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Preparation, Characterization, and Microbial Degradation of Specifically Radiolabeled [^{14}C]Lignocelluloses from Marine and Freshwater Macrophytes†

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Specifically radiolabeled [^{14}C-lignin]lignocelluloses were prepared from the aquatic macrophytes *Spartina alterniflora, Juncus roemerianus, Rhizophora mangle*, and *Carex walteriana* by using [^{14}C]phenylalanine, [^{14}C]tyrosine, and [^{14}C]cinnamic acid as precursors. Specifically radiolabeled [^{14}C-polysaccharide]lignocelluloses were prepared by using [^{14}C]glucose as precursor. The rates of microbial degradation varied among [^{14}C-lignin]lignocelluloses labeled with different lignin precursors within the same plant species. To determine the causes of these differential rates, [^{14}C-lignin]lignocelluloses were thoroughly characterized for the distribution of radioactivity in nonlignin contaminants and within the lignin macromolecule. In herbaceous plants, significant amounts (8 to 24%) of radioactivity from [^{14}C]phenylalanine and [^{14}C]tyrosine were found associated with protein, although very little (3%) radioactivity from [^{14}C]cinnamic acid was associated with protein. Microbial degradation of radiolabeled protein resulted in overestimation of lignin degradation rates in lignocelluloses derived from herbaceous aquatic plants. Other differences in degradation rates among [^{14}C-lignin]lignocelluloses from the same plant species were attributable to differences in the amount of label being associated with ester-linked subunits of peripheral lignin. After acid hydrolysis of [^{14}C-polysaccharide]lignocelluloses, radioactivity was detected in several sugars, although most of the radioactivity was distributed between glucose and xylose. After 576 h of incubation with salt marsh sediments, 38% of the polysaccharide component and between 6 and 16% of the lignin component (depending on the precursor) of *J. roemerianus* lignocellulose was mineralized to $^{14}CO_2$; during the same incubation period, 30% of the polysaccharide component and between 12 and 18% of the lignin component of *S. alterniflora* lignocellulose was mineralized.

Significant percentages of the particulate organic matter in freshwater wetlands and coastal marine ecosystems, such as salt marshes and mangrove swamps, are derived from vascular plants. Consequently, this organic detritus is composed primarily of the plant structural polymer lignocellulose (9, 13, 15, 16). Lignocellulose, a macromolecular complex which forms the bulk of higher plant biomass, consists of the aromatic heteropolymer lignin and the polysaccharides cellulose and hemicellulose. This fibrous component of plant tissue is recalcitrant to microbial degradation and is only slowly mineralized, converted to microbial biomass, or transformed into humic material.

Until recently, the kinetics of microbial transformations of lignocellulosic detritus in aquatic ecosystems have been difficult to characterize because of the long incubation periods required and the relative imprecision and insensitivity of methods for chemically determining changes in lignin and cellulose content in aquatic plant material. Recently, Maccubbin and Hodson (16) described a method which used incubations of specifically radiolabeled natural [^{14}C-lignin] lignocellulose and [^{14}C-cellulose]lignocellulose to determine rates of degradation and transformation of lignocellulosic particulate organic matter by salt marsh sediment microflora. The lignin fraction of lignocellulosic detritus derived from the cordgrass *Spartina alterniflora* was mineralized to CO_2 several times more slowly than the cellulose fraction, resulting in relative enrichment of the particulate organic matter in lignin. Degradation rates for both lignin and cellulosic components decreased logarithmically with incubation time. Preliminary results indicated that the decreases were due to increasing resistance to degradation of the remaining lignocellulose rather than to nutrient depletion during the month-long experiments. These conclusions were based on the assumption that mineralization of the labeled components of the lignocellulose closely tracks changes in bulk chemical composition of the lignocellulosic detritus. Subsequently, R. E. Hodson et al. (Mar. Biol., in press) tested the validity of this assumption by direct comparison of lignocellulose transformation rates determined by conventional chemical-gravimetric procedures and the radiotracer method. They found that both methods yielded essentially identical mineralization rates.

The radiotracer approach has advantages over the wet chemistry methods in several ways, including higher sensitivity, much shorter minimum incubation periods (days instead of months), smaller sample sizes, and the capability of tracing the flow of detritally derived carbon through microbial biomass into higher trophic levels in aquatic food webs. Of the available techniques, then, this would appear to be the method of choice for studying the transformation and fate of lignocellulosic particulate organic matter in aquatic environments. The method used to date for labeling *S. alterniflora* lignocellulose and following its rate of microbial degradation (15, 16) was adapted essentially unchanged from procedures described for labeling terrestrial woody plant lignocelluloses for forest litter decomposition studies (4, 5). Radiolabeling aquatic herbaceous plants, such as grasses and sedges, presents additional methodological problems in that these plants contain significantly higher percentages of

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protein than woody plants and their lignins differ significantly in composition from those of woody plants (17). These compositional differences will be reflected in the distribution of radiolabel in specifically labeled lignocellulose preparations, which in turn could cause errors in calculations of degradation rates. As reported by Maccubbin and Hodson (16), non-lignocellulosic material can account for significant percentages of total radiolabel in *S. alterniflora* lignocellulose prepared as originally described for woody plants; for instance, up to 34% of total label in lignin labeled with phenylalanine as precursor is solubilized upon hydrolysis with pepsin, suggesting that the material is protein. Subsequent experiments (unpublished data) indicated that under some conditions microbial mineralization of these contaminants can result in significant overestimates of the lignin mineralization rate. For the radiotracer approach to be widely applicable, these problems must be eliminated, and the method must be validated for lignocellulosic detritus derived from other important marine and freshwater plants. Until now, no attempts have been made to optimize labeling or experimental incubations for aquatic plants.

This report summarizes results of an intensive study to evaluate the extent of labeling of non-lignocellulosic components, determine the effect of labeled contaminants on the measurement of lignocellulose degradation rates, characterize the actual molecular components labeled in the preparations, and simplify incubation procedures for degradation experiments conducted under aquatic conditions. Several precursors of [^{14}C-lignin]lignocellulose were compared with respect to the efficiency and applicability of labeling the lignin component in four phylogenetically different aquatic plants: the black needle rush, *Juncus roemerianus*; the smooth cordgrass, *S. alterniflora*; the red mangrove, *Rhizophora mangle*; and the sedge, *Carex walteriana*.

MATERIALS AND METHODS

Preparation of radiolabeled lignocelluloses. *J. roemerianus* and short-form *S. alterniflora* plants used for radiolabeling were collected from Sapelo Island, Ga. *C. walteriana* plants used for radiolabeling were collected from the Okefenokee Swamp in southern Georgia. *R. mangle* cuttings used for radiolabeling were collected from Key Largo, Fla. The procedures used to prepare specifically radiolabeled lignocelluloses from *J. roemerianus, S. alterniflora, C. walteriana*, and *R. mangle* were adapted from methods described for terrestrial plants (4, 5). Branches of *R. mangle* (<1 cm in diameter) and the entire above-ground portions of *J. roemerianus, S. alterniflora*, and *C. walteriana* plants (<30 cm in height) were thoroughly washed in distilled water and placed in sterile test tubes containing 5 to 10 μCi of radioactive precursor in 1 ml of filter-sterilized water. Individual leaves from *R. mangle* were detached from the stem and placed in 10-ml beakers (two per beaker) containing 5 μCi of radioactive precursor in 1 ml of filter-sterilized water. The cuttings were placed under constant illumination and incubated until all of the liquid had been taken up. Additional sterile water was added to keep the cut ends immersed during a 72-h incubation period. Some plant cuttings were preferentially labeled in the lignin component with either L-[*U*-^{14}C]phenylalanine, L-[*U*-^{14}C]tyrosine, or side-chain labeled [3-^{14}C]cinnamic acid. Other cuttings were preferentially labeled in the cellulose-hemicellulose (polysaccharide) component with D-[*U*-^{14}C]glucose. After incorporation of radiolabel, cuttings were washed in distilled water and dried at 55°C for 48 to 72 h. Dried material was ground in a Waring blender to pass a 40-mesh screen (425 μm). An extract-free lignocellulose fraction was separated from unincorporated label and other plant components by serially extracting the plant material in a soxhlet apparatus with boiling ethanol (95%), ethanol-benzene (1:2, vol/vol), and water (2).

Characterization of radiolabeled lignocelluloses. The distribution of label between the lignin and polysaccharide components of *J. roemerianus* and *S. alterniflora* lignocelluloses was determined by Klason hydrolysis (2). Extract-free lignocelluloses (100 mg) were hydrolyzed with 72% sulfuric acid (2 ml) at 20°C for 2 h with continuous shaking. Samples were diluted with 56 ml of water, yielding a final acid concentration of 3%, and digested at 100°C for 3 h. A modified Klason hydrolysis was used to separate the lignin and polysaccharide components of *C. walteriana* and *R. mangle* lignocelluloses (7). Extract-free lignocelluloses (100 mg) were hydrolyzed with 72% sulfuric acid (2 ml) at 30°C for 1 h with continuous shaking. Samples were diluted with 56 ml of water and autoclaved at 15 lb/in^2 and 121°C for 1 h. During acid hydrolysis, polysaccharides are hydrolyzed, whereas most of the lignin condenses and remains insoluble. After acid hydrolysis, all samples were filtered (Whatman, Inc., Clifton, N.J.; GF/C) and thoroughly washed with hot water. The percentage of radioactivity remaining in the acid-insoluble lignin fraction was determined by combusting samples to $^{14}CO_2$ in an OX-300 Biological Oxidizer (R. J. Harvey Co., Hillsdale, N.J.) and collecting the $^{14}CO_2$ in a liquid scintillation medium (4). The percentage of radioactivity in the acid-soluble polysaccharide fraction was determined by assaying 1-ml samples of the filtrate in 10 ml of Scintiverse II (Fisher Scientific Co., Pittsburgh, Pa.) liquid scintillation medium. The distribution of radioactivity among sugars released during acid hydrolysis was determined by separation of the sugars via thin-layer chromatography and quantification of radioactivity associated with specific sugars (19).

Preparations of extract-free lignocellulose can have various amounts of protein associated with the lignocellulose as a contaminant (2). To determine the percentage of incorporated label associated with protein, lignocellulose samples (100 mg) were digested in a pepsin solution (4 ml, 1% solution in 0.1 N HCl) at 40°C for 20 h (2). Pepsin-treated lignocelluloses were washed thoroughly with hot water, and both the pepsin filtrate and the pepsin-insoluble fractions were assayed for radioactivity. Pepsin-treated and untreated lignocelluloses were also analyzed for total nitrogen content by combusting samples in a Hewlett-Packard model 185 carbon-hydrogen-nitrogen analyzer.

A common feature of grass lignins is the association of *p*-coumaric and ferulic acids esterified with the periphery of the lignin macromolecule (17). Samples (40 mg) of [^{14}C-lignin]lignocelluloses were hydrolyzed with 10 ml of 1 N NaOH at 20°C for 20 h (14). The mixture was filtered (Whatman GF/F), washed well with warm water, and acidified (pH 2) with 6 N HCl. The acidified filtrate was extracted with diethyl ether (10 ml, twice). The combined ether extracts were dried under a stream of nitrogen and dissolved in ethanol. Samples of the extract were chromatographed on cellulose thin-layer chromatography plates with *n*-butanol–acetic acid–water (62:15:23) (12). Ferulic, *p*-coumaric, and cinnamic acids were cochromatographed, and radioactivity from the corresponding spots of the [^{14}C]lignocellulose extracts was quantified.

Lignin is composed of three phenylpropane, cinnamyl alcohol derivatives in various concentrations depending on the plant species. To assess the distribution of label among the subunits of lignin, samples of *S. alterniflora* lignocellu-

lose labeled with [^{14}C]cinnamic acid were subjected to nitrobenzene oxidation (8). Five milliliters of 2 N NaOH, 0.5 ml of nitrobenzene, and 50 mg of [^{14}C]lignocellulose were added to a stainless steel Parr bomb and heated to 180°C for 2 h. After filtration (Whatman GF/F), the oxidation mixture was extracted (2 ml, twice) with dichloromethane to remove nitrobenzene. Phenolic aldehydes were removed from the mixture by acidifying to pH 2.5 (6 N NCl), saturating with sodium chloride, and extracting with dichloromethane (2 ml, twice). The combined solvent extracts were dried under a stream of nitrogen and dissolved in ethanol. The aldehydes were separated by thin-layer chromatography, and the radioactivity corresponding to each aldehyde was quantified (1).

Lignocellulose degradation experiments. Aerobic surface sediments were collected from Sapelo Island, Ga., in a high marsh area where *S. alterniflora* and *J. roemerianus* plants were interspersed. A seawater-sediment slurry consisting of 1 ml of sediment and 50 ml of filter-sterilized creek water (31‰) was used as an inoculum in incubations with *S. alterniflora* and *J. roemerianus* lignocelluloses. The seawater-sediment slurry (10 ml) was incubated with 10 mg of specifically radiolabeled lignocellulose (337 μm mean particle size) in 125-ml milk dilution bottles equipped with gassing ports. Samples were incubated in the dark with gentle shaking (125 rpm) at the in situ temperature of the sediment (28 to 32°C). The bottles were aerated with sterile, CO_2-free, humidified air for 15 min every 24 to 72 h. Oxygen tension measurements showed that the slurry remained well oxygenated (4.06 ml/liter) for these time periods. We compared rates of [^{14}C]lignocellulose mineralization for three different incubation procedures: stationary bottles oxygenated by continuous aeration, stationary bottles oxygenated by aeration for 15-min intervals every 24 h, and bottles incubated with shaking (125 rpm) and oxygenated by aeration for 15-min intervals every 24 h. After 284 h, all incubations had approximately the same mineralization rates and had mineralized the same total percentages of radioactivity. We chose the last method since the incubations were easy to maintain and had the least variation among replicates. Mineralization of the radiolabeled lignocelluloses was monitored by trapping the evolved $^{14}CO_2$ in a series of two scintillation vials containing liquid scintillation medium (16). Traps were changed after each aeration, and radioactivity was quantified in a Beckman LS-9000 liquid scintillation counter. Controls were killed with 5% Formalin and evolved no $^{14}CO_2$ during the incubation period. All incubations were done in triplicate.

To investigate the possible effects of nutrient depletion and buildup of toxic metabolites on lignocellulose degradation rates in long-term incubations, fresh seawater-sediment slurry was added to samples of aged [^{14}C]lignocelluloses. After 300 h of incubation with a seawater-sediment slurry, incubations containing specifically radiolabeled *S. alterniflora* lignocellulose were filtered onto 0.2-μm Nuclepore filters and washed with sterile creek water. Filters were dried (55°C) and kept frozen until the addition of a fresh seawater-sediment slurry. Upon the addition of 10 ml of a fresh seawater-sediment slurry, the aged [^{14}C]lignocellulose particles were scraped from the filters into milk dilution bottles. After replacement of the original seawater-sediment slurry with a fresh seawater-sediment slurry, bottles containing the aged [^{14}C-lignin]lignocellulose and [^{14}C-cellulose]lignocellulose were incubated for an additional 300 h.

Radiochemicals. D-[*U*-^{14}C]glucose and L-[*U*-^{14}C]phenylalanine were obtained from New England Nuclear Corp., Boston, Mass. L-[*U*-^{14}C]tyrosine and side-chain-labeled, [3-^{14}C]cinnamic acid were obtained from Amersham Corp., Arlington Heights, Ill.

RESULTS AND DISCUSSION

Validation of incubation procedure. Our previous aerobic laboratory incubations of [^{14}C]lignocelluloses with natural sediment slurries revealed that rates of degradation of both the lignin and polysaccharide components decrease logarithmically with time. Data from a typical incubation of salt marsh sediment with [^{14}C-lignin]lignocellulose and [^{14}C-polysaccharide]lignocellulose derived from *S. alterniflora*

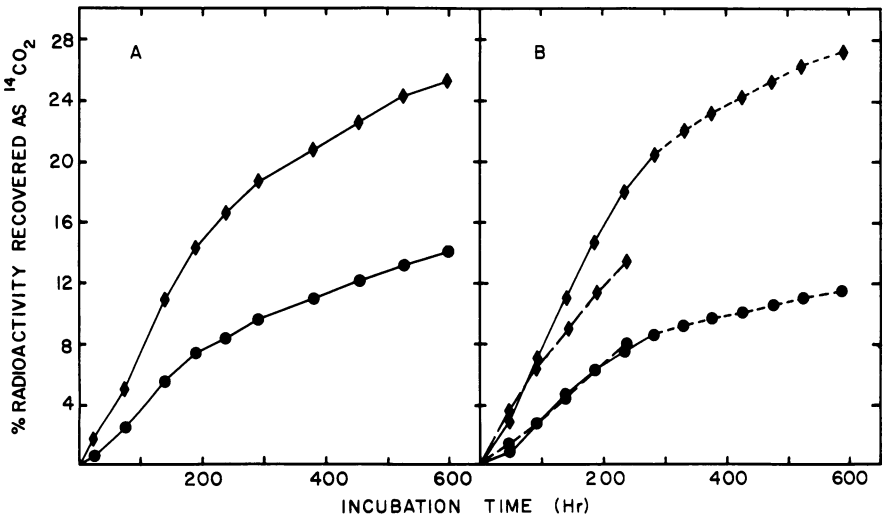

FIG. 1. Mineralization of [^{14}C]lignocellulose from *S. alterniflora* by salt marsh sediment microflora. (A) Typical data showing the kinetics of mineralization of [^{14}C-polysaccharide]lignocellulose (♦) and [^{14}C-lignin]lignocellulose (●). (B) Kinetics of mineralization of [^{14}C-polysaccharide]lignocellulose (♦) and [^{14}C-lignin]lignocellulose (●) with the original sediment slurry (——) and with the second sediment slurry (-----). To verify that the second sediment slurry contained an active lignocellulolytic microbial population, portions of the slurry were incubated with fresh [^{14}C]lignocellulose (— —). Each point represents the mean of three replicates.

are shown in Fig. 1A. The percent lignin or polysaccharide mineralized is a function of ln(t) after a given incubation period (t) (see Hodson et al., in press). We considered several hypotheses to explain the decrease in degradation rates: nutrient limitation, buildup of toxic metabolites (both artifacts of incubation), and actual increasing recalcitrance of the remaining lignocellulose to microbial degradation. To investigate the various possibilities, specifically labeled *S. alterniflora* lignocellulose was incubated for 285 h with a seawater-sediment slurry, recovered from the slurry by filtration, stored (−20°C) for 2 months, then added to a fresh seawater-sediment slurry and incubated for an additional 12 days. We considered that filtration of the [^{14}C]lignocellulose from the original seawater-sediment slurry would remove toxic metabolites and that reincubating the [^{14}C]lignocellulose in fresh seawater-sediment slurry would replace the natural concentrations of organic and inorganic nutrients that might be needed for optimal rates of degradation. Thus, if decreases in degradation rates were due to artifacts of the incubation procedures used rather than to increasing recalcitrance of the lignocellulose, rates should increase upon reinoculation in new slurry. During the first 285 h of incubation with the original seawater-sediment slurry, 8.6% of the [^{14}C-lignin]lignocellulose and 20.4% of the [^{14}C-polysaccharide]lignocellulose were mineralized (Fig. 1B). After replacement of the original slurry with fresh slurry, rates of mineralization of the lignin and polysaccharide components of the [^{14}C]lignocellulose did not return to the original rates observed during the first 285 h of incubation. Instead, rates of mineralization continued to decrease logarithmically (R^2 = 0.99) with time, indicating that decreases in rates of mineralization were due to increasing recalcitrance of the lignocellulose to microbial degradation rather than to nutrient limitation or accumulation of toxic metabolites. It is also possible that the slower mineralization rates during reincubation of the [^{14}C]lignocellulose with the second seawater-sediment slurry were due to a more inactive lignocellulolytic microbial population than the one present in the first slurry. To verify that the second slurry contained an active lignocellulolytic microbial population, an additional set of new slurries were incubated with fresh [^{14}C-lignin]lignocellulose and [^{14}C-polysaccharide]lignocellulose. The initial mineralization rates of the lignin and polysaccharide components of fresh [^{14}C]lignocellulose incubated with the second slurry were very similar to those observed during incubation with the first seawater-sediment slurry (Fig. 1B). We conclude that the kinetics of [^{14}C]lignocellulose degradation observed in month-long laboratory incubations are interpretable and are not artifacts of incubation methods.

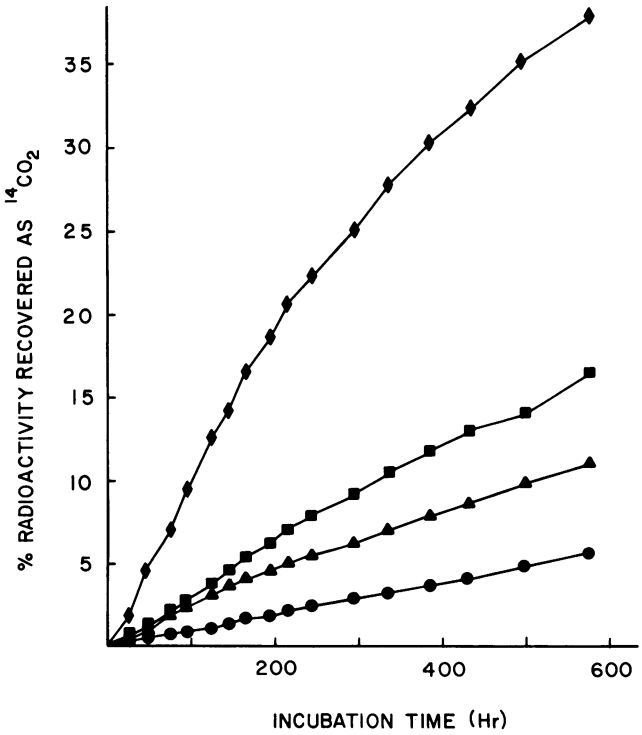

FIG. 2. Mineralization of [^{14}C]lignocellulose from *J. roemerianus* by salt marsh sediment microflora. Symbols: ♦, [^{14}C]glucose labeled; ■, [^{14}C]phenylalanine labeled; ▲, [^{14}C]tyrosine labeled; ●, [^{14}C]cinnamic acid labeled. Each point represents the mean of three replicates.

Microbial degradation of [^{14}C]lignocelluloses. One of the objectives of this study was to compare the kinetics of microbial degradation of [^{14}C-lignin]lignocelluloses which had been labeled with various precursors of lignin. Phenylalanine and cinnamic acid are universal precursors in the biosynthesis of lignin; tyrosine is a precursor in the biosynthesis of lignin in a limited number of plant genera, mainly in the grass family (17). All three precursors are readily available in radiolabeled form and are relatively inexpensive. If the precursors specifically label the lignin component of the lignocellulose identically, then rates of degradation of the resulting [^{14}C-lignin]lignocelluloses should also be the same. However, we found significant differences in degradation rates. For example, mineralization rates of *J. roemerianus* lignocellulose labeled in the lignin component with [^{14}C]cinnamic acid were significantly lower (two- to threefold) than rates of mineralization of *J. roemerianus* lignocellulose labeled with [^{14}C]phenylalanine or [^{14}C]tyrosine as precursor (Fig. 2). It seems likely that either the precursors were labeling components other than lignin or that they were not labeling the same subunits of the lignin component. However, all three [^{14}C-lignin]lignocelluloses were mineralized much more slowly than the glucose-labeled [^{14}C-polysaccharide]lignocelluloses. After 576 h of incubation, 2.3- to 6.8-fold more of the polysaccharide component of *J. roemerianus* lignocellulose was mineralized than the lignin component (depending on the lignin precursor) (Fig. 2).

Rates of mineralization of specifically radiolabeled lignocellulose from *S. alterniflora* are presented in Fig. 3. Although the total amount of label mineralized by the end of the experiment (576 h) did not vary as much among [^{14}C-lignin]lignocelluloses from *S. alterniflora* as it did with *J. roemerianus* [^{14}C-lignin]lignocelluloses, distinct differences in the kinetics of mineralization were observed between [^{14}C]cinnamic acid-labeled lignin and [^{14}C]phenyalanine-labeled or [^{14}C]tyrosine-labeled lignocellulose. During the first 100 h of incubation, [^{14}C]phenylalanine-labeled lignocellulose was mineralized faster than either [^{14}C]tyrosine-labeled or [^{14}C]cinnamic acid-labeled lignocelluloses. However, after 400 h of incubation, [^{14}C]cinnamic acid-labeled lignocellulose was mineralized at a faster rate than the other two [^{14}C-lignin]lignocelluloses. As with *J. roemerianus* lignocellulose, the polysaccharide component of *S. alterniflora* lignocellulose was mineralized at a faster rate than the lignin component; for example, after 576 h, 30% of glucose-labeled lignocellulose had been mineralized, which is equivalent to 1.6 and 2.5 times the mineralizations of [^{14}C]cinnamic acid-

labeled and [14C]tyrosine-labeled *S. alterniflora* lignocellulose, respectively (Fig. 3).

The [14C]phenylalanine and [14C]tyrosine used as precursors of [14C-lignin]lignocellulose were universally labeled, whereas the [14C]cinnamic acid lignin precursor was labeled in the side-chain carbon atom adjacent to the aromatic ring. This carbon has been shown in previous studies to be mineralized by microorganisms at approximately the same rate as the aromatic carbons of lignin (10). In contrast, terminal alcohol carbons of the side chain and carbons in methoxy substituents of the aromatic ring have been found to be mineralized at slightly higher rates (10). Thus, [14C]cinnamic acid-labeled [14C-lignin]lignocelluloses would be expected to be mineralized at rates indicative of degradation of the aromatic structures of the lignin macromolecule. [14C]phenylalanine-labeled and [14C]tyrosine-labeled lignocelluloses do contain labeled terminal alcohol carbons, but these make up only a minor percentage of total radiolabel, and thus, mineralization rates of such preparations also will be approximately representative of degradation of the aromatic structure of lignin. Thus, the observed differences in rates of mineralization of [14C-lignin]lignocelluloses labeled with the various precursors are not likely due to the different distributions of radiolabel in the precursors.

Distribution of label between lignin and polysaccharides. To investigate the causes of the observed differences in mineralization rates of [14C]lignocelluloses, we attempted to characterize the chemical components labeled by the various precursors. The distribution of label in [14C]lignocelluloses prepared from four phylogenetically different aquatic plants, *J. roemerianus, S. alterniflora, C. walteriana*, and *R. mangle*, is presented below for comparison.

The distribution of label between the lignin and polysaccharide components of the various [14C]lignocelluloses was determined by Klason hydrolysis. All the [14C-lignin]lignocelluloses contained the bulk of radioactivity in the acid-insoluble lignin fraction (Table 1). In contrast, when acid hydrolysis was performed on [14C-polysaccharide]lignocelluloses derived from *S. alterniflora, R. mangle*, and *C. walteriana*, the greater part of the label was found in the acid-soluble, presumably polysaccharide, fraction. However, in [14C-polysaccharide]lignocellulose derived from *J. roemerianus*, only 43.7% of the label was associated with the acid-soluble fraction. No complete separation of lignin from all other plant components has been described. Instead, with the methods presently available, some lignin is solubilized by the acid hydrolysis, and some protein and polyphenols may condense with the insoluble lignin fraction (17). Of the various precursors used to label the lignin component of *J. roemerianus, S. alterniflora*, and *C. walteriana* lignocelluloses, [14C]cinnamic acid yielded the highest percentage of label (76.9, 87.3, and 77.3%, respectively) in the acid-insoluble (lignin) fractions (Table 1). With *R. mangle* leaves labeled in the lignin component with [14C]cinnamic acid, 94.5% of the radioactivity was retained in the acid-insoluble fraction (Table 1). Thus, the potential for cross-labeling (labeling the polysaccharide component) when preparing [14C-lignin]lignocellulose, especially from nonwoody plant tissue, is minimized when [14C]cinnamic acid is used as precursor.

Although the percentages of acid-soluble label are greater for [14C]phenylalanine-labeled and [14C]tyrosine-labeled lignocelluloses than for [14C]cinnamic acid-labeled lignocelluloses, rates of mineralization were not correlated with the percentage of acid-soluble label in a particular [14C-lignin]lignocellulose preparation. For example, [14C]tyrosine-labeled

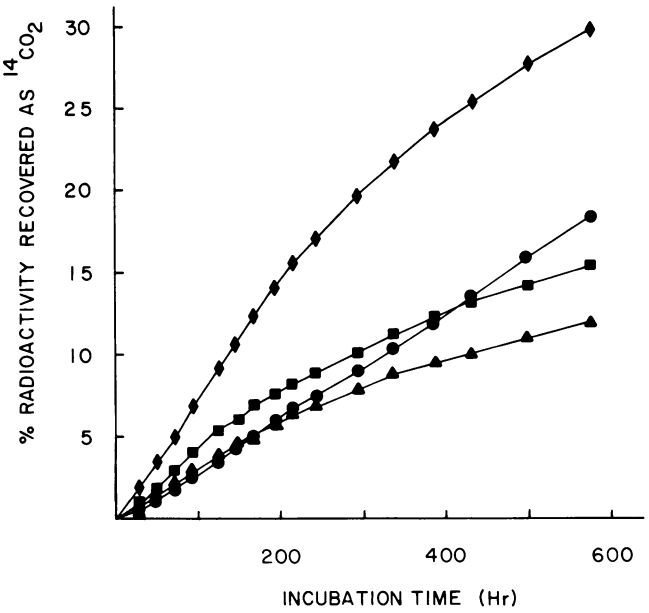

FIG. 3. Mineralization of [14C]lignocellulose from *S. alterniflora* by salt marsh sediment microflora. Symbols: ◆, [14C]glucose labeled; ■, [14C]phenylalanine labeled; ▲, [14C]tyrosine labeled; ●, [14C]cinnamic acid labeled. Each point represents the mean of three replicates.

J. roemerianus lignocellulose had a much higher percentage of acid-soluble radioactivity (31.5%) than [14C]phenylalanine-labeled lignocellulose did (16.4%), although [14C]phenylalanine-labeled lignocellulose was mineralized faster (Fig. 2).

With [14C-polysaccharide]lignocelluloses, between 44 and 84% of the label was associated with the acid-soluble (polysaccharide) fraction. The presence of significant percentages of acid-insoluble radioactivity indicates that cross-labeling of the lignin fraction can be appreciable in some plants when [14C]glucose is used as precursor of [14C-polysaccharide]lignocellulose (Table 1). When significant amounts of lignin are labeled with [14C]glucose, mineralization rates of polysaccharides may be underestimated.

We considered that radioactivity in the Klason acid-soluble fraction of various lignocelluloses could be associated with a variety of labeled compounds derived from cellulose and hemicellulose as well as acid-soluble lignin derivatives. Samples of the Klason filtrates from the various [14C]lignocelluloses were chromatographed by thin-layer chromatography to determine the amount of radioactivity in sugars. In [14C-lignin]lignocelluloses from *J. roemerianus, S. alterniflora, C. walteriana*, and *R. mangle*, less than 3% of the acid-soluble label was associated with sugars, suggesting that the acid-solubilized label was associated with acid-soluble lignin (6; data not shown). In [14C-polysaccharide] lignocellulose from *J. roemerianus*, 24 and 17% of the acid-soluble radioactivity were associated with glucose and xylose, respectively. In [14C-polysaccharide]lignocellulose prepared from *R. mangle* leaves, 22 and 6% of the acid-soluble radioactivity were located in glucose and xylose, respectively. A substantial percentage of the acid-soluble label (20%) was associated with an unidentified sugar. In [14C-polysaccharide]lignocellulose from *R. mangle* wood, 24 and 7% of the acid-soluble label were associated with glucose and xylose, respectively, and 9% of the label was

TABLE 1. Distribution of label between the polysaccharide (acid-soluble) and lignin (acid-insoluble) components of [^{14}C]lignocelluloses and the percentage of label solubilized during pepsin digestion

Lignocellulose	^{14}C-labeled precursor[b]	Sp act (dpm/mg)	Label (%)[a]		
			Acid-soluble	Acid-insoluble	Pepsin-soluble
J. roemerianus	Phe	14,347	16.4	83.6	24.1
	Tyr	2,550	31.5	68.5	8.4
	CinA	13,428	12.7	87.3	2.9
	Glucose	8,342	43.7	56.3	15.5
S. alterniflora	Phe	11,086	29.1	70.9	19.0
	Tyr	3,663	27.0	73.0	17.2
	CinA	9,601	23.1	76.9	3.1
	Glucose	4,342	61.9	38.1	21.9
C. walteriana	Phe	11,779	42.9	57.1	12.0
	CinA	7,103	22.7	77.3	2.5
	Glucose	2,827	77.4	22.6	22.9
R. mangle leaves	CinA	2,108	5.5	94.5	1.6
	Glucose	1,432	84.0	16.0	4.6
R. mangle wood	Phe	10,452	14.0	86.0	0.9
	Glucose	4,122	56.4	43.6	2.4

[a] Percentage recovered normalized to 100. Actual recoveries ranged from 73 to 128%; mean, 97.4%.

[b] Phe, Phenylalanine; Tyr, tyrosine; CinA, cinnamic acid.

associated with an unidentified sugar. Thus, in [^{14}C-polysaccharide]lignocelluloses from *J. roemerianus* and *R. mangle*, the cellulose fraction of the polysaccharides was preferentially labeled, although a significant amount of label was located in the hemicellulose fraction. In [^{14}C-polysaccharide]lignocellulose from *S. alterniflora*, 47 and 26% of the acid-soluble label were associated with xylose and glucose, respectively, indicating that hemicellulose was labeled to a greater extent than cellulose. We had previously found with an earlier batch of [^{14}C]glucose-labeled *S. alterniflora* lignocellulose that the cellulosic component was preferentially labeled and referred to the material as [^{14}C-cellulose]lignocellulose (16). However, in light of the present results this seems to be a misnomer, and we have devised [^{14}C-polysaccharide]lignocellulose as the proper term. Overall, analysis of the various [^{14}C-polysaccharide]lignocelluloses demonstrated that 45 to 75% of the acid-soluble radioactivity was associated with simple sugars derived from hydrolysis of polysaccharides.

Radiolabel associated with protein. Small amounts of protein remain in lignocellulose preparations after extraction of the plant material to remove non-lignocellulosic components (3). For most experimental purposes, it is desirable to minimize the labeling of protein when preparing [^{14}C]lignocelluloses. The amount of radioactivity associated with protein in our [^{14}C]lignocellulose preparations was determined by measuring the amount of radioactivity solubilized by digestion with a proteolytic solution containing pepsin. When [^{14}C]cinnamic acid was used as the precursor of [^{14}C-lignin]lignocellulose synthesis in *J. roemerianus, S. alterniflora, C. walteriana*, or *R. mangle*, only 3.1% or less of the incorporated label was solubilized by pepsin digestion (Table 1). In contrast, when [^{14}C]phenylalanine or [^{14}C]tyrosine was used as precursor in *J. roemerianus, S. alterniflora*, or *C. walteriana*, between 8.4 and 24.1% of incorporated radioactivity was solubilized by pepsin. Apparently, with herbaceous plants, [^{14}C]phenylalanine and [^{14}C]tyrosine can be incorporated into protein to a significant extent, whereas [^{14}C]cinnamic acid is only minimally incorporated into protein. Significant amounts (2.4 to 22.9%) of radioactivity were also incorporated into protein when [^{14}C]glucose was used as the precursor to label the polysaccharide component of lignocellulose in nonwoody plant materials (Table 1). In contrast, *R. mangle* wood lignocellulose labeled with [^{14}C]phenylalanine or [^{14}C]glucose contained less than 1 and 3%, respectively, of radioactivity as protein as indicated by pepsin digestion. These data indicate that lignocellulose from woody plant tissue, which is usually low in protein, can be labeled specifically in the lignin and polysaccharide moieties with [^{14}C]phenylalanine and [^{14}C]glucose, respectively, with little or no contamination from labeled protein.

Nitrogen analyses of lignocellulose preparations before and after pepsin treatment indicated that total nitrogen content decreased from 1.48 to 0.82% of plant dry weight for *J. roemerianus*, from 1.47 to 0.77% for *S. alterniflora*, and from 0.84 to 0.31% for *C. walteriana*. The total nitrogen content of *R. mangle* lignocelluloses was low and did not decrease after pepsin treatment. The fact that some nitrogen remains in lignocellulose after pepsin treatment may be due to unhydrolyzed protein or to nonprotein nitrogen, which has been shown to be a significant fraction (8 to 14%) of the nitrogen in living *S. alterniflora* and *J. roemerianus* plants (11). Any protein that is not removed during pepsin digestion is probably located below the surface of the lignocellulose particle and thus is not accessible to the enzyme. Therefore, during short-term incubations of pepsin-digested lignocelluloses with sediment, any labeled protein remaining within the particles is likely to be inaccessible to microbial enzymes and thus is not likely to affect the degradation rate.

Effects of protein mineralization on apparent lignin mineralization rates. Since radiolabeled protein was present in various amounts in the [^{14}C]lignocellulose preparations, we considered it possible that the differences observed in rates of microbial mineralization might be at least partially attributable to mineralization of protein. To examine this possibility, we compared rates of marine microbial mineralization of the variously labeled [^{14}C-lignin]lignocelluloses from *J. roemerianus* and *S. alterniflora* prepared exactly as described above with rates of mineralization of lignocelluloses prepared identically, except that they were subsequently subjected to pepsin hydrolysis to remove protein. Decreases in mineralization rates occurred when labeled protein was removed with pepsin treatment before incubation; the rates of mineralization of [^{14}C]phenylalanine-labeled and [^{14}C]tyrosine-labeled lignocelluloses from *J. roemerianus* decreased two- to threefold to rates almost identical to that of untreated [^{14}C]cinnamic acid-labeled lignocellulose (Fig. 4). No significant difference in the mineralization rates of pepsin-treated and untreated [^{14}C]cinnamic acid-labeled lignocellulose was observed; this finding is consistent with the low percentage of radioactivity solubilized by pepsin digestion (2.9%). Consequently, we may conclude that in short-term incubations, use of [^{14}C]phenylalanine-labeled and [^{14}C]tyrosine-labeled *J. roemerianus* lignocelluloses will significantly overestimate the rate of lignin degradation unless the lignocelluloses are first digested with pepsin to remove labeled protein. Protease treatment of [^{14}C-lignin]lignocellulose preparations was originally recommended by Crawford and Crawford (6). After the removal of labeled protein with pepsin, the three *J. roemerianus* [^{14}C-lignin]lignocelluloses were mineralized at the same rate, indicating that the three precursors labeled the lignin moiety similarly. Comparison of mineralization

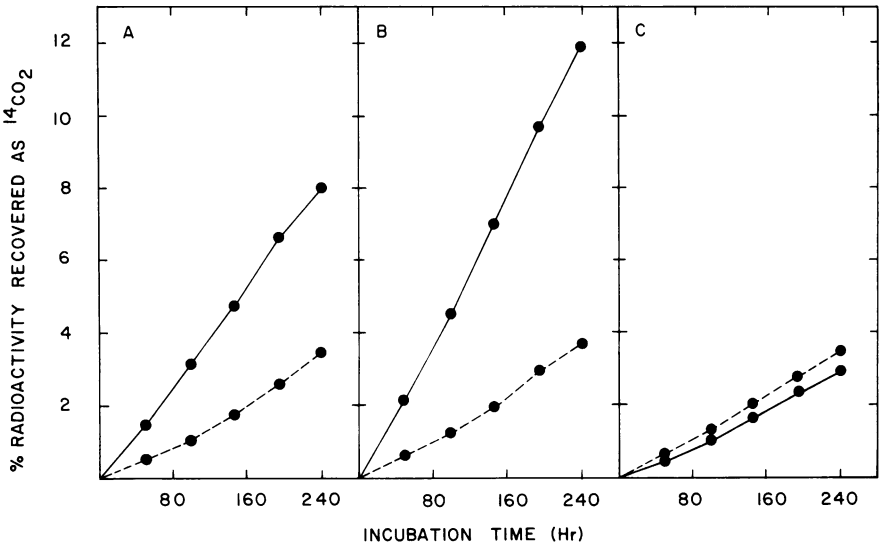

FIG. 4. Mineralization of [^{14}C-lignin]lignocellulose from *J. roemerianus* by salt marsh sediment microflora before (solid lines) and after (broken lines) pepsin treatment. (A) [^{14}C]phenylalanine labeled; (B) [^{14}C]tyrosine-labeled; (C) [^{14}C]cinnamic acid labeled. Each point represents the mean of three replicates.

rates of [^{14}C]lignocellulose with and without prior treatment with pepsin enables one to follow turnover rates of both lignin and protein.

[^{14}C-lignin]lignocelluloses isolated from *S. alterniflora* and labeled with [^{14}C]phenylalanine or [^{14}C]tyrosine were mineralized only slightly faster than *S. alterniflora* lignocellulose labeled with [^{14}C]cinnamic acid (Fig. 5). Pepsin-treated [^{14}C]phenylalanine-labeled and [^{14}C]tyrosine-labeled *S. alterniflora* lignocelluloses were mineralized 3.1 and 2.1 times more slowly, respectively, than the same lignocelluloses without prior pepsin treatment, indicating that the labeled protein was interfering with the determination of lignin mineralization rates. However, pepsin-treated [^{14}C]cinnamic acid-labeled *S. alterniflora* lignocellulose was mineralized only slightly slower (1.2 times) than the same lignocellulose without pepsin treatment, indicating there was little interference from labeled protein. This result is consistent with the very low percent solubilization of radioactivity (3.1%) resulting from pepsin hydrolysis. Unlike those from *J. roemerianus*, the variously labeled [^{14}C-lignin]lignocelluloses from *S. alterniflora* were not mineralized at the same rates after pepsin treatment; [^{14}C]cinnamic acid-labeled lignocellulose was mineralized 2.1 times faster than [^{14}C]phenylalanine-labeled lignocellulose and 1.5 times faster than [^{14}C]tyrosine-labeled lignocellulose. These differences in mineralization rates after the removal of labeled protein suggest that [^{14}C]cinnamic acid labeled the lignin of *S. alterniflora* in a manner different from that of [^{14}C]phenylalanine and [^{14}C]tyrosine.

No direct comparison was made of rates of microbial mineralization of pepsin-treated and untreated *C. walteriana* lignocelluloses labeled with [^{14}C]phenylalanine and [^{14}C]cin-

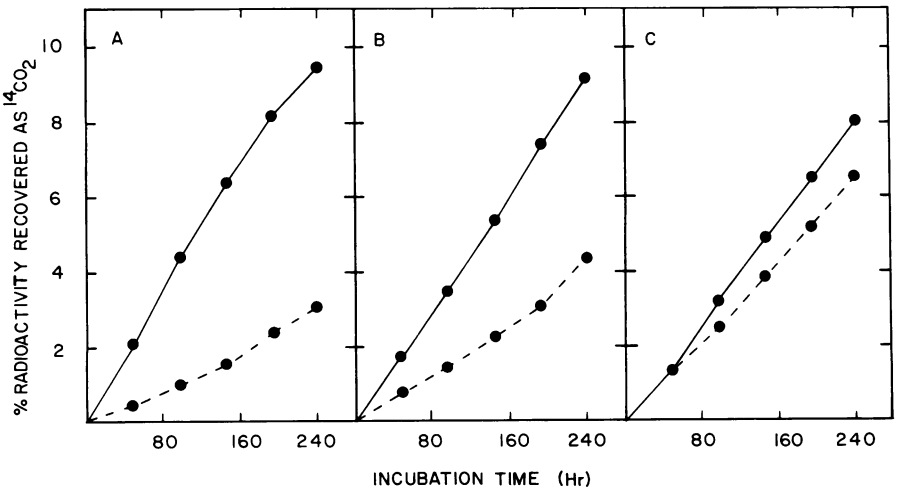

FIG. 5. Mineralization of [^{14}C-lignin]lignocellulose from *S. alterniflora* by salt marsh sediment microflora before (solid lines) and after (broken lines) pepsin treatment. (A) [^{14}C]phenylalanine labeled; (B) [^{14}C]tyrosine labeled; (C) [^{14}C]cinnamic acid labeled. Each point represents the mean of three replicates.

TABLE 2. Incorporation of [^{14}C]lignin precursors into cinnamic acid esters (ferulic, *p*-coumaric, and cinnamic acids) and the percentage of label solubilized during hydrolysis with 1 N NaOH

Lignocellulose	^{14}C-labeled precursor[a]	Label (%)		
		Solubilized	Ether-soluble	In esters
J. roemerianus	Phe	35.9	9.0	5.5
	Tyr	54.2	2.8	2.0
	CinA	30.2	10.0	5.0
S. alterniflora	Phe	36.3	7.4	5.0
	Tyr	79.8	6.3	5.4
	CinA	66.2	19.2	10.2
C. walteriana	CinA	74.6	13.7	11.8
R. mangle leaves	CinA	32.4	4.5	2.5
R. mangle wood	Phe	16.8	0.6	0

[a] See Table 1, footnote *b*, for abbreviations.

namic acid. However, [^{14}C]phenylalanine-labeled lignocellulose was mineralized approximately two times faster than [^{14}C]cinnamic acid-labeled lignocellulose when incubated with sediment from the Okefenokee Swamp (data not shown). Since *R. mangle* plant material is low in protein and very little radiolabel was found associated with protein, comparative incubations of pepsin-treated and untreated lignocelluloses were not performed.

The presence of radiolabeled protein in [^{14}C]lignocellulose preparations does not always interfere with measurement of lignin degradation. Maccubbin and Hodson (16) labeled *S. alterniflora* lignocellulose in the lignin component with [^{14}C]phenylalanine, and although a significant percentage (34%) of the radioactivity was solubilized by pepsin digestion, they found no difference between the mineralization rates of pepsin-treated and untreated material. In fact, the [^{14}C]phenylalanine-labeled *S. alterniflora* lignocellulose used in the present study was mineralized 1.5 times faster than the [^{14}C]phenylalanine-labeled lignocellulose used by Maccubbin and Hodson (16), whereas the [^{14}C]glucose-labeled *S. alterniflora* lignocelluloses used in both studies were mineralized at nearly identical rates by the salt marsh sediment microflora.

Distribution of radiolabel within the lignin component of lignocelluloses. From data shown above, it can be concluded that the three lignin precursors labeled the lignin component of *J. roemerianus* lignocellulose similarly and that the differences in the rates of mineralization among [^{14}C-lignin]lignocelluloses not extracted with pepsin were due to the various degrees of labeling of protein by [^{14}C]phenylalanine and [^{14}C]tyrosine. In contrast, the three lignin precursors apparently did not label the lignin component of *S. alterniflora* lignocellulose similarly. Pepsin-digested [^{14}C]cinnamic acid-labeled lignocellulose was mineralized faster than either of the other [^{14}C-lignin]lignocelluloses after pepsin treatment, indicating that the radioactivity in the lignin macromolecule labeled with [^{14}C]cinnamic acid was distributed differently than it was in lignin labeled with the other two precursors tested.

Herbaceous plants, especially grasses, are known to contain cinnamic acid derivatives esterified to the periphery of the lignin macromolecule (17). Ferulic and *p*-coumaric acids are the most commonly found cinnamic acids esterified with lignins, and they typically make up 5 to 10% of grass lignins (14, 18). These bound cinnamic acids possibly function as linkage units between core lignin and structural carbohydrates of plant cell walls and as precursors of further core lignin biosynthesis (17). Saponification of lignocelluloses with weak alkali solubilizes some core lignin and releases esterified cinnamic acids of peripheral lignin (17). We subjected the variously labeled [^{14}C-lignin]lignocelluloses to this procedure to determine whether or not differences in the degree of labeling of cinnamic acid derivatives could account for the observed differences in biodegradation rates. After removal from the lignocelluloses, the cinnamic acids were then separated by thin-layer chromatography, and the radioactivity in each acid was determined. As shown in Table 2, in *J. roemerianus* [^{14}C-lignin]lignocelluloses labeled with [^{14}C]phenylalanine or [^{14}C]cinnamic acid, approximately the same percentage (5.5 and 5.0%, respectively) of the radioactivity was in cinnamic acid esters (sum of ferulic, *p*-coumaric, and cinnamic acids). This finding is consistent with the fact that [^{14}C]phenylalanine-labeled and [^{14}C]cinnamic acid-labeled [^{14}C-lignin]lignocelluloses were mineralized at nearly identical rates after removal of contaminating labeled protein from the [^{14}C]phenylalanine-labeled preparation. [^{14}C]tyrosine-labeled lignocellulose had a lower percentage (2.0%) of radioactivity in cinnamic acid esters, but it was also mineralized after pepsin treatment at a rate similar to that of the other [^{14}C-lignin]lignocelluloses from *J. roemerianus*.

In [^{14}C-lignin]lignocellulose from *S. alterniflora* labeled with [^{14}C]cinnamic acid, 10.2% of the radioactivity was located in cinnamic acid esters, whereas in [^{14}C]phenylalanine-labeled and [^{14}C]tyrosine-labeled lignocelluloses only 5.0 and 5.4%, respectively, of the radioactivity was located in cinnamic acid esters. It is likely, therefore, that the greater percentage of labeled cinnamic acid esters in [^{14}C]cinnamic acid-labeled *S. alterniflora* lignocellulose may contribute to its faster mineralization rate relative to [^{14}C]phenylalanine-labeled or [^{14}C]tyrosine-labeled lignocelluloses after pepsin removal of contaminating labeled protein. However, it has not been established previously that the cinnamic acid esters of the so-called peripheral lignin are microbially degraded at higher rates than the core lignin.

The [^{14}C]cinnamic acid-labeled *J. roemerianus* lignocellulose was mineralized two to three times slower than [^{14}C]cinnamic acid-labeled *S. alterniflora* lignocellulose by the natural assemblage of salt marsh sediment microflora. Neither of the [^{14}C]cinnamic acid-labeled lignocelluloses contained a significant amount of contaminating labeled protein, but the [^{14}C]cinnamic acid-labeled *S. alterniflora* lignocellulose contained approximately twice as much label in esterified cinnamic acids of peripheral lignin than [^{14}C]cinnamic acid-labeled *J. roemerianus* lignocellulose. Since esterified cinnamic acids appear to be more easily biodegradeable than core lignin, the observed differences in the mineralization rates of [^{14}C]cinnamic acid-labeled *J. roemerianus* and *S. alterniflora* lignocelluloses may in part be due to the greater relative percentage of labeled peripheral lignin in *S. alterniflora* [^{14}C-lignin]lignocellulose. Pepsin-treated [^{14}C]phenylalanine-labeled and [^{14}C]tyrosine-labeled *J. roemerianus* and *S. alterniflora* lignocelluloses were mineralized at almost identical rates during short-term incubations. Esterified cinnamic acids accounted for approximately the same percentage of total radioactivity in these [^{14}C-lignin]lignocellulose preparations. The polysaccharide component of *J. roemerianus* lignocellulose was mineralized at a slightly faster rate than the polysaccharide component of *S. alterniflora* lignocellulose; the polysaccharide components of both plants were mineralized faster than the lignin components. The fact

that the polysaccharide components of *J. roemerianus* and *S. alterniflora* lignocelluloses are degraded by salt marsh sediment microflora more rapidly than the lignin components results in the detritus from these plants being gradually enriched in lignin and thus increasingly recalcitrant to microbial degradation.

[^{14}C]cinnamic acid-labeled *C. walteriana* lignocellulose contained a high percentage of radioactivity (11.8%) in cinnamic acid esters, whereas in [^{14}C-lignin]lignocelluloses prepared from *R. mangle* leaves and wood, much lower percentages of the radioactivity were associated with labeled cinnamic acid esters in the peripheral lignin. In [^{14}C]cinnamic acid-labeled lignocellulose from *R. mangle* leaves, 2.5% of the label was associated with ester groups. *R. mangle* lignocellulose prepared from wood labeled with [^{14}C]phenylalanine contained no detectable label in esters. These results are consistent with the fact that lignins of woody plant tissues generally lack these ester groups (18).

Grass lignins consist of three phenylpropane subunits, coniferyl, sinapyl and *p*-coumaryl alcohols, in various proportions depending on the particular plant species. Upon nitrobenzene oxidation, these three phenylpropane subunits yield vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde, respectively. Gardner and Menzel (8) found that living *S. alterniflora* plants yielded vanillin–*p*-hydroxybenzaldehyde–syringaldehyde at a ratio of 2.4:1.0:2.2. To determine whether the various monomeric subunits of *S. alterniflora* lignin were labeled proportionately by [^{14}C]cinnamic acid, [^{14}C-lignin]lignocellulose was subjected to nitrobenzene oxidation, the oxidation products were separated by thin-layer chromatography, and the radioactivity in each of the resulting aromatic aldehydes characteristic of lignin was quantified (1). We found that the radioactivity in [^{14}C]cinnamic acid-labeled *S. alterniflora* lignocellulose was distributed among vanillin, *p*-hydroxybenzaldehyde, and syringaldehyde at a similar ratio (2.3:1.0:1.6), indicating that the label was adequately distributed throughout the lignin molecule.

In conclusion, this study has demonstrated that the specific radiolabeling approach is applicable to studies of the transformations and fate of detrital lignocelluloses in freshwater and marine sediments, using aerobic laboratory incubations lasting up to 1 month. The particular precursor chosen to label the lignin component will depend on the type of plant material being labeled. Precursors, such as [^{14}C]phenylalanine, which are commonly used to label lignin in woody plants label significant amounts of protein in herbaceous plants; unless removed from [^{14}C-lignin]lignocellulose preparations, the labeled protein can interfere with determinations of lignin degradation rates. Our experience with preparing specifically radiolabeled lignocelluloses derived from a wide variety of aquatic macrophytes has led us to conclude that herbaceous plants, which normally contain a higher proportion of protein than woody plants, can be successfully radiolabeled in the lignin component by using [^{14}C]cinnamic acid as precursor and that very little label is incorporated into protein. The polysaccharide component of lignocellulose is preferentially labeled with [^{14}C]glucose, although some of the label is incorporated into protein and lignin. When significant amounts of lignin are labeled by [^{14}C]glucose, mineralization rates of polysaccharides may be underestimated. Finally, we conclude that as suggested by Crawford and Crawford (6), each time [^{14}C]lignocellulose is prepared, the material should be chemically characterized before use to ensure that the observed rates of degradation accurately reflect those of the lignin or polysaccharide components.

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LITERATURE CITED

1. **Brand, J. M.** 1966. Studies on grass lignins. I. Separation and quantitative determination of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde by thin-layer chromatography. J. Chromatogr. **21**:424–429.
2. **Browning, B. L.** 1967. Methods of wood chemistry, vol. 2. Interscience Publishers Inc., New York.
3. **Crawford, D. L.** 1978. Lignocellulose decomposition by selected *Streptomyces* strains. Appl. Environ. Microbiol. **35**:1041–1045.
4. **Crawford, D. L., and R. L. Crawford.** 1976. Microbial degradation of lignocellulose: the lignin component. Appl. Environ. Microbiol. **31**:714–717.
5. **Crawford, D. L., R. L. Crawford, and A. L. Pommeto III.** 1977. Preparation of specifically labeled ^{14}C-(lignin)- and ^{14}C-(cellulose)-lignocelluloses and their decomposition by the microflora of soil. Appl. Environ. Microbiol. **33**:1247–1251.
6. **Crawford, R. L., and D. L. Crawford.** 1978. Radioisotopic methods for the study of lignin biodegradation. Dev. Ind. Microbiol. **19**:35–49.
7. **Effland, M. J.** 1977. Modified procedure to determine acid-insoluble lignin in wood and pulp. TAPPI **60**(10):143–144.
8. **Gardner, W. S., and D. W. Menzel.** 1974. Phenolic aldehydes as indicators of terrestrially-derived organic matter in the sea. Geochim. Cosmochim. Acta **38**:813–822.
9. **Godshalk, G. L., and R. G. Wetzel.** 1978. Decomposition of aquatic angiosperms. II. Particulate components. Aquat. Bot. **5**:301–327.
10. **Haider, K., J. P. Martin, and E. Rietz.** 1977. Decomposition in soil of ^{14}C-labeled coumaryl alcohols: free and linked into dehydropolymer and plant lignins and model humic acids. Soil. Sci. Soc. Am. J. **41**:556–561.
11. **Haines, E. B., and R. B. Hanson.** 1979. Experimental degradation of detritus made from the salt marsh plants *Spartina alterniflora* Loisel., *Salicornia virginica* L., and *Juncus roemerianus* Scheele. J. Exp. Mar. Biol. Ecol. **40**:27–40.
12. **Hartley, R. D.** 1973. Carbohydrate esters of ferulic acid as components of cell-walls of *Lolium multiflorum*. Phytochemistry **12**:661–665.
13. **Heald, E.** 1971. The production of organic detritus in a south Florida estuary. Univ. Miami Sea Grant Program Sea Grant Tech. Bull. **6**:1–110.
14. **Higuchi, T., Y. Ito, M. Shimada, and I. Kawamura.** 1967. Chemical properties of milled wood lignin of grasses. Phytochemistry **6**:1551–1556.
15. **Hodson, R. E., A. E. Maccubbin, and R. Benner.** 1982. Microbial degradation of natural and pollutionally-derived lignocellulosic detritus in wetland ecosystems. U.S. Department of the Interior OWRT project report no. A-082-GA. U.S. Department of the Interior, Washington, D.C.
16. **Maccubbin, A. E., and R. E. Hodson.** 1980. Microbial degradation of detrital lignocelluloses by salt marsh sediment microflora. Appl. Environ. Microbiol. **40**:735–740.
17. **Sarkanen, K. V., and C. H. Ludwig (ed.).** 1971. Lignins: occurrence, formation, structure, and reactions. Wiley Interscience, New York.
18. **Shimada, M., T. Fukuzuka, and T. Higuchi.** 1971. Ester linkages of *p*-coumaric acid in bamboo and grass lignins. TAPPI **54**:72–78.
19. **Vomhof, D. W., and T. C. Tucker.** 1965. The separation of simple sugars by cellulose thin-layer chromatography. J. Chromatogr. **17**:300–306.