Table I. Kernel Quality (C.V. Big Z)

Ethylene μl/l.	% by weight		
	Fancy	Standard	Amber
0	91	9	0
0.10	61	39	0
0.25	57	53	0
0.50	34	53	13
0.75	14	77	9
1.00	18	78	4
2.50	9	91	0
5.00	0	73	27
7.50	0	100	0
10.00	0	83	17

centrations substantially below the one-half maximum concentration requirement for shuck dehiscence. The change in color of pecan kernels to exogenous ethylene could be due to a direct effect of ethylene on kernel color and/or an indirect effect, e.g. enhancing the rate of physiological processes which in turn affect pigment synthesis.

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Cycocel Plant Growth Regulant. Fate of Carbon-14 Chlorocholine Chloride in Sugarcane Grown in Hawaii

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Studies of the biochemical behavior of ^{14}C-labeled chlorocholine chloride in sugarcane using thin-layer chromatography showed evidence of no other radioactive material in the foliage than the labeled chlorocholine chloride. Only very low concentrations of radioactivity were found in the sugarcane stalks. Radioisotope analysis and chemical analysis for chlorocholine chloride in the stalk and in foliage were in good agreement.

Cycocel Plant Growth Regulant (registered trademark of American Cyanamid Company), 2-chloroethyl trimethylammonium chloride (also referred to as CCC and chlorocholine chloride), has shown promise as a ripener for sugarcane. Indications from field studies are that foliage sprays at late stages of maturity bring about reductions in the vegetative growth and an increase in the sugar content in the cane.

The purpose of the present study was to determine the metabolic fate of this compound in the sugarcane plant to provide the basis for residue analytical methodology responding to all of the toxicologically significant components of the residue. The metabolism of chlorocholine chloride labeled with carbon-14 had been studied in wheat plants (Blinn, 1967) where the compound was not significantly converted by metabolic processes. Faust and Bier (1967), working with nitrogen-15 labeled chlorocholine chloride in cereal plants, were also unable to find any labeled conversion products of CCC. Willemot and Belzile (1970) in their study with alfalfa leaflets showed slow conversion of Cycocel-^{14}C to phosphatidylcholine (0.5% in 5 hr). By contrast, Jung and EL-Fouly (1966) and EL-Fouly and Ismail (1969) showed that chlorocholine chloride from aqueous extracts of wheat and cotton plants was quickly converted into choline. Also, Stephan and Schütte (1970), working with 5- to 10-day-old barley embryos, separated from their roots and placed in beakers containing ^{14}C-labeled chlorocholine chloride, showed that 10 to 20% of the radioactivity isolated was present as choline. Belzile et al. (1972) found chlorocholine chloride to be weakly metabolized by winter barley to choline and unidentified compounds.

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MATERIALS AND METHODS

Radiolabeled Chlorocholine Chloride. Chlorocholine-*1,2-*^{14}C chloride was obtained from New England Nuclear Corporation, Boston, Mass. It had a specific activity of 6 mCi/mmol. The radiochemical purity was checked by two-dimensional thin-layer chromatography with a solvent system consisting of acetonitrile–water–acetic acid (60:40:2) and the radiopurity of the radiotracer was found to be 98%.

Application to Sugarcane. Carbon-14 labeled chlorocholine chloride was applied to mature sugarcane plants (approximately 18 months of age) in Hawaii. Applications were made by representatives of the Hawaiian Sugar Planters Association. Two experiments were conducted, one in May 1972 and the other in October 1972. For the May experiment, the plants were sprayed with undiluted radioactive chlorocholine chloride and each plant received 0.67 mg of the plant growth regulant. For the October experiment, the ^{14}C-labeled chlorocholine chloride was mixed with 33 parts of unlabeled chlorocholine chloride and this mixture was applied at a rate of 4 lb of active ingredient per acre to ten isolated and tagged stalks representing one stool (5 ft × 5 ft microplot). Both applications were made with a chromatograph sprayer. No cultural practices were conducted

Table I. Residues of Chlorocholine Chloride (ppm) in Sugarcane Plants after Foliar Treatment with 0.67 mg of Chlorocholine-^{14}C Chloride in May 1972[a]

Plant part		Weeks after treatment: 0 Radioanal.[b]	0 GLC[c]	1 Radioanal.	1 GLC	2 Radioanal.	2 GLC	4 Radioanal.	4 GLC	8 Radioanal.	8 GLC
Leaves	Methanol extract	0.22	–[d]	0.17	0.11	–	–	–	–	0.03	–
	Marc	0.01	–	0.02	–	–	–	–	–	0.02	–
	Total	0.23	–	0.19	0.11	–	–	–	–	0.05	–
Stalks	Methanol extract	<0.01	–	<0.01	<0.05	–	<0.05	–	<0.05	0.01	<0.05
	Marc	<0.01	–	<0.01	–	–	–	–	–	<0.01	–
	Total	<0.01	–	<0.01	<0.05	–	<0.05	–	<0.05	0.01	<0.05

[a] Treated at rate of 0.12 lb of active ingredient per acre. [b] Radioanalysis average of three samples. [c] GLC analysis average of two samples. [d] Not determined.

during the period between application and harvest except for weekly irrigation by furrow. Samples were harvested at 0, 1, 2, 4, and 8 weeks after application. Each sample of cane was separated into (a) adhering green leaves and tops and (b) stalks (millable cane). Each sample of plant parts was grossly chopped separately, frozen, and shipped by air freight to our laboratories at Princeton under Dry Ice.

Extraction Procedure. Chlorocholine chloride is very soluble in water, methanol, and ethanol but quite insoluble in nonpolar solvents. Of these, methanol in a solvent-to-sample ratio of about 12 to 1 for leaves and 8 to 1 for stalks was successful in extracting 90% of the radioactivity from 100 g of leaves and 85% from 500 g of stalks. Samples were finely chopped, placed in a Mason jar with 400 ml of methanol, and minced by means of an Omni-mixer for 10 min. The resultant homogenate was filtered by suction through a 600-ml Buchner-type sintered-glass funnel (medium porosity). The procedure was repeated three times and the resultant extracts were combined and aliquots taken for radioassay. The remaining extracts were evaporated to dryness in a rotating film evaporator under vacuum at 40°C.

Determination of Radioactivity. The radioactivity recovered in the methanol extracts was determined by direct liquid scintillation counting in a Packard Tri-Carb scintillation spectrometer. Samples were dissolved in a scintillator solution (2700 ml of dioxane, 500 ml of anisole, 500 ml of 1,2-dimethoxyethane, and 300 ml of N.E.N. Liquifluor). Efficiency of counting was determined by internal standardization using a commercial standard of toluene-^{14}C. Samples of the marc were combusted in a Packard Tri-Carb sample oxidizer (Model 305, Packard Instrument Co., Downers Grove, Ill.) by weighing about 500 mg of each sample on a filter paper (Whatman, 7 cm) and compressing it into a pellet prior to combustion.

Radioactive spots on thin-layer chromatograms were located by means of radioautography using Kodak Royal Pan professional-grade photographic film.

Charcoal–Alumina Cleanup. The residues from the extracts of sugarcane or leaves were dissolved in 2 ml of distilled water and 2 ml of methanol. Shaking was required until the dissolution of each residue was complete. Then, 40 g of 80–200 mesh Fisher adsorption alumina (Grade A-540) was added to each solution and the resulting slurry was thoroughly mixed using a glass stirring rod until a dry flowable powder resulted. Each mixture was then transferred to a 500-ml beaker and the flask rinsed successively with three 50-ml portions of 25% methanol in acetonitrile and the rinses combined with the mixture. About 20 g of Fisher technical animal charcoal powder (Grade C-263) was then added to each mixture; the resulting slurry was then stirred for 10 min and filtered. The filter cakes were each washed with two 25-ml portions of 25% methanol in acetonitrile; the resultant filtrates from each sample were combined in a 500-ml pear-shaped flask and evaporated to dryness using a rotary film evaporator. In order to determine if any loss occurred by absorption on charcoal or alumina, an aliquot of the extract (before evaporation) was radioassayed by direct liquid scintillation counting and a portion of the charcoal and alumina was combusted in the oxidizer.

Thin-Layer Chromatography. The silica gel thin-layer chromatographic system most useful for separation of chlorocholine chloride from its reported metabolites, choline chloride and betaine, was acetonitrile–water–acetic acid (60:40:2) used for one-dimensional chromatography. One-dimensional chromatography was accomplished by allowing the solvent system to migrate twice, as double development minimized streaking. All thin-layer chromatography was performed on 20 cm × 20 cm plates precoated with silica gel scored for a 15-cm solvent development. Authentic chlorocholine-*1,2*-^{14}C chloride was mixed with control extracts and the mixtures were chromatographed near the sample spot on the same plate. Unlabeled chlorocholine chloride, choline chloride, and betaine were placed on plates separately and as a mixture in a single spot. After a chromatogram had been developed, the air-dried plates were sprayed with Dragendorff's reagent (Stahl, 1969) to give a colored spot.

Gas Chromatography. The isolated chlorocholine chloride from sugarcane leaves and stalks was demethylated and derivatized by reaction with sodium benzenethiolate in anhydrous butanone (Tafuri et al., 1970) and the derivative formed was determined by gas chromatography (Higham et al., 1975). The instrument was a Hewlett-Packard Model 402 equipped with a Micro-Tek Model MT/FPD-11 (Melpar, Inc., Model FDP-100) flame photometric detector and 394-nm sulfur filter. The column was a 120 cm × 4 mm i.d. U-shaped borosilicate glass tube packed with 15% butane-1,4-diol succinate on Gas-Chrom Q (80–100 mesh).

The operating conditions were: temperatures, flash heater 230°C, column 190°C, detector 210°C; flow rates in ml/min, hydrogen 150, air 100, oxygen 20, nitrogen 80. The retention time of the chlorocholine derivative under these conditions was 1.5 min.

RESULTS AND DISCUSSION

Analysis of the sugarcane leaves and sugarcane stalks for the May study, which were treated with chlorocholine-^{14}C chloride at the rate of 0.67 mg/plant, is shown in Table I. The concentration of Cycocel as calculated from radioactivity recovered from the leaves decreased over the 8 weeks of the study dropping from 0.24 to 0.05 ppm. Meanwhile, the amount increased in sugarcane stalks from 0.002 to

Table II. Residual Radioactivity (ppm) in Sugarcane Plants 8 Weeks after Foliar Treatment with Mixture of ^{14}C-Labeled and Nonlabeled Chlorocholine Chloride in October 1972[a]

Plant part		Radio-anal.	GLC anal.
Leaves	Methanol extract	18.8	20.4
	Marc	2.0	–[b]
	Total	20.8	–
Stalks	Methanol extract	0.75	0.59
	Marc	0.11	–
	Total	0.86	–

[a] Treated at rate of 4 lb of active ingredient per acre. [b] Not determined.

0.013 ppm. The limited number of chemical assays with the GLC method was in agreement with the radioassays as is evident from the data in Table I. Because this experiment used such a low treatment rate compared to actual field conditions, another experiment was conducted in October using a dosage rate of diluted chlorocholine-^{14}C chloride equivalent to 4 lb of active ingredient per acre, which corresponds to the rate for commercial usage. Table II shows the distribution of radioactivity found in leaves and stalks 8 weeks after application. The residues of extracted radioactivity found were 18.8 ppm in leaves and 0.75 ppm in stalks. When these same samples were analyzed by the chemical procedure, the values of 20.4 and 0.59 ppm were found. Only negligible amounts of radioactivity were found in the marc.

Removal of plant pigment from sugarcane leaves before thin-layer chromatography was achieved by treatment of the methanol–acetonitrile solution with animal charcoal. Only 3% of the radioactivity was adsorbed by the charcoal and alumina, while the bulk of the radioactivity was recovered in the filtrate. The nature of the adsorbed radioactivity is not known but presumed to be parent Cycocel.

Investigations with various organic solvents using silica gel plates revealed that acetonitrile–water–acetic acid (60:40:2) gave the best separation of chlorocholine chloride from its reported metabolites (choline chloride and betaine) using one-dimensional chromatography.

An example of the autoradiograms obtained from thin-layer analysis of leaf extracts and mixtures of control extracts with authentic chlorocholine-^{14}C chloride is shown in Figure 1. Only one spot was observed demonstrating that only the labeled chlorocholine chloride was present.

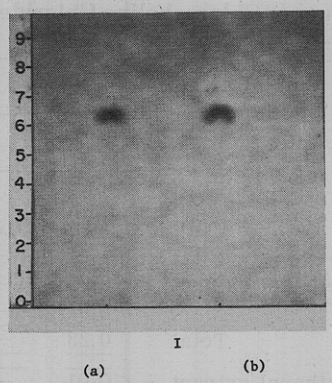

Figure 1. Thin-layer chromatography of the methanol-extractable radioactivity found in sugarcane leaves 8 weeks after treatment (second experiment, Oct 1972): (a) sample; (b) fortified control.

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Indole-3-acetic Acid. Mass Spectra and Chromatographic Properties of Amino Acid Conjugates

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Twenty amino acid conjugates of indole-3-acetic acid were synthesized and characterized by mass spectrometry and paper and thin-layer chromatography. Molecular ions and a base peak of *m/e* 130 were observed for most conjugates. The mass spectral fragments of high *m/e* (>175) are few in number, predictable, and correlated with specific amino acid derivatives.

A number of plant species convert indole-3-acetic acid (IAA) into several ether-insoluble (at neutral pH) metabolites. One of these metabolites has been identified as the amino acid conjugate, indole-3-acetylaspartic acid (IAA-Asp). Indole-3-acetylysine (IAA-Lys) has also been isolated from certain strains of *Pseudomonas savastanoi* (Hutzinger and Kosuge, 1968). IAA-Asp has been identified in most cases through its chromatographic properties, color tests, and, in a few instances, biological activity (Andreae and

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