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Preparation of Specifically Labeled ^{14}C-(Lignin)- and ^{14}C-(Cellulose)-Lignocelluloses and Their Decomposition by the Microflora of Soil[1]

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Microbial decomposition of lignocellulose in soil was studied using radioisotope techniques. Natural lignocelluloses containing ^{14}C in either their lignin or cellulose (glucan) components were prepared by feeding plants L-[U-^{14}C]phenylalanine or D-[U-^{14}C]glucose, respectively, through their cut stems. Detailed chemical and chromatographic characterization of labeled lignocelluloses from three hardwood and three softwood species showed that those labeled by the [^{14}C]glucose incorporation method contained specifically labeled cellulosic components, whereas those labeled by the [^{14}C]phenylalanine incorporation method contained specifically labeled lignin components. Microbial degradation of these differentially labeled lignocelluloses was followed by monitoring $^{14}CO_2$ evolution from selected soil samples incubated with known amounts of radiolabeled lignocelluloses. The lignin components of the six woods were shown to be decomposed in soil 4 to 10 times more slowly than their cellulosic components. These rates of mineralization were comparable to the generalized patterns previously reported in the literature. The present technique, however, was thought to be simpler, more sensitive, and less prone to interference than methods previously available.

The rates at which the microflora of different soils mineralize the individual components of lignocellulosic plant materials have never been determined accurately despite the fact that considerable research has been devoted to this subject. Research has provided only generalized information showing that soils, over time, tend to become enriched with the more recalcitrant lignaceous materials found in decomposing plant material, whereas cellulosic materials are more readily and completely recycled (4, 9). Lignocellulosic materials from different plants are known to decay at different rates under similar environmental conditions, probably because of differences in their structure and composition (4). Lignocellulose decomposition has usually been measured by procedures that fractionate decomposing plant material, stepwise, into water-soluble, organic-soluble, acid-soluble (cellulosic carbohydrate), and acid-insoluble (lignin) components (6). These methods require large samples and can give only rough approximations of the rates of lignin and cellulose decomposition in natural systems or in flask culture studies with specific microorganisms. In our laboratories we have recently utilized a radioisotopic technique for monitoring microbiological lignin degradation (2). Natural lignocelluloses, containing ^{14}C in their lignin components, were prepared by feeding plants L-[U-^{14}C]phenylalanine through their cut stems. Microbial decomposition of lignin was followed in different soils by monitoring evolution of $^{14}CO_2$ from soil samples incubated with specific amounts of ^{14}C-lignin-labeled lignocelluloses. This new and sensitive assay has opened the way for in-depth studies of the microbial ecology of lignin degradation in nature (2).

In the present paper we report further studies on the preparation of labeled lignocelluloses and on their decomposition by the microflora of soil. Three hardwood and three softwood lignocelluloses were labeled in their lignin components using the [^{14}C]phenylalanine incorporation method. In addition, cellulosic components of the same woods were labeled using a new [^{14}C]glucose incorporation technique. Each of the labeled lignocelluloses was incubated under defined conditions with selected soil samples, and the rates of decomposition of ^{14}C-lignin- and ^{14}C-cellulose-labeled substrates were moni-

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tored by trapping and quantifying $^{14}CO_2$ released as a result of microbial attack on the lignocelluloses. The rates of lignin versus cellulose decomposition were compared for all six lignocelluloses.

MATERIALS AND METHODS

Preparation and characterization of labeled lignocelluloses. The lignin components of six lignocelluloses were selectively labeled with ^{14}C by feeding twigs aqueous solutions of L-[U-^{14}C]phenylalanine (50 μCi/twig) through their cut stems (2). The cellulosic components of the lignocelluloses were selectively labeled by substituting aqueous solutions (50 μCi) of D-[U-^{14}C]glucose for phenylalanine. Hardwood species labeled were white oak (*Quercus albus*), red maple (*Acer rubrum*), and black gum (*Nyssa sylvatica*). Softwoods labeled were Virginia pine (*Pinus virginianum*), hemlock (*Tsyga canadensis*), and red cedar (*Juniperus virginiana*). Labeled lignocelluloses were milled to pass a 40-mesh screen and freed of extractives as previously described (2). Care was taken to remove all extractable radioactivity from each wood. Extractive-free lignocelluloses were analyzed for distribution of ^{14}C by a modified Klason fractionation procedure (2, 8).

Supernatants from the Klason procedure contained free wood sugars as a result of the acid hydrolysis of the carbohydrate fractions of the woods. The three predominant sugars (glucose, mannose, and xylose) were separated from each by descending paper chromatography according to the procedure of Moore and Johnson (8). Isolated sugars were cut approximately quantitively from the chromatograms and analyzed for radioactivity. For each supernatant, the percentage of ^{14}C in each sugar, compared to the total ^{14}C applied to the chromatogram, was calculated.

Counting of radioactivity. ^{14}C was quantified by liquid scintillation techniques as previously described (2, 3).

Microbial decomposition of labeled lignocelluloses. The extent of decomposition of ^{14}C-lignin- and ^{14}C-cellulose-labeled lignocelluloses was followed in two soil samples taken from a heavily forested area of White Oak Canyon, Shenandoah National Park, Va. Sample number 1 was taken from the humus fraction of a moist soil beneath a virgin stand of hemlocks. Sample number 2 was taken from the humus fraction of a moist soil beneath a stand of mixed hardwoods, mostly oak and maple. Both sites had thick litter layers and contained substantial amounts of decomposing lignocellulose.

Each soil sample was divided into 12 subsamples for the biodegradation study. After moistening with sterile distilled water (1 ml/2 g of soil, wet weight), each subsample was incubated with a different labeled lignocellulose preparation in a sterile flask. A given subsample received a total of 75,000 dpm of labeled substrate, and the final lignocellulose-to-soil ratio was 10 mg of labeled lignocellulose per g of soil. Flasks were incubated at room temperature and were continuously flushed with sterile, humidified, CO_2-free air. All exit gases were passed continuously through scintillation vials containing CO_2-trapping/counting fluid (2). Vials were changed every 24 h, and trapped $^{14}CO_2$ present was quantified by liquid scintillation techniques as previously described (2, 3). Biodegradation of labeled substrate was followed by monitoring the percentage of total ^{14}C evolved as $^{14}CO_2$ from each sample versus time. Controls sterilized by autoclaving or by addition of sodium azide did not evolve $^{14}CO_2$.

RESULTS AND DISCUSSION

Before decomposition studies were begun, each of the ground, extracted lignocelluloses was analyzed for its specific radioactivity by combustion of 1- to 2-mg amounts to $^{14}CO_2$ (2) and for distribution of radioactivity between carbohydrate and lignin fractions by the Klason procedure. These analyses are summarized in Tables 1 and 2. The lower specific radioactiv-

TABLE 1. *Distribution of ^{14}C between the carbohydrate and lignin fractions of six lignin-labeled lignocelluloses*

Lignocellulose source	Sp act (dpm/mg)	^{14}C acid insoluble (%)	^{14}C acid soluble (%)	Total ^{14}C recovered (%)[a]
Hardwoods				
Black gum	6,000	48	46	94
Red maple	1,900	70	26	96
White oak	6,900	45	40	85
Softwoods				
Hemlock	6,500	52	36	88
Red cedar	6,000	42	32	74
Virginia pine	4,400	56	35	91
Average		52	36	88

[a] [(Total dpm acid soluble + total dpm acid insoluble)/total dpm of lignocellulose] × 100.

TABLE 2. *Distribution of ^{14}C between the carbohydrate and lignin fractions of six carbohydrate-labeled lignocelluloses*

Lignocellulose source	Sp act (dpm/mg)	^{14}C acid insoluble (%)	^{14}C acid soluble (%)	Total ^{14}C recovered (%)[a]
Hardwoods				
Black gum	550	19	50	69
Red maple	1,170	29	80	109
White oak	660	28	40	68
Softwoods				
Hemlock	3,020	26	67	93
Red cedar	1,020	19	69	88
Virginia pine	860	22	109	131
Average		24	69	93

[a] [(Total dpm acid soluble + total dpm acid insoluble)/total dpm of lignocellulose] × 100.

ities for the glucose-labeled lignocelluloses as compared to phenylalanine-labeled lignocelluloses were a result of more pronounced removal of incorporated ^{14}C during extraction of the ground wood with water (2).

In all cases a higher percentage of radioactivity was localized in the Klason lignin fraction than in the carbohydrate fraction of lignocelluloses labeled by the phenylalanine method (Table 1). The values for percentage of ^{14}C present in the acid-soluble fractions ("carbohydrate") are higher than those previously reported (2), but can still be accounted for as acid-soluble lignin components. Klason fractionation of these lignocelluloses gives only a rough approximation of the distribution of ^{14}C between carbohydrate and lignin. The Klason acid hydrolysis procedure, although it is the only commonly used lignin assay, has a serious drawback since considerable 72% sulfuric acid-soluble lignin is present in most lignocelluloses. Migita and Kawamura (7), for example, reported that the percentage of acid-soluble lignin varied between 5 and 20% of the Klason lignin for tropical hardwoods and between 25 and 60% for temperate hardwoods. The values observed here for acid-soluble ^{14}C probably result from the relatively high and variable content of these plants of acid-soluble lignin.

There is a reversed pattern of label distribution when [^{14}C]glucose is substituted for [^{14}C]phenylalanine as the substrate for incorporation (Table 2). For all six lignocelluloses, the acid-soluble fractions contained most of the radioactivity. Consequently, most of the ^{14}C was localized in the cellulosic components of these lignocelluloses, even when recognizing the shortcomings of the Klason procedure.

It is important that distribution of ^{14}C be analyzed thoroughly for any labeled lignocellulose before it is used as a substrate in microbial studies, due to the inherent variability of different plant species in their response to added chemicals. The Klason fractionations support the conclusions that these lignocelluloses labeled by the glucose incorporation method contain specifically labeled carbohydrate, whereas those labeled with phenylalanine contain specifically labeled lignin. Further support for these conclusions comes from analyses of the individual wood sugars present in the acid-soluble fractions of each lignocellulose. Supernatant solutions from all 12 Klason fractionations were chromatographed to separate the three dominant wood sugars (glucose, mannose, and xylose) one from another. After separation, the amounts of radioactivity in each sugar were determined, and comparisons were made between sugars from phenylalanine- versus glucose-labeled supernatant solutions. In all cases it was shown that only supernatants from lignocelluloses labeled by glucose incorporation contained labeled sugar after acid hydrolysis of the polymeric carbohydrates, and of the three wood sugars examined, only glucose contained label. All of the ^{14}C detected in the sugars would, therefore, be localized in acid-hydrolyzed glucose, an indication that labeling was in primarily the cellulosic (glucan) fraction of the carbohydrate in these lignocelluloses.

Differential labeling with [^{14}C]phenylalanine or [^{14}C]glucose, then, produced two types of specifically labeled lignocelluloses, those that were either ^{14}C-lignin or ^{14}C-cellulose (glucan) labeled. By adding these labeled substrates to soil, under defined conditions, and monitoring mineralization to $^{14}CO_2$, it was possible to compare the rates of decomposition of cellulose and lignin in natural soil habitats. In addition, the availability of labeled, natural lignocelluloses made it possible to look independently at the decomposition rates for each component of a particular lignocellulose without first physically fractionating it into its carbohydrate and lignin components.

Table 3 compares the extent of mineralization in soil of the lignin and cellulose fractions of each of the six lignocelluloses. In both soils examined, the extent of $^{14}CO_2$ evolution after 700 h of incubation was similar. Markedly different levels of mineralization were observed for lignin versus cellulose fractions of each lignocellulose. The cellulosic components were decomposed at rates averaging 4 to 10 times faster than decomposition rates for the lignin components, with variability depending on the particular soil sample and source of lignocellulose. These data show that the cellulosic components

TABLE 3. *Extent of mineralization of the lignin and cellulose components of six lignocelluloses after 700 h of incubation in soil*

Lignocellulose source	^{14}C (%) recovered as $^{14}CO_2$			
	Lignin labeled		Cellulose labeled	
	1[a]	2	1	2
Hardwoods				
Black gum	5.9	10.0	22.3	38.6
Red maple	2.2	3.1	19.0	22.0
White oak	2.2	5.5	19.1	22.1
Softwoods				
Hemlock	4.1	6.5	38.2	45.0
Red cedar	2.1	3.0	24.7	29.7
Virginia pine	1.9	1.8	21.7	29.5

[a] Soil sample.

of lignocelluloses would be completely recycled in damp forest soils within relatively short times, since minimal values for recovery of ^{14}C ranged from 19 to 45% after 700 h of incubation at room temperature. Lignin components, on the other hand, would likely accumulate over time in these soils where recoveries ranged from only 2 to 6.5% in 700 h, excluding one black gum lignin component which gave a 10% recovery. Previous studies with pure cultures have shown that the minimal recovery of added ^{14}C-labeled lignin as $^{14}CO_2$ should exceed about 2% before lignin decomposition can be considered highly significant, though this is a conservatively high figure (2). This indicates that in one or two of the present samples, lignin mineralization over the time span studied was marginally significant. Our observations are in agreement with past observations that lignaceous materials accumulate in soils and are important in processes such as humification (4, 5, 9). The oxidation rates observed account for only that cellulose or lignin that was completely oxidized to $^{14}CO_2$. Solubilization of labeled materials, microbial incorporation of ^{14}C into cellular mass, or other bioconversions such as humification were not accounted for in this system. Therefore, these percent recovery values must be considered as minimal decomposition values (2).

Both cellulose and lignin oxidation rates increased significantly after lag periods where CO_2 evolution proceeded more slowly. A typical rate curve for mineralization of specifically labeled hemlock lignocelluloses is shown in Fig. 1. Similar patterns were observed for the other woods. Lag periods were very pronounced for the ^{14}C-labeled lignin oxidation curves, whereas rates of $^{14}CO_2$ evolution from soils incubated with ^{14}C-cellulose-labeled lignocellulose increased rapidly, after short lags, to maximal linear rates. The lags are probably the result of the mechanism of decomposition of lignocellulose by microorganisms (3). Considerable preliminary attack on the polymeric structure, particularly of lignin, must occur before appreciable oxidation to $^{14}CO_2$ is possible. Recently, other workers have observed similar patterns for the decomposition of ^{14}C-labeled synthetic lignins in soil (5).

The values of using specifically labeled ^{14}C-lignocellulose in microbiological decomposition studies include the fact that it is an easier technique and is a far more sensitive assay for mineralization than the previously available fractionation procedures. Due to its increased sensitivity and the fact that substrates can be differentially labeled, this ^{14}C technique is more amenable to studies on the microbial ecology of lignocellulose decomposition. In addition, this ^{14}C technique is not prone to the inaccuracies of the Klason procedure that are caused by the presence of acid-insoluble contaminants in the assayed materials. Microbial cell mass, for example, interferes with the Klason assay (1). The ^{14}C technique should, therefore, be especially useful in pure culture studies designed to measure the lignin- and cellulose-decomposing abilities of particular microorganisms.

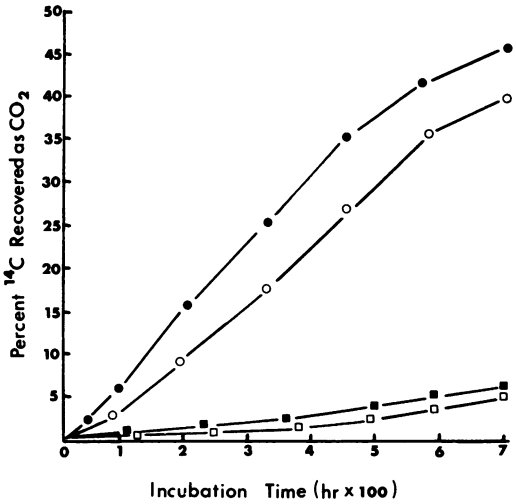

FIG. 1. *Decomposition of ^{14}C-labeled hemlock lignocellulose by the microflora of soil. ^{14}C-lignin-labeled lignocellulose: Soil sample no. 1* (□); *soil sample no. 2* (■). *^{14}C-cellulose-labeled lignocellulose: Soil sample no. 1* (○); *soil sample no. 2* (●).

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