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Preparation and microbial decomposition of synthetic [^{14}C]lignins

(lignin biodegradation/wood decay/dehydrogenative polymerizate)

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ABSTRACT **A definitive assay for microbiological and biochemical research on the biodegradation of lignin was developed using radioactive synthetic lignins specifically labeled in the side chains, aromatic rings or in the methoxyl groups. The [^{14}C]lignins were prepared by oxidative polymerization with peroxidase and H_2O_2 of specifically labeled coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol). The synthetic polymers were shown by spectroscopic and chemical methods to contain the same intermonomer linkages found in natural lignins. Incubation of the [^{14}C]lignins with known lignin-degrading fungi and with a forest soil resulted in $^{14}CO_2$ evolution.**

Lignin is a major structural component of vascular plants and makes up a large fraction of the carbon in the biosphere. During the past 30 years the structure and biosynthesis of this complex aromatic polymer have been elucidated (1, 2), but despite numerous investigations through the years, the microbial and biochemical processes of lignin decomposition have yet to be well defined (3). A major hindrance to the solution of these problems has been lack of a representative substrate and a specific and sensitive assay for lignin biodegradation.

Recent spectroscopic (4) and chemical (G. E. Miksche, unpublished results) studies have corroborated and extended earlier evidence (2) that synthetic lignins ("dehydrogenative polymerizates," "DHPs") prepared by the enzymatic polymerization of *p*-hydroxycinnamyl alcohols contain essentially the same structural subunits as natural lignins and, therefore, can be considered to be suitable model polymers. These recent studies support the suggestion (5) that such lignins, labeled with ^{14}C, have potential in biodegradation research. We describe here the preparation and characterization of synthetic [^{14}C]lignins and their use in providing an unequivocal assay for lignin biodegradation.

MATERIALS AND METHODS

Spruce Lignin. Milled wood lignin (6) was prepared from Engelmann spruce wood as previously described (7).

Synthesis of [*Side chain*-^{14}C]Lignin. Coniferaldehyde methoxymethyl ether was synthesized by the reaction at pH 9.5–10 of 5.88 g (0.03 mole) of vanillin methoxymethyl ether with 1.45 g of acetaldehyde (containing 44 mg (1 mCi) of [*1,2*-^{14}C]acetaldehyde) (Schwarz/Mann, Orangeburg, N.Y.) (8). The product was purified by silica gel column chromatography, with benzene, then 2% (v:v) ethyl ether in benzene, as eluting solvent. The final yield was 3.3 g (49%). Hydrolysis (8) and recrystallization from ether gave coniferaldehyde in 95% yield (melting point 82–3°; ref. 8, 82.5°). The proton magnetic resonance (PMR) spectrum agreed with published data (9).

Abbreviations: PMR, proton magnetic resonance; NMR, nuclear magnetic resonance; UV, ultraviolet; IR, infrared; MW, molecular weight.

Coniferaldehyde was reduced to coniferyl alcohol (**Ia**) at room temperature with $NaBH_4$ in 50% (v:v) aqueous ethanol. Extraction and work-up gave a crystalline product in quantitative yield, the purity of which was established by thin-layer chromatography, gas chromatography, and PMR. Recrystallization from 1,2-dichloroethane provided white needles of melting point 74.5–75° (corrected); (ref. 10, 74–6°). Analysis: calculated OCH_3, 17.2%; found, 17.1%. The PMR spectrum agreed with published data (11). Ultraviolet (UV) spectrum in 95% ethanol 292 nm, $\epsilon = 5.85 \times 10^3$; 262 nm, $\epsilon = 1.45 \times 10^4$.

Coniferyl alcohol [1.7 g (9.45 mmol)] and 80 mg of peroxidase (EC 1.11.1.7, Type II, Sigma, St. Louis, Mo.) were dissolved in 400 ml of degassed sodium phosphate buffer (0.01 M) at pH 7.5. This solution and 9.45 mmol of H_2O_2 in 400 ml of degassed buffer were added simultaneously and separately to 200 ml of well-stirred degassed buffer containing 20 mg of peroxidase and 30 mg (0.18 mmol) of vanillyl alcohol, over a 16 hr period with a proportioning pump. All solutions were kept under N_2 and the reaction vessel was kept dark. After final addition the mixture was stirred an additional 10 hr. The insoluble polymer (1.8 g) was separated by centrifugation at 5°, twice suspended in 150 ml of water and recovered by centrifugation, then finally resuspended in 100 ml of water and stored frozen at −20°.

Unlabeled synthetic lignin prepared by the procedure used for the side chain-labeled material was used for elemental analyses, molecular weight measurements, and carbon-13 nuclear magnetic resonance (^{13}C-NMR) studies.

Synthesis of [*Ring*-^{14}C]Lignin. Ring-labeled **Ia** was synthesized from 840 mg (10 mmol) of phenol (containing 500 μCi of [*U*-^{14}C]phenol) (Amersham/Searle, Arlington Heights, Ill.), using published methods (10–14), with modifications as follows. *(a)* The intermediate 2-(2-methoxyphenoxy)-5-nitrobenzophenone was purified by recrystallization so that subsequent hydrolysis with piperidine gave guaiacol (75% yield from phenol) that did not require distillation (compare ref. 12). *(b)* Vanillin, prepared by formylation of the guaiacol, was purified directly by preparative thin-layer chromatography on Brinkmann 2.0 mm silica HF_{254} plates by developing twice in benzene/acetic acid/water, 4:2:1 (v:v:v); purification via the bisulfite addition product (13) was thereby eliminated. *(c)* Ethyl ferulate was synthesized in 94% yield by condensing vanillin with ethyl hydrogen malonate, and was purified by column chromatography on silica gel with 30–60° petroleum ether/ethyl ether, 3:1 (v:v) as solvent.

The ethyl [^{14}C]ferulate was reduced to coniferyl alcohol (**Ia**) with $LiAlH_4$ at −10 to −15° during 12 hr (10, 11). The product was purified by silica gel column chromatography with benzene/ethyl ether (9:1 to 9:2, v:v) as solvent. The compound crystallized in 78% yield after evaporation of solvents. Analytical data were the same as given for the side chain-labeled coniferyl alcohol.

The ring-labeled **Ia** was polymerized by the same procedure used for the side chain-labeled material.

Synthesis of [*Methoxy*-^{14}C]Lignin. Diazo[^{14}C]methane, prepared from 0.83 mCi of *N*-[^{14}C]methyl-*N*-nitroso-*p*-toluenesulfonamide (New England Nuclear, Boston, Mass.), was used to methylate 3.35 g of 2-(2-hydroxyphenoxy)-5-nitrobenzophenone (12). Synthesis of coniferyl alcohol from the product, 2-(2-[^{14}C]methoxyphenoxy)-5-nitrobenzophenone, was the same as with ring-labeled material. Polymerization was as described above.

Characterization Procedures. UV spectra of lignins in 50% aqueous dioxane were made with a Beckman DK 2-A instrument. Infrared (IR) spectra were taken on a Beckman IR-12 spectrophotometer, with lignins in KBr pellets (1.5 mg of dry lignin in 200 mg of KBr). The procedures of Lüdemann and Nimz (15) were followed for ^{13}C-NMR, using 20% solutions of lignins in completely deuterated dioxane/D_2O, 9:1 (v:v), and a Varian XL-100 spectrometer (Fourier transform method).

Degradative acid hydrolysis ("acidolysis") was performed by the method of Lundquist (16, 17), and the procedures for oxidative degradation ($KMnO_4$, H_2O_2) of methylated samples were those developed by Larsson and Miksche (17, 18).

Number average molecular weight ($\overline{M_n}$) was determined by vapor pressure osmometry at 37° with dimethylformamide as solvent (19) by Galbraith Laboratories, Knoxville, Tenn.

Analytical gel filtration of the lignins was carried out on a 12 × 750 mm column of Sephadex LH-20 in dimethylformamide; the flow rate was 18 ml/hr and the exclusion volume was 21.0 ml. A variety of lignin-related compounds of MW 168-1070 was used to calibrate the column. Elution volumes of the compounds of MW greater than 300 were closely molecular weight dependent, and the column had a projected exclusion limit of MW 1450.

Specific activity of the [^{14}C]lignins was determined with a Packard 3003 Tri-Carb liquid scintillation spectrometer. Aqueous lignin suspensions (10–100 μl) containing 10–20 mg/ml were added to 10 ml of scintillation fluid consisting of 10 g of 2,5-diphenyloxazole (PPO), 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP), and 100 g of naphthalene per liter of dioxane. Samples were counted until probable error was ≤1%. Efficiency and specific activity were determined by means of internal standardization with [^{14}C]toluene (5.00 × 10^5 dpm/ml, ICN Pharmaceuticals, Irvine, Calif.).

Biodegradation of [^{14}C]Lignins. Miami silt loam soil was collected from the "A" horizon in the University of Wisconsin Arboretum, Madison. Soil samples were put in sterile 125 ml reaction vessels equipped with gas ports for continuous aeration and effluent monitoring. Reaction vessels contained 0.5 g (wet weight) of soil, 2.0 ml of sterile distilled water, and approximately 0.3 ml of a [^{14}C]lignin suspension. Control vessels contained 2 ml of 37% (w:v) aqueous formaldehyde in place of the water. Reaction vessels were aerated with humidified, CO_2-free air at a rate of 10 ml/min and incubated at 37°.

The white-rot (lignin-degrading) fungi *Coriolus versicolor* (L. ex Fr.) Quel. (isolate Mad. 697) and *Phanerochaete chrysosporium* Burds., *sp. nov.* (isolate ME 446) and the brown-rot (polysaccharide-degrading) fungi *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. (isolate Mad. 617) and *Poria cocos* (Schw.) Wolf (isolate FP-97438-Sp) were obtained from the Center for Forest Mycology Research in Madison, Wis. These wood-destroying fungi were incubated with the labeled lignins in closed 125 ml erlenmeyer flasks that contained 2 g of a 1:1 mixture of ground aspen and spruce wood (20 mesh = 7.9 openings/cm), 2 ml of a filter-sterilized basal medium (below), and 3 ml of lignin suspension (640 μg, 5 × 10^4 dpm). Lignins in dimethylformamide solution (20–30 mg/ml) were added aseptically to sterile distilled water to give a fine suspension; no contamination problem was encountered. Lignins were adjusted with unlabeled synthetic lignin to a specific activity of 7.8 × 10^4 dpm/mg. Vessels were inoculated with 2 ml of a homogenized, washed mycelial suspension prepared from mycelia grown on basal medium with 10 g/liter glucose, and were maintained at 28° except with *Phanerochaete chrysosporium* (39°). Basal medium contained per liter of distilled water: NH_4NO_3, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; asparagine, 1 g; 10 ml of trace salts mixture (20) and 5 ml of vitamin solution (20). Reaction vessels were incubated in the dark and flushed thoroughly with sterile air at 3-day intervals.

The $^{14}CO_2$ evolved during incubation of individual reaction vessels containing soil was collected in 3 ml of 5% (w:v) KOH. At various time intervals the CO_2 traps were acidified with 6 ml of 2 N H_2SO_4 and sparged with air to transfer the $^{14}CO_2$ into scintillation vials that contained 10 ml of scintillation fluid consisting of equal parts of a toluene cocktail (4.0 g PPO and 0.1 g POPOP per liter toluene) and an anhydrous methanol/ethanolamine (4:1 v:v) solution. Trapping and transfers were shown to be quantitative. CO_2 in the effluent gas from the wood meal cultures was trapped directly in 10 ml of the above scintillation fluid. Flushing of the cultures and trapping of the $^{14}CO_2$ were shown to be quantitative.

RESULTS

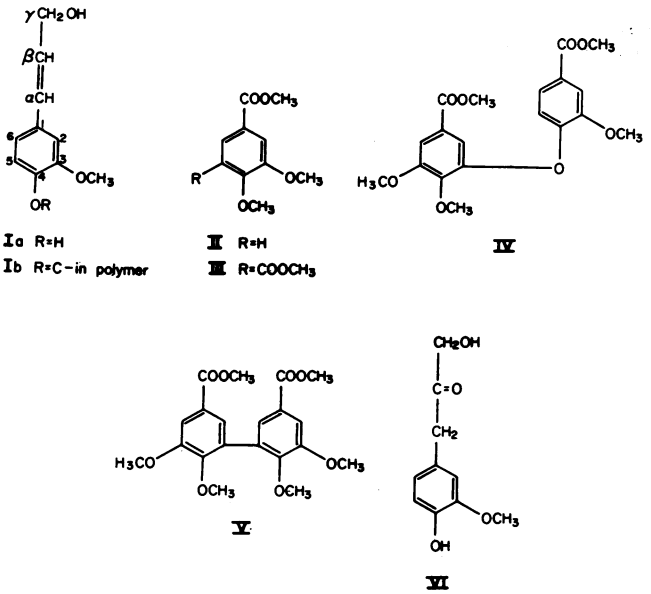
Synthesis and General Properties of Labeled Lignins. [β,γ-^{14}C]Coniferyl alcohol, [*ring-U*-^{14}C]coniferyl alcohol and [*methoxy*-^{14}C]coniferyl alcohol were prepared in overall yields of 46, 31, and 32% from vanillin methoxymethyl ether, phenol, and guaiacol, respectively. Polymerization of the labeled coniferyl alcohols gave synthetic lignins in quantitative yields with specific activities of 1.8 × 10^5, 1.4 × 10^5, and 7.8 × 10^4 dpm/mg for the side-chain-, ring-, and methoxy-labeled polymers, respectively.

The synthetic lignins were cream-colored powders when dried from aqueous suspension. They were insoluble in water, but were soluble in formamide, dimethylformamide, dimethyl sulfoxide, and in dioxane/water mixtures (1:1–19:1, v:v). These characteristics were the same as those for the milled wood lignin from spruce wood.

The number average molecular weights ($\overline{M_n}$) for two preparations of the synthetic lignin were 1490 and 1600. This corresponds to an average number of units per polymer molecule ($\overline{x_n}$) of 8.1, assuming a unit weight of 185. Analytical gel filtration on Sephadex LH-20 in dimethylformamide indicated that the [^{14}C]lignins contained less than 1% of material of MW less than 750, i.e., smaller than tetrameric. Approximately 90% of the synthetic lignin was excluded in the void volume.

Comparisons of Synthetic and Natural Lignins. Elemental and methoxyl analysis gave values for the synthetic lignins that were close to those for the spruce lignin: synthetic (unlabeled)-C, 63.2%; H, 5.8%; OCH_3, 16.3%; spruce milled wood lignin: C, 62.9%; H, 6.1%; OCH_3, 15.1%.

Percent yields of products II–V on oxidative degradation of methylated samples, and of product VI on acidolytic degradation, were as follows for synthetic (ring-labeled) and spruce lignin, respectively: II (3.8, 7.8), III (2.1, 1.4), IV (1.1, 0.8), V (2.8, 1.1), and VI (3.0, 4.5).



The IR spectra of synthetic and natural lignins were very similar; both had distinct bands at 3430, 2900, 1665, 1600, 1505, 1460, 1425, 1270, 1220, 1145, 1090, 1035, 860, and 820 cm^{-1}. A band at 970 cm^{-1} in the synthetic lignin spectrum appeared as a slight shoulder in the spruce lignin spectrum, and a shoulder at 1725 cm^{-1} in the natural lignin was absent from the synthetic.

In contrast to the IR spectra, the UV spectra of synthetic and spruce lignins differed substantially. Maxima and absorptivities for the lignins were as follows: spruce milled wood lignin (280 nm, a = 18 $l \cdot g^{-1} \cdot cm^{-1}$), synthetic lignin (273 nm, a = 31 $l \cdot g^{-1} \cdot cm^{-1}$).

The ^{13}C-NMR spectra of synthetic and natural lignins (Fig. 1) corresponded closely to published spectra (4), permitting assignment of the peaks. Comparison of the spectra documented the similarity of the two lignins in essential structural features (see refs. 4 and 21), and also specifically identified certain of the differences. The spectroscopic and chemical dissimilarities between the synthetic and natural lignins indicate differences in the frequency of four types of normal lignin constituents or substructures. *(a)* A higher proportion of coniferyl alcohol end groups (**Ib**) in the synthetic polymers is shown by the differences in the UV spectra, by the distinct IR band at 970 cm^{-1} in the synthetic lignin spectrum (1), and by differences in the ^{13}C-NMR spectra: relatively stronger peaks at 115.9, 128.0, 130.7, and 144.9 ppm in the synthetic lignin spectrum (4, 15). *(b)* The presence of more β-β linked units (VII) in the synthetic lignin is suggested by a peak at 72.1 ppm (4, 22). *(c)* A higher proportion of phenylcoumaran structures (VIII) is indicated by the more intense peaks at 88.2 and 144.7 ppm in the ^{13}C-NMR spectrum of the synthetic lignin (4, 15). *(d)* A greater frequency of 5–5 linked units (biphenyl structures, IX) is suggested by the higher yield of product V on chemical degradation of the synthetic lignin (23). The IR absorbance of 1725 cm^{-1} in the spruce lignin spectrum is due to a small amount (17) of carboxyl or ester groups, which are perhaps artifacts of the isolation procedure.

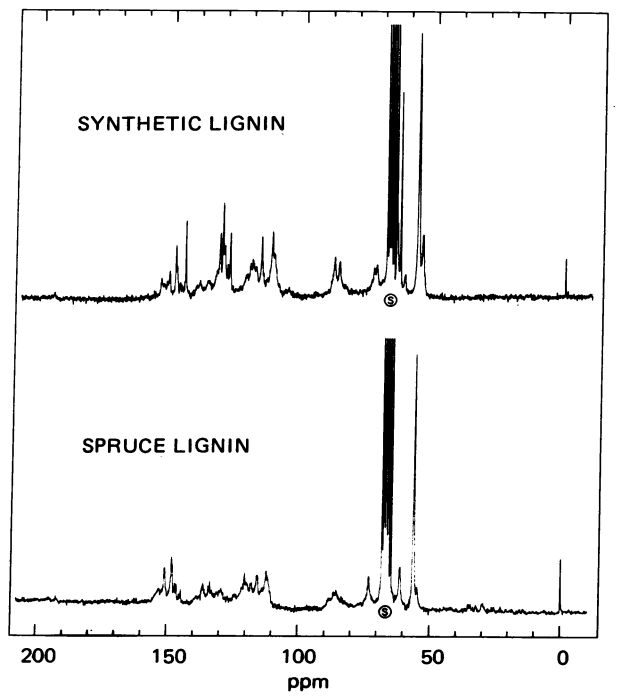
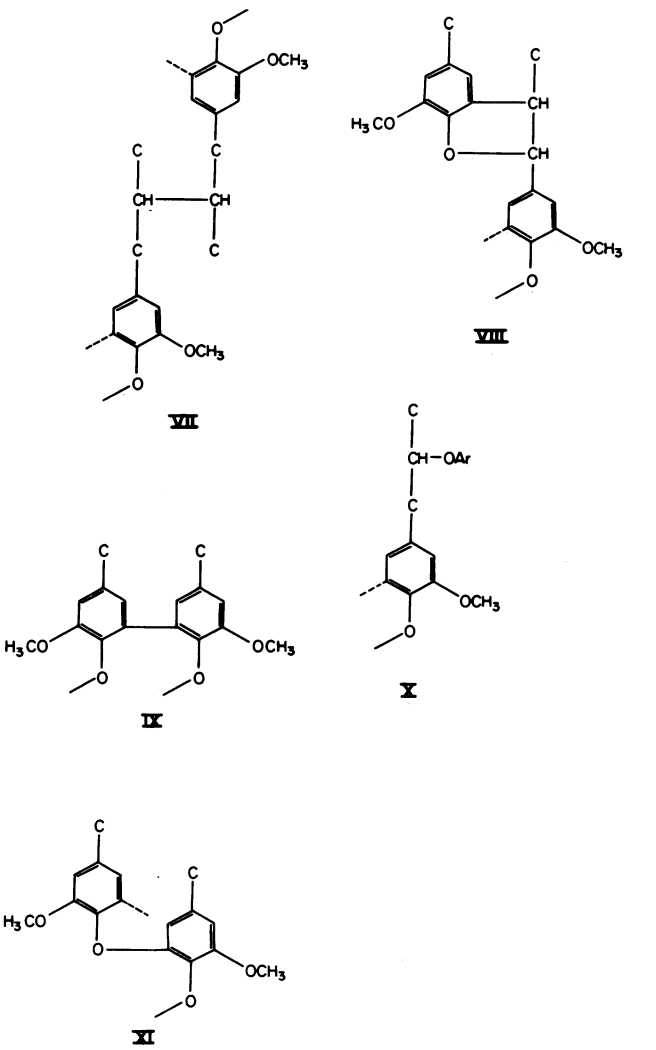


FIG. 1. ^{13}C-NMR spectra of synthetic and spruce lignins. Samples were run as 20% solutions in deuterated dioxane/D_2O (9:1, v:v). Ⓢ = methylene carbon signal of solvent. Scale is ppm downfield from tetramethylsilane.



Microbial Oxidation of Synthetic Lignins. Degradation of side-chain-, ring-, and methoxy-labeled lignins by the white-rot wood-destroying fungus *C. versicolor* is depicted in Fig. 2. During an initial lag period of about 150 hr the

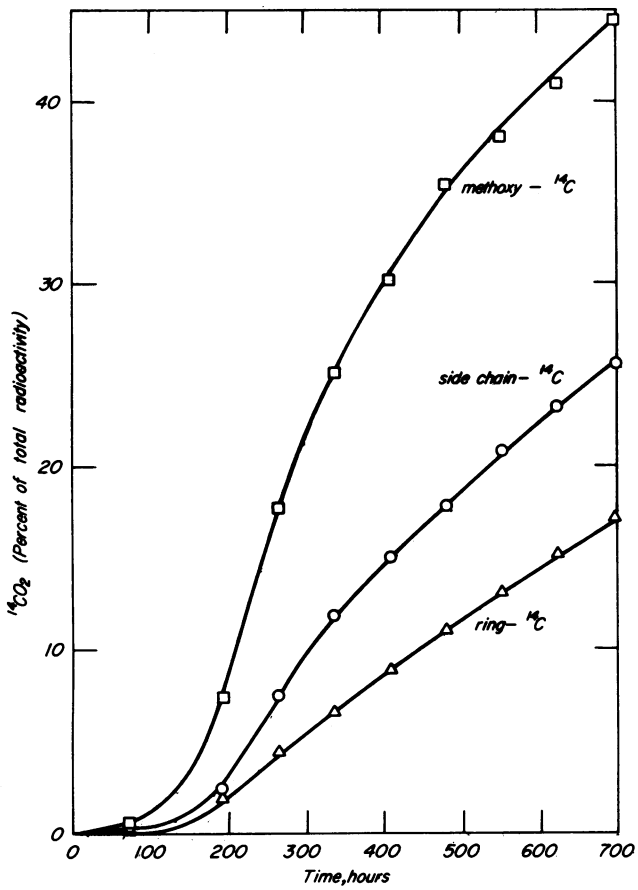


FIG. 2. Degradation of labeled lignins by the wood-destroying fungus *Coriolus versicolor*. Culture vessels contained wood meal, lignin, and a nutrient supplement (see *text*).

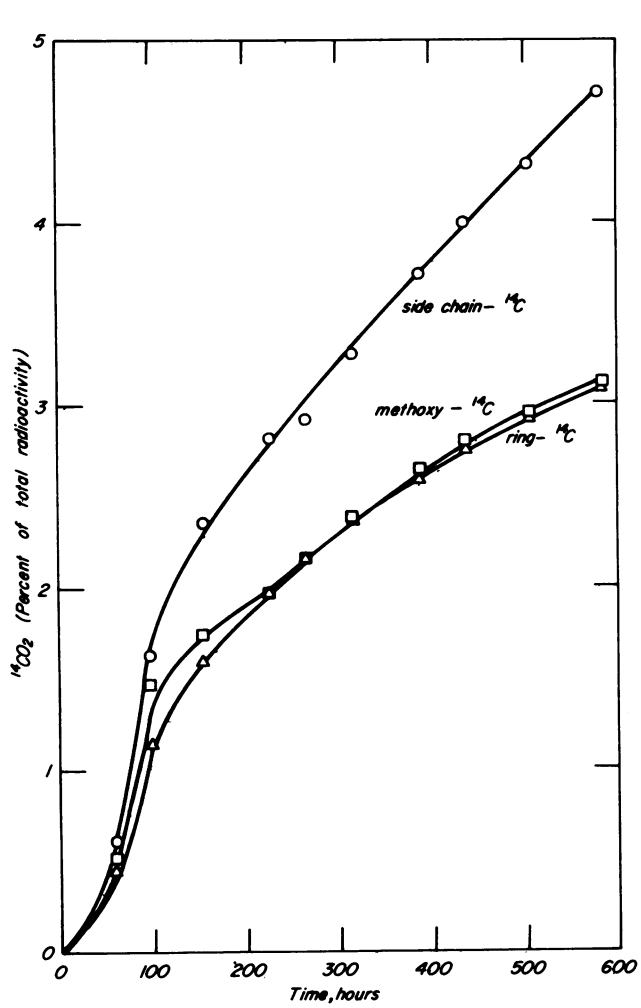


FIG. 3. Degradation of labeled lignins in forest soil. Aerated culture vessels contained soil, water, and ^{14}C-lignin ([*side-chain*-^{14}C], 1.75 mg; [*ring*-^{14}C], 1.4 mg; [*methoxy*-^{14}C], 2.7 mg).

fungus became established in the wood meal medium. After this, degradation rates accelerated rapidly and then became essentially linear. No $^{14}CO_2$ was released from control (non-inoculated or formaldehyde-killed) flasks. Throughout the 700 hr culture period the methoxy-C was converted to $^{14}CO_2$ much more rapidly than side-chain-C, which was converted substantially faster than ring-C. The same result was obtained with another white-rot fungus, *Phanerochaete chrysosporium*.

Table 1. $^{14}CO_2$ produced on fungal decomposition in wood meal cultures of synthetic lignins labeled in the side chains, aromatic rings, or methoxyl groups

Fungus	Percent loss in dry weight of wood meal after 600 hr*	Percent of total radioactivity recovered as $^{14}CO_2$ after 600 hr†		
		Side chain	Aromatic ring	Methoxyl
White-rot:				
Coriolus versicolor	12	22	15	40
Phanerochaete chrysosporium	14	20	15	33
Brown-rot:				
Gloeophyllum trabeum	15	5	2	8
Poria cocos	8	2	1	4

* Not corrected for mycelium in decayed wood.
† Culture conditions same as given in Fig. 3.

The percentages of the three labeled lignins that were converted to $^{14}CO_2$ in 600 hr in the wood meal medium by two white- and two brown-rot fungi are given in Table 1. The fungi grew well under these conditions, and the extents to which the wood was decayed after 600 hr were very similar for *G. trabeum* and the two white-rot fungi (Table 1). The low degradation of the side-chain- and ring-labeled lignins by the brown-rot fungi is fully in accord with the observation that brown-rot fungi, which destroy the polysaccharides in wood, do not substantially deplete the lignin (3, 24). Also, the data are consistent with the demonstration (25) that *G. trabeum* (= *Lenzites trabea* Pers ex Fries L.) demethylates lignin.

Fig. 3 illustrates degradation of the three labeled lignins incubated in the presence of a forest soil. Compared to the rates observed with the fungal cultures, evolution of $^{14}CO_2$ by the soil microflora was slow. Preliminary experiments with certain other soils have shown much greater rates of conversion in some, and essentially no degradation in others. Controls gave no degradation.

DISCUSSION

The chemical and physical comparisons of synthetic and natural lignins show that they contain the same types of

major intermonomer linkages. Biodegradation studies demonstrate that the synthetic polymers are recognized by the lignin-degrading enzyme system of known lignin-degrading fungi. The relative resistance of the synthetic lignins to brown- but not white-rot fungi (Table 1) parallels observations with natural lignins (3). These various chemical, physical, and biological observations indicate, therefore, that the synthetic [^{14}C]lignins are appropriate substrates for biodegradation studies, as suggested by Haider and Grabbe (5).

The major intermonomer linkages found in natural lignins are shown in substructures VII, VIII, IX, X, and XI. These are formed by β–β, β–5, 5–5, β–O–4, and 4–O–5 coupling, respectively, during the free radical polymerization of coniferyl alcohol (1). The presence of substructures IX, X, and XI in the synthetic lignins is documented by the identification of the chemical degradation products V, VI, and IV, respectively, whereas substructures VII and VIII are indicated by ^{13}C-NMR spectroscopy (15).

Even though the synthetic and spruce lignins are qualitatively similar, our chemical and physical comparisons show that the polymers differ in the relative proportions of coniferyl alcohol end groups (**Ib**) and three normal substructures: VII, VIII, IX. These differences are probably the result of a greater amount of monomer-monomer, as opposed to monomer-polymer, coupling in the synthetic reaction than occurs in spruce tissue (see ref. 1). The fine physiological control that undoubtedly limits monomer–monomer coupling in plants has not yet been duplicated synthetically.

Although natural lignins have not been exactly duplicated synthetically, recent studies have demonstrated that between plant species lignins differ in the relative frequency of certain substructures. The differences are especially great between angiosperms and gymnosperms (21, 26), and among different species of angiosperms (27). The natural variation in substructure frequency is mainly a reflection of the relative proportions of the coniferyl and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohol precursors. Because natural lignins differ in substructure frequency, it is to be expected that lignin-degrading organisms can cope with the variation, although the rates of decomposition might be expected to vary.

In future use of the assay method described here, it will be desirable to establish a minimum value in terms of the percent of lignin converted to $^{14}CO_2$ in order to ascribe lignin-degrading activity to a given organism or mixed culture. We have obtained no chemical or physical evidence that the preparations contain spurious components or low molecular weight fractions that might be especially easily metabolized. We have found, however, that in certain soils somewhat less than 1% of the synthetic lignin is converted to $^{14}CO_2$ more rapidly than the remaining 99%; this suggests that the minimum is less than 1% conversion to $^{14}CO_2$.

It should be noted that certain microorganisms or mixed microflora can in all likelihood degrade only specific structures in lignin, so that the amount of $^{14}CO_2$ evolved in this assay will depend on whether the label is in the ring, side chain, or methoxyl. Furthermore, some microorganisms may degrade most of the lignin, as occurs in humification, without converting it substantially to $^{14}CO_2$. Also, as the lignin is degraded, much of the ^{14}C will be incorporated into cell mass rather than into $^{14}CO_2$. For these reasons $^{14}CO_2$ evolution provides a minimum value for lignin biodegradation. Other microbially caused changes in the [^{14}C]lignins, such as in solubility properties or in molecular weight distribution, can provide additional information.

Synthetic ^{14}C-labeled lignins prepared as described here will permit investigation of such hitherto unresolved problems as *(a)* the role of various bacteria and fungi in lignin biodegradation, *(b)* the cultural and nutritional parameters affecting biodegradation, *(c)* the turnover of lignin in nature, including the conversion of lignin to humic substances, and *(d)* the biochemistry and molecular biology of microbial decomposition.

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