± Values are 95% confidence limits; 3 replicates except for the NaOH extrn. where there were 6.

TABLE 5 - COMPOUNDS DETECTED IN SOIL

Compound	Amount Recovered as ug. 4-Amino-3,5,6-trichloro-picolinic Acid Equivalent						Total ug.	% of Total
	80% EtOH	1.5N NH_3	HCl Hydrolysis		NaOH Extraction			
			EtOAc	H_2O	EtOAc	H_2O		
4-Amino-3,5,6-trichloro-picolinic acid	40.1	22.8	0	0	0	0	62.9	54
4-amino-3,5-dichloro-6-hydroxypicolinic acid	1.7	0	0	0	0	0	1.7	1
Unknowns	1.0	6.1	27.7	6.9	2.8	1.6	46.1	41
Non-extractable unknown in soil	--	--	--	--	--	--	4.7	4

In summary, it appears that the biochemical transformations imposed on 4-amino-3,5,6-trichloropicolinic acid, whether by means of microorganisms in soil or by plants, does not result in a complex maze of degradation products closely related to the herbicide but, rather, it yields a spectrum of simpler compounds more nearly related to materials which are normal to a living system. As a consequence of this, the only compound one finds in major quantities is the herbicide itself.

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