J. Agric. Food Chem. 1991, 39, 2285-2289

Characterization of ^{14}C Residues in the Grain of Rice Plants Grown in Soil Treated with [*phenyl*-^{14}C]-2-(Diphenylmethoxy)acetic Acid Methyl Ester

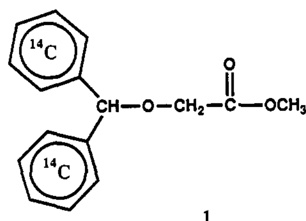
Ronn G. Nadeau,*,‡ Robert K. Howe,‡ Thomas J. Burnett,§ and Brian D. Lange§

Environmental Science Technology Department, Monsanto Agricultural Company, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198, and Pan-Agricultural Laboratories, Inc., Madera, California 93638

A study was conducted on the uptake of ^{14}C residues in grain from field-grown rice plants treated shortly after planting with [*phenyl*-^{14}C]-2-(diphenylmethoxy)acetic acid methyl ester ([^{14}C]DME) and nonradioactive butachlor. Treatment rates of 0.061 and 0.16 kg/ha [^{14}C]DME resulted in residues of 0.015 and 0.060 ppm in the polished rice. Extraction of powdered polished rice with an optimized solvent resulted in only 3.9% extraction of radioactivity. Treatment of the extracted rice with α-amylase gave a soluble fraction containing 80% of the radioactivity and a nonsolubilized material. The soluble fraction was treated with α-glucosidase to convert maltose to glucose, yielding a material called the starch hydrolysate. HPLC experiments on the starch hydrolysate and its product from acetylation indicated that [^{14}C]glucose was the only detectable radioactive compound present, and ^{1}H NMR analysis of the acetylation product showed that it consisted of a mixture of the α and β isomers of D-glucose pentaacetate. The results indicated that most of the radioactivity in the polished rice was incorporated in natural constituents, mainly in glucose units of starch.

INTRODUCTION

2-(Diphenylmethoxy)acetic acid methyl ester (DME, 1) is a safener used with butachlor herbicide in paddy rice. As part of a study to determine the extent of uptake of the safener into rice from plants grown under use con-



ditions, rice plants were grown in field plots treated with [^{14}C]DME and nonradioactive butachlor at Pan-Agricultural Laboratories, Inc. (Madera, CA). The resulting polished rice contained low levels of radioactive residues, and extraction of the powdered polished rice with organic/aqueous solvents removed only 3.9% of its radioactivity. Therefore, a method was sought to solubilize the unextractable radioactivity and determine its identity. It was of particular interest to learn whether the radioactivity resided in [^{14}C]DME-related metabolites or in naturally occurring compounds formed by incorporation of small ^{14}C degradates. Faced with the same question, other workers hydrolyzed residue-containing extracted rice (Ku et al., 1978), barley (Rouchand et al., 1979), and wheat (Wargo et al., 1975) with strong acid, converted the resulting glucose to the glucosazone, and recrystallized the derivative to constant specific radioactivity. Instead, we enzymically hydrolyzed the extracted rice and used HPLC to analyze the resulting glucose and its pentaacetate derivative. The results showed that a high percentage of the grain radioactivity resided in glucose units of starch.

* Address correspondence to this author.

‡ Monsanto Agricultural Co.

§ Pan-Agricultural Laboratories.

MATERIALS AND METHODS

Chemicals, Materials, and Solvents. [*phenyl*-^{14}C]DME (MW = 256.3) was prepared in-house and had a specific radioactivity of 11.2 mCi/mmol (93 900 dpm/μg). Its chemical and radiochemical purities were >98%. [^{14}C-UL]-D-Glucose, purchased from Sigma Chemical Co. (St. Louis, MO), was >98% radiochemically pure and had a specific radioactivity of 8.7 mCi/mmol. A sample having a concentration of 245 dpm/10 μL was prepared in water/acetonitrile (90:10). The α and β isomers of D-glucose pentaacetate were purchased from Aldrich Chemical Co. (Milwaukee, WI). The following enzymes were purchased from Sigma: α-amylase, type I-A from porcine pancreas; protease, Pronase E from *Streptomyces griseus*; α-glucosidase, type III from yeast. Rice seed, variety L202, was obtained from Norvil-Davis Elevator (Firebaugh, CA).

Whatman glass microfiber filters, 4.25-cm diameter, and Chemstrip 2GP urine test strips were purchased from Fisher Scientific Co. (St. Louis, MO). AG 1-X4 (formate form) and AG 50-X4 (H^+ form) resins, both 20–50 mesh, were purchased from Bio-Rad Laboratories (Richmond, CA). HPLC solvents and materials such as buffer salts, pyridine, and acetic anhydride were purchased from Fisher Scientific. Atomlight scintillation cocktail was purchased from NEN Research Products (Boston, MA). Other reagents and solvents (ACS grade) were obtained from commercial suppliers and were used without further purification.

Plant Material. The rice plants were grown outdoors in test plots at Pan-Agricultural Laboratories. Each plot consisted of an individual paddy excavated 4 ft by 10 ft by 20 in. deep, lined with plastic, and filled to 1 ft with clay soil. Two treatment plots (low and high rates) and one control plot were used. The rice seed (variety L202) was soaked for 48 h, kept moist to germinate for 36 h, and broadcast by hand onto water-saturated soil at a rate of 90 lb of dry seed/acre. At 3 days after planting, when coleoptiles were just emerging, test mixtures were applied using a precalibrated nozzle pressurized to 40 psi with CO_2. The test was designed to deliver butachlor/[^{14}C]DME at rates of 0.75 kg/ha:0.075 kg/ha (low rate) and 1.5 kg/ha:0.15 kg/ha (high rate). Filter paper disks were used to monitor the application rates. Immediately following the application, the disks were transferred to scintillation vials for analysis by LSC.

The plots were flooded at 10 days after treatment and were maintained at a constant water level of about 2 in. for the duration

0021-8561/91/1439-2285$02.50/0 © 1991 American Chemical Society

of the growth period. At 144 days after treatment, the rice plants were harvested, yielding 3743, 3286, and 2118 g of rough rice from the low-treatment, high-treatment, and untreated control plots, respectively. The rough rice samples were processed to produce polished rice using a McGill 2 sample sheller (Rapsco, Brookshire, TX), and the polished rice was analyzed by combustion and shipped to Monsanto for further analysis.

Combustion Analysis. Samples were combusted in triplicate at Pan-Agricultural Laboratories, using a Harvey OX500 oxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ). $^{14}CO_2$ was trapped in Harvey carbon-14 cocktail, and the samples were analyzed using an LKB Rack-Beta liquid scintillation counter (Pharmacia LKB Biotechnology, Inc., Gaithersburg, MD). Samples of rice from the untreated plot were fortified with [^{14}C]-benzoic acid to check for recoveries during combustion, showing that recoveries were between 96 and 100%. A minimum quantifiable level, in terms of micrograms of [^{14}C]DME equivalents per fresh weight gram of rice (parts per million), was calculated according to the method of Currie (1968); the minimum quantifiable level was 0.0034 ppm, based on a rice combustion sample weight of 0.25 g.

Liquid Scintillation Counting Analyses (LSC). LSC analyses were performed with Tracor Analytic Mark III Model 6881 counters interfaced with TRACE, a computer hardware and software system developed at Monsanto. The TRACE system collected data from the counters and performed data reduction and statistical analysis. The counting time was 20 min/pass, and three passes were made. Long counting times were employed because very low levels of radioactivity (e.g., 50 counts above background) had to be accurately measured. Duplicates were employed wherever possible, and in those cases duplicate analyses had relative standard deviations of below 5%.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra were obtained using a Varian VXR-300 spectrometer with an operating frequency of 300 MHz. Chemical shifts were referenced using the resonance frequency of the solvent peak and are reported in parts per million from tetramethylsilane. Samples were dissolved in 99.9% $CDCl_3$ (Merck) and were analyzed in 5-mm tubes (Wilmad Glass Co., Buena, NJ).

Powdering the Polished Rice. A sample (276.1 g) of polished rice from the high treatment rate plot was mixed in a Waring blender with approximately 150 g of dry ice and blended for 10 min. This resulted in a fine powder (powdered polished rice) weighing 265.2 g. This material was used in the experiments described below.

Extractability Test on Powdered Polished Rice. Six samples of powdered polished rice (0.50 g each) were weighed into three pairs (duplicates) of 20-mL screw-capped test tubes. To the tubes were added 5-mL portions of acetonitrile/water, 25:75, 50:50, or 75:25. The tubes were shaken for 30 min and centrifuged at 1000 rpm for 5 min, and the extracts were decanted into scintillation vials. Atomlight scintillation cocktail (15 mL) was added, and the samples were analyzed by LSC.

Extraction of Powdered Polished Rice. To 5.000 g of the powdered polished rice in a 150-mL centrifuge tube was added 40 mL of 25:75 acetonitrile/water (see Figure 1). The tube was shaken for 30 min and centrifuged for 10 min at 5000 rpm, and the supernatant was decanted into a tared flask. A second acetonitrile/water extraction was conducted in the same way, and a third extraction was conducted using 0.05 M potassium phosphate buffer. The phosphate buffer extract was isolated by filtration using a Büchner funnel and a glass microfiber filter. The extracted-rice filtercake was dried in air overnight and weighed. The extracts were analyzed by LSC, and the extracted rice was analyzed by combustion (see data in Table I).

Enzymic Treatments. *α-Amylase.* To a 150-mL centrifuge tube was added 1.000 g (5790 dpm) of extracted rice, 100 mL of 0.05 M (pH 7.0) phosphate buffer, and 0.40 mL of α-amylase preparation (see Figure 1). The mixture was gently shaken in a 30 °C water bath for 31 h and filtered using a Büchner funnel and a tared glass microfiber filter. The filtrate (α-amylase hydrolysate) was weighed and analyzed by LSC, and the filtercake was air-dried overnight (see data in Table II). The hydrolysate was stored at -20 °C until used as described below.

Protease. To the post-α-amylase filtercake in a 150-mL centrifuge tube were added 100 mL of 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.2) and 0.300 g of protease enzyme, and the mixture was gently shaken at 30 °C for 16 h. The mixture was filtered, weighed, and analyzed by LSC, and the filtercake was weighed (see data in Table II).

α-Glucosidase. The α-amylase hydrolysate was adjusted to pH 6.0 with a few drops of phosphoric acid. To the solution, which weighed 101.2 g, was added 0.5 mL of α-glucosidase preparation (see Figure 1). The mixture was gently shaken at 25 °C for 20 h, at which time it gave a response, using Chemstrip 2GP glucose reagent strips, commensurate with the amount of glucose expected in the mixture. The hydrolysate, referred to as the starch hydrolysate solution, was stored frozen until the next step.

Removal of Salts and Protein from the Starch Hydrolysate Solution. A desalting and deproteining column was

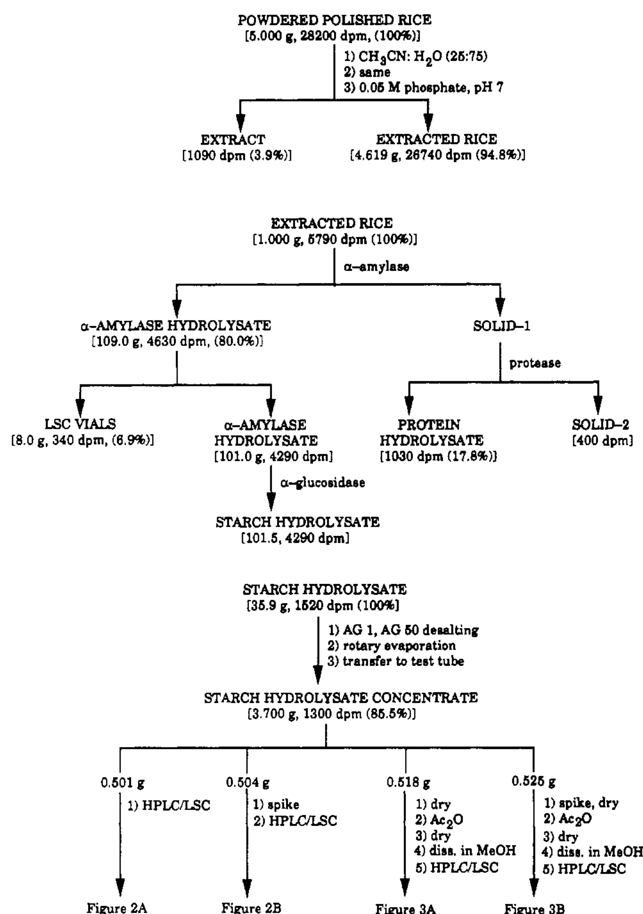


Figure 1. Extraction of powdered polished rice, enzymic treatments of extracted rice, and preparation of the starch hydrolysate concentrate for HPLC/LSC analyses.

Table I. Distribution of Radioactivity during Extraction of Powdered Polished Rice

analysis	dpm (%)	dry wt, g
powdered polished rice	28 200 (100)	5.000
first CH_3CN/H_2O (25:75)	680 (2.4)	
second CH_3CN/H_2O	190 (0.7)	
50 mM phosphate, pH 7	220 (0.8)	
extracted rice	26 740 (94.8)	4.619
total	27 830 (98.7)	

Table II. Distribution of Radioactivity during Enzymic Treatments of Extracted Rice

analysis	dpm (%)	dry wt, g
extracted rice	5790 (100)	1.000
α-amylase hydrolysate	4630 (80.0)	
post-α-amylase filtercake		0.119
protease hydrolysate	1030 (17.8)	
postprotease filtercake	400 (6.9)	0.051
total	6060 (104.7)	

Table III. dpm Accounting during HPLC/LSC Analysis of Starch Hydrolysate Concentrate (SHC) and Acetylated SHC

	nonspiked SHC	[^{14}C]glucose-spiked SHC	acetylated nonspiked SHC	acetylated [^{14}C]glucose-spiked SHC
wt of SHC sample used, g	0.501	0.504	0.518	0.525
dpm in SHC sample used	176	177	182	185
dpm of spiked [^{14}C]glucose	0	245	0	245
total dpm in the sample	176	422	182	430
dpm left in vial/syringe (%)	15 (8.5)	26 (6.2)	48 (26)	88 (21)
net dpm injected	161	396	134	342
dpm recovered in peak (%)	151 (94)	352 (89)	121 (90)	300 (88)

prepared by placing a mixture of 10 g each of AG 1-X4 (formate) and AG 50-X4 (H^+) in a 9 in. by 1 in. diameter Bio-Rad glass column and flushing it with 300 mL of water. To the column was added 35.9 g (1520 dpm) of the starch hydrolysate solution (see Figure 1). The eluent and a 15-mL water wash were collected in a tared 250-mL round-bottom flask. Water was removed by rotary evaporation at 45 °C under high vacuum, and the glassy residue (0.315 g) was transferred to a tared 20-mL screw-capped test tube with water. The resulting solution, called the starch hydrolysate concentrate, weighed 3.700 g. LSC analysis showed that it contained 1300 dpm, representing an 85.5% recovery based on the radioactivity in the starting amount (35.9 g) of starch hydrolysate solution.

Acetylation of Nonspiked and [^{14}C]Glucose-Spiked Starch Hydrolysate Concentrate. Two portions of the starch hydrolysate concentrate, one of which was spiked with 245 dpm (10 μL) of the [^{14}C]glucose stock solution, were prepared in tared vials (see Figure 1). The solutions were brought to dryness with a stream of nitrogen overnight, after which time the samples each weighed 0.039 g. To each vial were added 1.0 mL of pyridine and 1.0 mL of acetic anhydride, and the vials were capped and placed in a gently shaking water bath at 50 °C for 2 h. Pyridine and acetic anhydride were removed using a stream of nitrogen overnight, resulting in dry residues ready for further analysis.

High-Pressure Liquid Chromatography/LSC Analysis (HPLC/LSC). The HPLC instrumentation consisted of a Waters Model U6K injector (Waters, Milford, MA), a Waters Lambda-Max Model 481 LC spectrophotometer, Waters Model 6000A HPLC pumps, and a Waters Model 680 automated gradient controller. A Beckman Ultrasphere ODS semipreparative column (25 cm by 1.0 cm diameter) was used. The HPLC conditions were as follows: solvent A, 1% HOAc; solvent B, acetonitrile; flow rate, 3 mL/min; start at 100% A and hold for 5 min; go linearly to 100% B in 15 min and hold until 30 min. Radioactive peaks were detected by collecting the HPLC eluent at 0.3-min intervals and analyzing the fractions by LSC.

Analysis of Nonspiked and [^{14}C]Glucose-Spiked Starch Hydrolysate Concentrate. First, a blank run was made in which 0.5 mL of water was injected, and eluent fractions were collected and analyzed by LSC to determine whether the HPLC system was sufficiently clean for the low dpm runs which were to follow. The result showed that none of the collected fractions contained significant radioactivity above background. Two portions of the starch hydrolysate concentrate, weighing 0.501 and 0.504 g, the latter spiked with 245 dpm (10 μL) of the [^{14}C]glucose stock solution, were analyzed (see Figure 1). After each sample injection, the small amount of solution that remained in the 1-mL syringe was washed into the vial that had contained the sample, and the sample was analyzed by LSC to determine how much of the sample had *not* been injected. The resulting value was subtracted from the dpm known to be in the vial at the start to ascertain the dpm-injected value (Table III). After 100 fractions were collected during the 30-min runs, the fractions were analyzed by LSC, and the results were converted to histograms (Figure 2). In each case, the contents of the three LSC vials that contained the radioactive peak were pooled in a single vial for reanalysis by LSC; the results are shown in Table III.

Analysis of Acetylated Nonspiked and Acetylated [^{14}C]Glucose-Spiked Starch Hydrolysate Concentrate. Each of the dry acetylation product residues (see above) was dissolved in 200 μL of methanol, drawn into a 1-mL syringe, and injected. The HPLC/LSC analyses were conducted according to the same methods as described above for nonderivatized starch hydrolysate concentrates. The results are shown in Figure 3 and Table III.

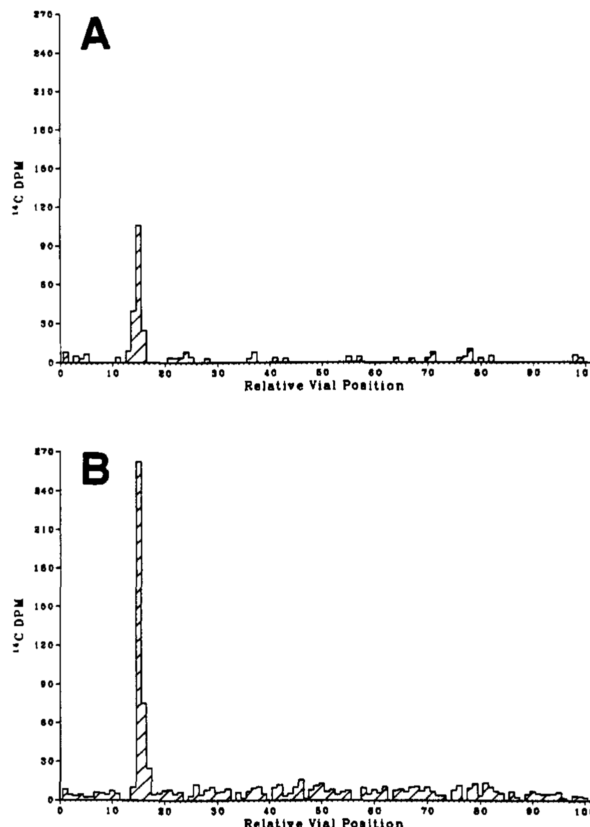


Figure 2. HPLC/LSC analyses of nonspiked (A) and [^{14}C]glucose-spiked (B) starch hydrolysate concentrate (SHC).

RESULTS AND DISCUSSION

Plant Material. On the basis of data from combustion of filter paper disks placed in the paddies just prior to spraying, the actual [^{14}C]DME treatment rates were 0.061 and 0.16 kg/ha, compared with target rates of 0.075 and 0.15 kg/ha, in the low and high treatment rate plots, respectively. The polished rice from plants grown in these paddies had specific radioactivities (on a fresh weight basis) of 1400 and 5640 dpm/g, corresponding to residues (DME equivalent) of 0.015 and 0.060 ppm. Since these residues were so low, the ensuing experiments were conducted using only the rice from the high treatment rate plot.

Extractions. Extractability tests on small samples of powdered polished rice, using three ratios of acetonitrile/water, showed that acetonitrile/water (25:75) removed approximately twice as much radioactivity as did either of the other solvents. This solvent was used for extraction of a larger portion of the powdered polished rice, but only 3.9% of the rice radioactivity was removed (see Table I and Figure 1).

Enzymic Treatments and HPLC/LSC Analyses. Treatment of a portion of the extracted rice with α-amy-

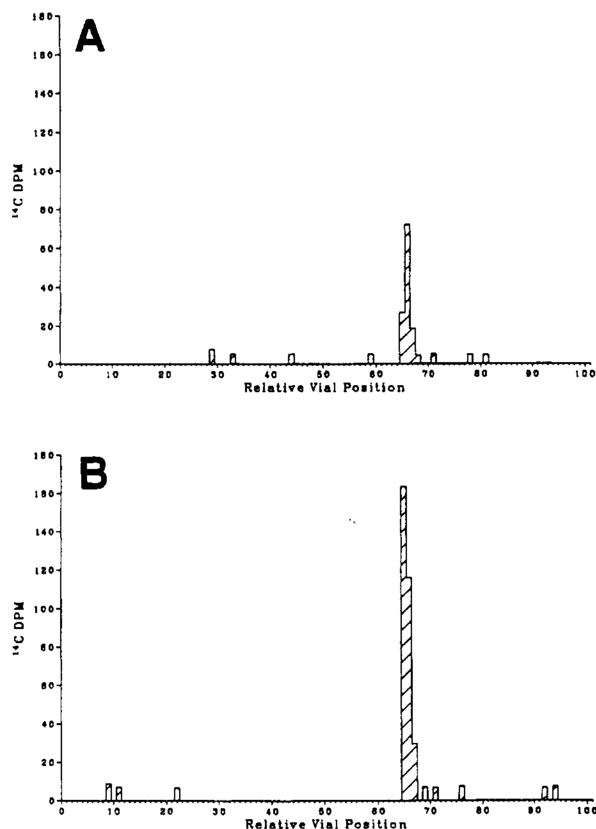
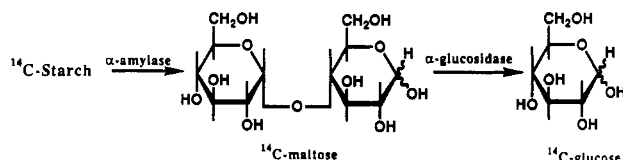


Figure 3. HPLC/LSC analyses of the acetylated derivatives of nonspiked (A) and [^{14}C]glucose-spiked (B) starch hydrolysate concentrate (SHC).

Scheme I



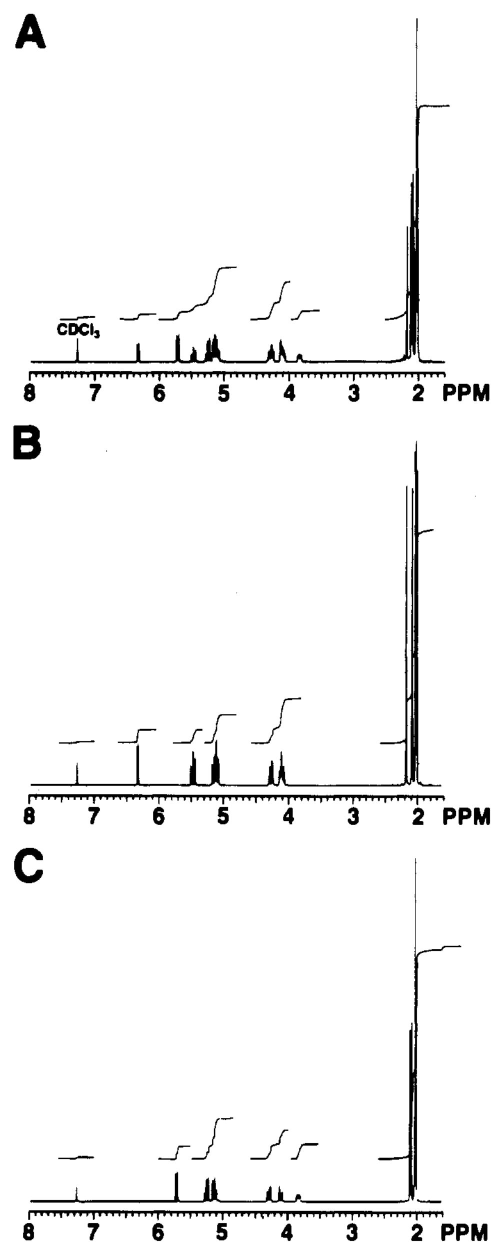


Figure 4. ^{1}H NMR spectra of (A) the acetylated derivative of the starch hydrolysate, (B) α-D-glucose pentaacetate, and (C) β-D-glucose pentaacetate.

lase resulted in solubilization of 80.0% of its radioactivity, and treatment of the nonsolubilized radioactive residue with protease resulted in solubilization of most of the remaining radioactivity (Table II). These results were consistent with the hypothesis that most of the rice radioactivity resided in natural products, since the percentage of starch in polished rice is approximately 80% (Juliano and Bechtel, 1985). However, these results by themselves did not prove that the radiolabel was part of the starch structure. Unresolved was the question of whether the radiolabel was in [^{14}C]glucose units or in recognizable fragments of [^{14}C]DME molecules that were bonded to or occluded in the starch.

To answer the question, the product obtained from treatment of the starch with α-amylase, known to consist mainly of maltose, was treated further with α-glucosidase to convert the maltose to glucose (Scheme I). The enzymically formed glucose was compared with purchased [^{14}C]glucose in chromatographic analyses designed to test for the presence of starch-origin [^{14}C]glucose (see Materials and Methods). The HPLC/LSC chromatograms from the nonspiked and spiked starch hydrolysate both displayed a radioactive peak eluting shortly after the void volume (vials 14–16), and the peak in the spiked analysis was enhanced to the expected degree (see Figure 2 and Table III). Similarly, treatment of nonspiked and spiked portions of the starch hydrolysate with acetic anhydride in pyridine produced derivatized materials whose HPLC/LSC analyses each displayed a single well-retained radioactive peak (vials 65–67), and the peak in the spiked case was enhanced to the expected degree (see Figure 3 and Table III). These results indicated that the radioactive compound produced from enzymic hydrolysis of the rice was exclusively [^{14}C]glucose.

NMR Analyses. Comparison of the ^{1}H NMR spectrum of the starch-origin glucose pentaacetate (Figure 4A) with the ^{1}H NMR spectra of the authentic α- (Figure 4B) and β- (Figure 4C) D-glucose pentaacetates showed that the starch-origin pentaacetate consisted of a 40:60 (α:β) mixture of isomers.

There are precedents in the literature for incorporation of pesticide radiolabels into starch glucose units. Wargo et al. (1975) demonstrated incorporation of the ring-labeled carbon-14 of nitrofen into glucose units of rice and wheat starch. Ku et al. (1978) showed incorporation of the carbon-14 label of mephosfolan insecticide into glucose units of rice starch. Rouchaud et al. (1979) showed that

the tritium label from triforine fungicide was incorporated into glucose units of barley starch.

In the above-cited studies, the starch was hydrolyzed with hydrochloric acid to glucose, the glucose was converted to the glucosazone derivative, and the glucosazone was recrystallized to constant specific radioactivity. Although the "enzyme/HPLC" method presented here was not directly compared with the "acid/glucosazone recrystallization" method, the former offers the following potential advantages: mild hydrolysis conditions would not destroy pesticide metabolites; experiments can be performed on relatively small amounts of test material; it may be more quantitative and faster than the older method.

CONCLUSIONS

Nonextractable radioactive residues in polished rice from [^{14}C]DME-treated plots were enzymically solubilized, and chromatographic analyses showed that the starch hydrolysate radioactivity resided in [^{14}C]glucose. The protein hydrolysate radioactivity was not further investigated but likely contained [^{14}C]amino acids. Incorporation of ^{14}C into starch glucose units must have proceeded via extensive catabolism of [^{14}C]DME, either in the soil or in the plants, to compounds involved in the glucose synthesis pathway.

ACKNOWLEDGMENT

We thank Dr. Jeff Lawrence, Pharmacology and Toxicology Research Laboratory (PTRL), for helpful advice on enzymic hydrolysis of plant materials.

LITERATURE CITED

Currie, L. A. Limits for Qualitative Detection and Quantitative Determination. *Anal. Chem.* **1968**, *40*, 586–593.

Juliano, B. O.; Bechtel, D. B. In *Rice: Chemistry and Technology*; Juliano, B. O., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1985; p 17.

Ku, C. C.; Kapoor, I. P.; Rosen, J. D. Metabolism of Cytrolane (Mephosfolan) Systemic Insecticide [(Diethoxyphosphinyl)-dithioimidocarbonic Acid, Cyclic Propylene Ester] in a Simulated Rice Paddy. *J. Agric. Food Chem.* **1978**, *26*, 1352–1357.

Rouchaud, J.; Moons, C.; Meyer, J. A. Characterisation of Bound Residues of [3H]Triforine in Barley Grain Grown in the Field. *Pestic. Sci.* **1979**, *10*, 509–518.

Wargo, J. P.; Honeycutt, R. C.; Adler, I. L. Characterization of Bound Residues of Nitrofen in Cereal Grains. *J. Agric. Food Chem.* **1975**, *23*, 1097–1101.

Received for review April 22, 1991. Accepted September 19, 1991.

Registry No. DME, 41858-19-9; butachlor, 23184-66-9; starch, 9005-25-8.