Tracer Study of 6-Chloropicolinic Acid in Corn

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The initial and principal metabolite resulting from N-Serve [2-chloro-6-(trichloromethyl)-pyridine] degradation by plants has been shown to be 6-chloropicolinic acid. This paper describes a further study of N-Serve metabolism in corn. 6-Chloro-Cl^{36}-picolinic acid is used as a starting point instead of N-Serve in order to detect compounds arising as a result of subsequent metabolic transformation of the picolinic acid derivative. The following radioactive substances were detected: unchanged 6-chloropicolinic acid, 2-chloropyridine, inorganic chloride, at least four lipid-like materials from which the picolinic acid derivative was liberated by incubation with pancreatic lipase, and an intractable substance which was felt to be an amide of 6-chloropicolinic acid with protein. This last material has not been identified. The synthesis of 6-chloro-Cl^{36}-picolinic acid is also described.

THE nutrient conserver 2-chloro-6-(trichloromethyl)pyridine (N-Serve, trade-mark of The Dow Chemical Co.) is a compound proposed for use as a nitrogen-conserving agent (*2*). When used in this manner, it would be applied to soil along with fertilizers containing ammonium ions to inhibit microbial oxidation and subsequent leaching loss.

Redemann and coworkers (*4*) have shown that the principal residual compound to be expected in a variety of plants growing in soil treated with N-Serve is 6-chloropicolinic acid. It has also been demonstrated in the authors' laboratory that these plants evolve radioactive carbon dioxide at a slow rate. Clearly, the remaining compound, after decarboxylation, is no longer labeled. A radioactive label at some other position in the molecule seemed to be necessary in order to discover what became of the decarboxylated material. Accordingly, the work described herein was carried out, utilizing 6-chloro-Cl^{36}-picolinic acid. Since the aim of this work was to describe the fate of N-Serve following the formation of its primary metabolite, the authors felt justified in using this metabolite as a starting material. Furthermore, the acid is very readily taken up from the soil by plants, and a working concentration of the labeled compound could be expected to appear in the plant as the result of a simple soil application.

Materials and Methods

6-Chloro-Cl^{36}-Picolinic Acid. Lithium chloride-Cl^{36} was generated from lithium carbonate (66 mg., 1.80 meq.) and hydrochloric acid-Cl^{36} (2.88*N*, 0.625 ml., 12.4 μc. per mmole).

The lithium chloride-Cl^{36} and redistilled phosphorus oxychloride (1.6 ml., 52.5 meq. of Cl) were heated for 4 hours at 50° C. in a sealed tube with occasional agitation. At the end of the heating period, the phosphorus oxychloride-Cl^{36} was transferred, by means of a vacuum manifold, to a tube containing 6-hydroxypicolinic acid (101 mg., 0.727 mmole). The tube was vented with dry air, sealed, and then heated for 2 hours at 100°. The cooled tube was opened and the contents were hydrolyzed by pouring onto ice. After the hydrolysis mixture had been gently warmed on the steam bath, the pH of the mixture was adjusted to 3 with aqueous ammonia. A 3-hour continuous extraction with ether yielded 86 mg. (75%) of white crystals of 6-chloro-Cl^{36} picolinic acid. The melting point of the product from test runs was 190° [(*1*), 190°]; its infrared spectrum was identical to that of authentic 6-chloropicolinic acid.

The radioactive product, submitted to paper chromatography using 1-butanol–1.5*N* ammonia (1/1, v./v.), had an R_f of 0.34, identical with that of an authentic sample of 6-chloropicolinic acid. Two additional solvent systems, described by Redemann and coworkers (*5*), were used to substantiate this identification. Its radiochemical purity was 97%. The remaining 3% of the radioactivity had an R_f of 0.89 and was probably the amide of 6-chloropicolinic acid. The specific activity of the 6-chloro-Cl^{36}-picolinic acid was 0.41 μc. per mmole.

Application Details. Twenty-six milligrams of 6-chloro-Cl^{36}-picolinic acid were dissolved in 35 ml. of water containing a very small amount of ammonia. This solution was poured onto the surface of a mixture of equal volumes of a sandy loam soil and Sponge Rock planted to Golden Cross Bantam corn. The rate corresponds to 1 pound per acre and is equivalent to 1.6 pounds of N-Serve per acre. Subsequently, the corn was grown in the greenhouse under maintenance which avoided leaching of the soil. This treatment was four times the recommended rate for an N-Serve application.

Radioautograph. A 25-day-old corn seedling was removed from the soil and the roots were well washed with tap water. The plant was dried between blotters in a botanical specimen press at 60°. The dried sample was covered with 1/2-mil polyethylene film, placed in intimate contact with x-ray film for 31 days, and the film was processed in the usual manner.

The radioautograph is shown in Figure 1.

Sampling. The corn crop was thinned after 25 days, and the harvested thinnings were used in the metabolism study. In evaluating the significance of the various metabolites found in this study, it should be kept in mind that the tissues were sampled near the time when the residue content of the plant was close to its maximum value in terms of specific activity, rather than at maturity.

Fractionation Procedure. Figure 2 is a flow diagram of the fractionation scheme.

Ninety-eight grams of plant tissue were ground with a meat grinder and the tissue, A, along with the water washings, was freeze-dried. The lapsed time between the sampling and freezing the tissue was 15 minutes. This procedure yielded 9.2 grams of dried leaf and stem tissue, A2, and 88 ml. of water, A1.

The water condensate from freeze-drying, A1, was evaporated to incipient dryness under reduced pressure, after first being made acid to Congo Red with 6*N* hydrochloric acid. A portion of the resulting solid was counted, and a count rate significantly different from background was obtained. This and all subsequent counting data are recorded in Table I. All the counting was done in 1-inch diameter, nickel-plated dishes with a thin, end-window GM tube.

The result with A1 indicates that a small quantity of a volatile, radioactive

Figure 1. Radioautograph of 25-day-old corn plant grown in soil containing 6-chloro-Cl^{36}-picolinic acid

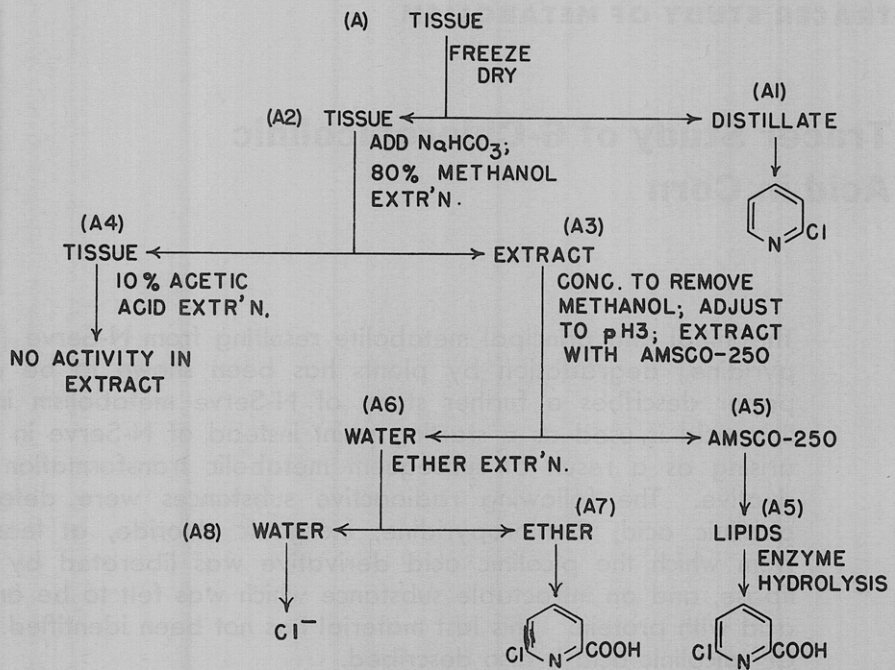

Figure 2. Fractionation procedure used to isolate metabolites from corn plants

chlorine-containing base was present. The identity of this volatile material was checked by isotope dilution. The total quantity of acidified concentrate from freeze-drying, A1, was redissolved in water and neutralized to pH 7 with sodium hydroxide. Nonradioactive 2-chloropyridine was added as carrier, and the mercuric chloride salt was precipitated from solution. The derivative was then recrystallized to constant specific activity, strongly suggesting that the radioactive material in A1 was 2-chloropyridine.

The dried plant tissue, A2 (9.18 grams), was chopped into small pieces and three replicates were counted. The material was then mixed with a small amount of sodium bicarbonate to inhibit esterification of 6-chloropicolinic acid and extracted continuously with 80% aqueous methanol for 6 hours. Examination of the methanol-insoluble material, A4, will be considered first.

Continuous extraction of A4 with 10% aqueous acetic acid for 17 hours failed to remove any radioactivity. The radioactivity remaining in the tissue at this point is felt to represent intractable conjugated 6-chloropicolinic acid, perhaps as an amide with protein. Other work with similarly constituted aromatic acids (*3*) allows us to offer this suggestion, since the authors find in these cases that the acid is liberated by prolonged acid hydrolysis. In the present work, this point was not investigated further.

The methanol-soluble extract, A3, was evaporated under reduced pressure to remove methanol. Water was added, the pH was adjusted to 3 with hydrochloric acid, and the mixture was extracted with three portions of Amsco 250 hydrocarbon solvent. The dried extracts, A5, were found to contain radioactivity.

Column chromatography of extract A5 was carried out on an 18 × 2.3 cm. column of silicic acid using petroleum ether, chloroform, and alcohol-free ether as eluents, in that order. Ten-milliliter fractions of eluate were collected.

Table I. Distribution of Radioactivity in Fractions from Figure 2

Fraction[a]	Total Wt. or Vol.	Wt. or Vol. Counted	Total Counts	Net Count Rate, C.P.M.[b]	Counts in Total Sample[c]	Counts Relative to A2, %
A1	1.4 ml.	0.1 ml.	33,294	1.1 ± 0.4	15.4	[d]
A2	9.18 g.	126[e] mg.	1,000	7.7 ± 1.8[e]	70.8	100
A4	5.19 g.	145[e] mg.	1,000	1.3 ± 1.0[e]	6.7	9.5
A5	11.0 ml.	0.1 ml.	4,096	1.4 ± 1.1	154	23.2[f]
A7	0.5 ml.	0.1 ml.	4.096	33.2	166	25.1[f]
A8	3.4 ml.	0.1 ml.	4,096	8.2	279	42.2[f]

[a] See Figure 2 for identity of fractions.

[b] Backgrounds counted as follows: A1, A2, A4. 25,000 total counts = 26.1 c.p.m.; A5, 23,584 total counts = 24.1 c.p.m.; A7, A8. 1000 total counts = 26.2 c.p.m. Confidence limits represent 95% level of significance except where otherwise noted.

[c] $\frac{\text{Net ct. rate} \times \text{total vol.}}{\text{vol. ctd.}}$ = cts. in total sample; or, net ct. rate × total wt. = cts. in total sample. In the case of A2 and A4, samples were counted at infinite thickness. This is an arbitrary measure of total activity in each fraction and serves to determine distribution of radioactivity.

[d] Total counts in A1 were not included in subsequent calculations of distribution, but by comparing the value, 15.4, with the corresponding value for A5, 154, it is clear that A1 encompasses no more than 2% of total.

[e] Average of 3 replicates.

[f] Values calculated as follows: A3 determined by difference (A2-A4) to be 90.5%. Since the proportion of A5, A7, and A8 to their total can be easily calculated, it is simple to multiply each by 0.905.

The results of this separation are presented in Figure 3, where it can be seen that at least four different radioactive constituents are present in extract A5.

The constituent obtained in largest quantity from the column chromatography just described was hydrolyzed enzymatically by incubation at 37° for 20 hours with an aqueous solution of bile salts and pancreatic lipase. The enzymatic hydrolysis product was worked up and the radioactivity present was identified as 6-chloropicolinic acid by means of paper chromatography, using the solvent systems described under preparation of 6-chloro-Cl^{36}-picolinic acid. The only other radioactive material present was unhydrolyzed lipid to the extent of 29%.

The chromatographic scheme used here has been described by Strain (*6*) for the separation of lipids into different structural groups—for example, simple esters of long-chain fatty alcohols tend to come off the column immediately with hydrocarbon solvents, sterol esters are removed with the chloroform, and the triglycerides are eluted with the ether. Perhaps, here, there is a similar pattern in which one of the carboxylic acids involved is 6-chloropicolinic acid.

The acidified, aqueous phase remaining from the hydrocarbon solvent extraction, A6, was continuously extracted with alcohol-free ether for 5 hours. The

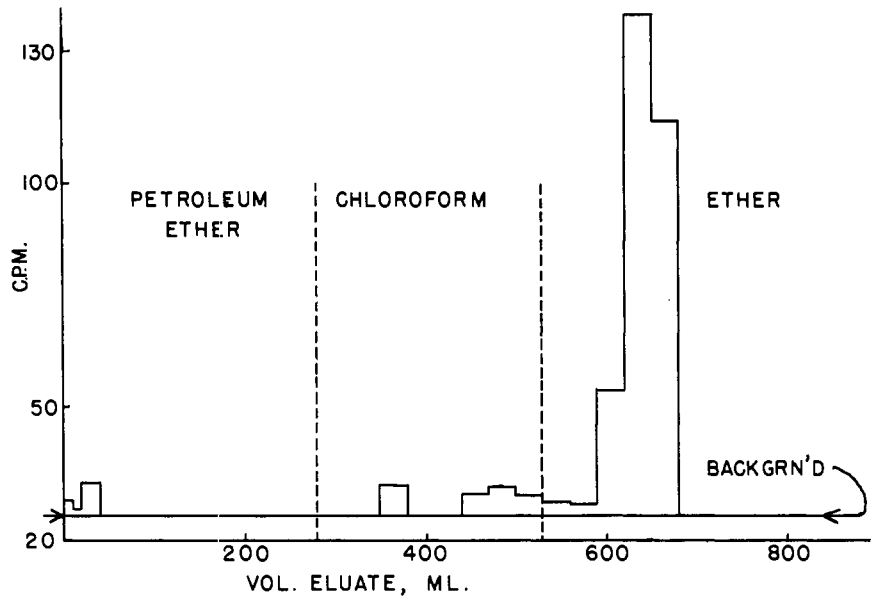

Figure 3. Elution pattern of radioactive lipids from silica gel column

radioactivity in the ether extract, A7, was identified as 6-chloropicolinic acid by means of paper chromatography, again using the three solvent systems described. No other radioactive compounds were present.

The aqueous phase remaining after the ether extraction, A8, also contained radioactivity. This was shown to be inorganic chloride-Cl^{36} by means of paper chromatography using 1-butanol–1.5*N* ammonia (1/1, v./v.), R_f 0.10, and also by preparation and counting of a silver chloride plate. The latter was resuspended in hot, dilute nitric acid and replated until constant specific activity was reached.

Discussion

Our results show that 6-chloropicolinic acid is taken up by the corn plant from soil. A small portion is converted to 2-chloropyridine, some is esterified, and a small part, the authors suggest, is conjugated with insoluble protein. In addition, some of the 6-chloropicolinic acid and/or subsequent metabolites are dehalogenated to yield chloride ion. This pattern of metabolism is shown in Figure 4. It is likely that these reactions also take place in soil through the medium of microbial action, and the proportion of metabolites supplied to the plant by soil is not known.

2-Chloropyridine is present in the corn plant to only a very small extent at the early stage of growth in this experiment. Redemann and coworkers (*4*) were able to find only a small amount of residual N-Serve in their experiments, even after only 2 weeks. Since these two compounds are very similar in terms of volatility, it seems improbable that 2-chloropyridine will be detected in corn at maturity. If the treatment in this experiment had been normal—that is, $^1/_4$ of the rate actually applied—this compound would probably not have been found.

The quantity of 6-chloropicolinic acid appearing as lipid and protein conjugates is substantial. Any scheme for residue determination should take this into account by including a hydrolysis step.

We can only speculate about the structure of the compounds formed when chlorine is removed from the metabolites shown in Figure 4. In the area of plant metabolism, our experience suggests that the initial reaction is one in which chlorine is replaced by hydroxyl. The resulting 2-pyridinols are relatively unstable entities in plant biological environs and would be expected to disappear quickly.

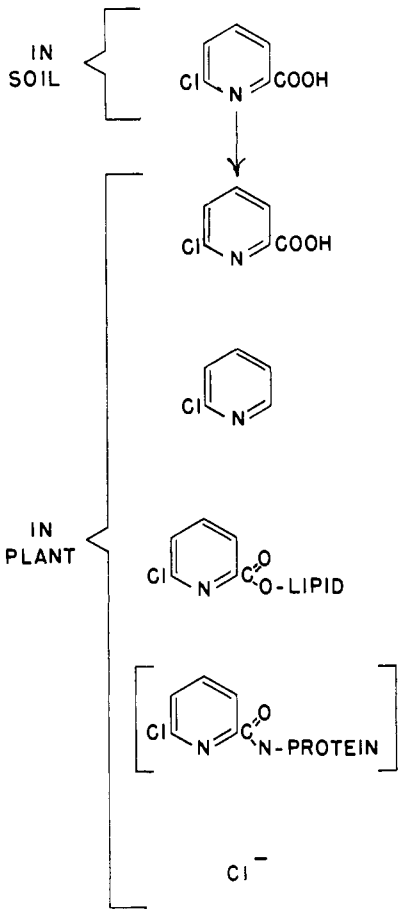

Figure 4. Summary of metabolites found in 25-day-old corn plants grown in soil containing 6-chloro-Cl^{36}-picolinic acid

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